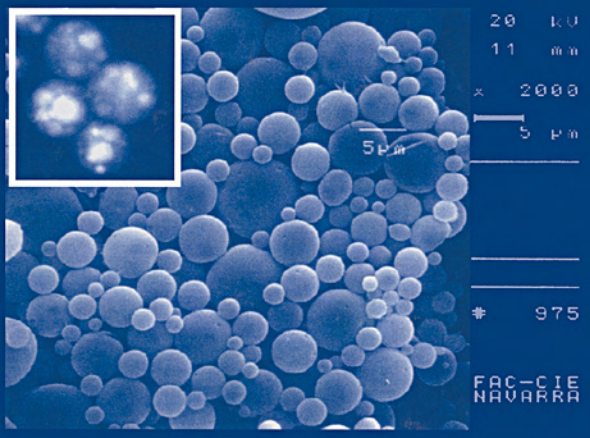
Brucella

Molecular and Cellular Biology

Edited by:

Ignacio López-Goñi and Ignacio Moriyón



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Edited by

Ignacio López-Goñi

Ignacio Moriyón

Universidad de Navarra,
Pamplona, Spain

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Horizon Bioscience 32 Hewitts Lane Wymondham Norfolk NR18 0JA England
This edition published in the Taylor & Francis e-Library, 2005.

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British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

ISBN 0-203-01745-5 Master e-book ISBN

ISBN: 1-904933-04-1 (Print Edition)

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Dedication

Ramón Díaz García was born in San Martín de Trevejo, a small village in the mountains of Northern Extremadura, Spain, in 1935. Son of a rural physician, he was witness to the harsh socioeconomic conditions prevailing in some countryside areas of Spain after the 1936–1939 devastating Civil War and developed both a compassionate attitude towards human suffering and an avid curiosity on the causes of human diseases. He obtained his Medical Degree at the University of Navarra in 1962, and his Ph.D. degree at this same University in 1965. His Ph.D. dissertation thesis, entitled "Analysis by immunoelectrophoresis and hemagglutination of the genus *Brucella*", was connected with the high prevalence of brucellosis to be found in Spain at the time. In the same year, he moved to the University of Wisconsin-Madison (U.S.A.) to work as a postdoctoral student for Joseph Wilson, and there he established a lifelong and fruitful cooperation with the late Lois Jones and with David Berman. In Wisconsin, he made seminal contributions to our understanding of the structure and biological activities of *Brucella* lipopolysaccharide and helped to establish the taxonomic position of *B. ovis* and *B. canis* within the genus at a time when this was the matter of some hot (and mostly forgotten) disputes. At the end of 1968, Ramón Díaz moved back to Spain to teach Microbiology at the Medical School of the University of Navarra but shortly afterwards Michel Plommet, who was Head of the Department of Reproductive Pathology of the INRA station at Nouzilly (France), offered him a position as a researcher. He thus moved to France in 1970, where he helped Michel Plommet to shape the INRA brucellosis research team. At INRA, he initiated the characterization of the *Brucella* antigens involved in the skin test reaction, identified the rough lipopolysaccharide of *Brucella*, and performed experiments that demonstrated the congenital transmission of brucellosis. He was also the first to identify the antigens involved in the cross-reactivity between *Y. enterocolitica* O:9 and smooth brucellae. He moved back to the University of Navarra in October 1973 and became Head of the Department of Microbiology. Here, he made the observation that *Brucella* native haptens polysaccharides differentiate infected from strain 19 vaccinated cattle in precipitation reactions. This led him to develop a simple immunoprecipitation assay that was used as the reference confirmatory test during the brucellosis eradication campaign in Navarra and that was considered by the veterinarians involved as a decisive tool in the final success of the campaign twenty years ago. He was also first to demonstrate the usefulness of protein antigens in the diagnosis of human brucellosis and their value in the differentiation of human brucellosis and yersiniosis. In later years, he generously helped younger researchers and professors of the Department, never seeking

personal credit for this obscure but very valuable work. Ramón Díaz is the author or co-author of over one hundred papers and book chapters on *Brucella* and brucellosis. He is to retire in 2005 and, with the wholehearted agreement of all co-authors, this book is dedicated to him as a token of our deep gratitude.

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Contributors

Eric Anderson
Department of Microbiology and Immunology
East Carolina University School of Medicine
Greenville
North Carolina 27834
USA
Cynthia L.Baldwin
Department of Veterinary and Animal
Sciences
University of Massachusetts
Amherst
Massachusetts 01003
USA
Montserrat Barberán
Unidad de Sanidad
SIA-DGA
50080 Zaragoza
Spain
Richard H.Borschel
Department of Bacterial Diseases
Walter Reed Army Institute of Research
503 Robert Grant Avenue
Silver Spring
MD 20910–7500
USA
Bryan H.Bellaire
Department of Microbiology and Immunology
Louisiana State University Health Sciences
Center
Shreveport
Louisiana 71130–3932
USA
José M.Blasco
Unidad de Sanidad
SIA-DGA

50080 Zaragoza
Spain
Apurba K.Bhattacharjee
Department of Bacterial Diseases
Walter Reed Army Institute of Research
503 Robert Grant Avenue
Silver Spring
MD 20910–7500
USA
Stephen Boyle
Center for Molecular Medicine and Infectious
Diseases
Virginia-Maryland Regional College of
Veterinary Medicine
Virginia Tech
Blacksburg
VA 24061
USA
Betsy J.Bricker
National Animal Disease Center
Agricultural Research Service
United States Department of Agriculture
Ames
Iowa 50010 USA
Axel Cloeckaert
Unité BioAgresseurs
Santé et Environnement
Institut National de la Recherche
Agronomique
37380 Nouzilly
France
Juan D.Colmenero
Unidad de Enfermedades Infecciosas
Departamento de Medicina Internal
Hospital Universitario “Carlos Haya”
29010 Malaga
Spain
Raquel Conde
Departamento de Microbiología y
Parasitología
Universidad de Navarra 31008
Pamplona
Spain
Xavier De Bolle
Microbiology Laboratory
Research Unit in Molecular Biology

University of Namur
B-5000 Namur
Belgium
Rose M.Delrue
Microbiology Laboratory
Research Unit in Molecular Biology
University of Namur
B-5000 Namur
Belgium
Vito G.DelVecchio
Vital Probes Inc.
1300 old Plank Road
Mayfield
PA 18433
USA
Eric Depiereux
Research Unit in Molecular Biology
University of Namur
B-5000 Namur
Belgium
Maite Estevan
Departamento de Microbiología y
Parasitología
Universidad de Navarra
31008 Pamplona
Spain
Carlos Gamazo
Departamento de Microbiología y
Parasitología
Universidad de Navarra
31008 Pamplona
Spain
Juan M.García-Lobo
Departamento de Biología Molecular
Facultad de Medicina
Universidad de Cantabria-CIB/CSIC
39011 Santander
Spain;
Cynthia Gibas
Department of Biology
College of Science
Virginia Tech
Blacksburg
VA 24061
USA
Radhika Goenka

Department of Vet and Animal Sciences
University of Massachusetts
Amherst
Massachusetts 01003
USA
M.Mar Goñi
Departamento de Tecnología Farmacéutica
Universidad de Navarra
31008 Pamplona
Spain
David González
Departamento de Microbiología y
Parasitología
Universidad de Navarra 31008
Pamplona
Spain
Jean-Pierre Gorvel
Centre d'Immunologie INSERM-CNRS
Université de la Méditerranée de Marseille-
Luminy
Parc scientifique et technologique de Luminy
Marseille 13288
France
M.Jesús Grilló
Unidad de Sanidad
SIA-DGA
50080 Zaragoza
Spain
Shirley M.Halling
Bacterial Diseases of Livestock Research Unit
National Animal Disease Center
Agricultural Research Service
United States Department of Agriculture
Ames
Iowa 50010 USA
David L.Hoover
Department of Bacterial Diseases
Walter Reed Army Institute of Research
503 Robert Grant Avenue
Silver Spring
MD 20910–7500
USA
Nora Iñón de Iannino
Instituto de Investigaciones Biotecnológicas
Instituto Tecnológico de Chascomús (IIB-
INTECH

CONICET)
Universidad Nacional de General San Martín
Buenos Aires
Argentina
Juan M.Irache
Departamento de Tecnología Farmacéutica
Universidad de Navarra
31008 Pamplona
Spain
Maite Iriarte
Departamento de Microbiología y
Parasitología
Universidad de Navarra 31008
Pamplona
Spain
Mina J.Izadjoo
Department of Bacterial Diseases
Walter Reed Army Institute of Research
503 Robert Grant Avenue
Silver Spring
MD 20910–7500
USA
Véronique Jubier-Maurin
INSERM U-431
Université Montpellier II
34095 Montpellier
France
Stephan Köhler
INSERM U-431
Université Montpellier II
34095 Montpellier
France
Christophe Lambert
Research Unit in Molecular Biology
University of Namur
B-5000 Namur
Belgium
Jean-Jacques Letesson
Microbiology Laboratory
Research Unit in Molecular Biology
University of Namur
B-5000 Namur
Belgium
Jean-Pierre Liautard
INSERM U-431
Université Montpellier II

34095 Montpellier
France
Séverine Loisel
INSERM U-431
Université Montpellier II
34095 Montpellier
France
Ignacio López-Goñi
Departamento de Microbiología y
Parasitología
Universidad de Navarra 31008
Pamplona
Spain
Clara M.Marín
Unidad de Sanidad
SIA-DGA
50080 Zaragoza
Spain
Lorea Manterola
Departamento de Microbiología y
Parasitología
Universidad de Navarra
31008 Pamplona
Spain
Daniel Monreal
Departamento de Microbiología y
Parasitología
Universidad de Navarra 31008
Pamplona
Spain
Pilar Morata
Departamento de Bioquímica y Biología
Molecular
Facultad de Medicina
29071 Malaga
Spain
Ignacio Moriyón
Departamento de Microbiología y
Parasitología
Universidad de Navarra 31008
Pamplona
Spain
Edgardo Moreno
Programa de Investigación en Enfermedades
Tropicales (PIET)
Escuela de Medicina Veterinaria

Universidad Nacional
304–3000 Heredia
Costa Rica
Cesar V.Mujer
Vital Probes Inc.
1300 Old Plank Road
Mayfield
PA 18433
USA;
Maribel Murillo
Departamento de Microbiología y
Parasitología
Universidad de Navarra
31008 Pamplona
Spain
Mikeljon P.Nikolich
Department of Bacterial Diseases
Walter Reed Army Institute of Research
503 Robert Grant Avenue
Silver Spring
MD 20910–7500
USA
Shen Q.Pan
Department of Biological Sciences
National University of Singapore
117543 Singapore
James T.Paulley
Department of Microbiology and Immunology
East Carolina University School of Medicine
Greenville
North Carolina 27834
USA
María I.Queipo-Ortuño
Departamento de Bioquímica y Biología
Molecular
Facultad de Medicina
29071 Malaga
Spain
R.Martin Roop II
Department of Microbiology and Immunology
East Carolina University School of Medicine
Greenville
North Carolina 27834
USA
Félix J.Sangari García
Departamento de Biología Molecular

Facultad de Medicina
Universidad de Cantabria-CIB/CSIC
39011 Santander
Spain
Rodolfo A.Ugalde
Instituto de Investigaciones Biotecnológicas
Instituto Tecnológico de Chascomús (IIB-
INTECH
CONICET)
Universidad Nacional de General San Martín
Buenos Aires
Argentina
Nieves Vizcaino
Departamento de Microbiología y Genética
Universidad de Salamanca
37007 Salamanca
Spain
Masahisa Watarai
Department of Applied Veterinary Science
Obihiro University of Agriculture and
Veterinary Medicine
Obihiro
Hokkaido 080–8555
Japan

Foreword

Brucellosis is the term used to describe a group of closely related diseases caused by the members of the genus *Brucella* in animals and human beings. This term has largely replaced others like Malt fever, Mediterranean fever, undulant fever, Bang's disease, etc., that were originally used to describe the human *Brucella* infections associated with a given area, some of the symptoms, or the disease in animals. Starting from the work of Evans, it progressively became clear that closely related bacteria caused all of these diseases, and Meyer and Shaw created the genus *Brucella* in 1920 to accommodate these microorganisms (Meyer and Shaw, 1920). Originally, the genus had two species, *B. abortus* and *B. melitensis.* Nine years later, Huddleson designated the isolates from pigs (which up to that point were considered to be *B. abortus* variants) as a new and different species, *B. suis* (Huddleson, 1929). The genus membership remained stable until 1956 when Buddle described *B. ovis* (Buddle, 1956). In the following year, Stoenner and Lackman described *B. neotomae*, the desert woodrat *(Neotoma lepida)* brucella (Stoenner and Lackman, 1957), and Carmichael and Bruner described *B. canis* in 1968 (Carmichael and Bruner, 1968). To these six species, usually referred to as the classical species, we have to add the marine mammal isolates whose taxonomical status is discussed in Chapter 1 of this book in the light of modern tools.

Brucellosis is a complex disease but the basic facts concerning this infection are simple. The range of primary hosts of *Brucella* (i.e. those in which the disease perpetuates) is large and encompasses several domestic or semi-domestic animals (ruminants, swine, dogs, reindeer), cetaceans, pinnipeds, some wild rodents and, to a minor extent, other species of warm-blooded vertebrates. In domestic ruminants, the disease is manifested mostly as abortions and infertility. Humans usually acquire brucellosis from domestic animals and are not themselves a source of contagion. The human disease is insidious and debilitating, sometimes grave, rarely deadly, and may leave severe sequelae.

Brucellosis is an extremely important disease around the world. This is because the domestic or semi-domestic species afflicted with brucellosis are widely used for meat, milk, hair, wool, hides, fertiliser, fuel, for carrying burdens or for cultivating the soil. Moreover, these animals are generally in parts of the world where animal and/or human health services are scarce or nonexistant. According to FAO records, about 97% (720 million) of goats, 70% (730 million) of sheep, 80% (1,100 million) of cattle and 77% (726 million) of pigs are to be found in non-industrialised countries (http://apps.fao.org/page/collections?subset=agriculture). This picture is aggravated by the fact that eradication of brucelosis has been achieved only in a handful of developed

countries. Eradication has been linked mostly to the availability of proficient animal health services, good animal management, intensive breeding, and control of movement and trade of animals, conditions all necessary to implement successful vaccination campaigns. Wherever flocks are large, or animals are bred extensively, and transhumance or other migration practices are necessary, eradication becomes an extremely difficult task, even with the best means available. Indeed, it is not possible to estimate the actual incidence of brucellosis in non-industrialised countries but it was estimated that over 60% of flocks in Southern European Mediterranean countries were infected only thirty years ago. We do not know whether brucellosis is currently present at these levels in non-industrialised countries since maintenance of the disease depends on many factors. However assuming that the disease level is similar to that found in the Southern European Mediterranean countries thirty years ago, this would suggest that the disease is very important in the less favoured parts of the world, and that millions of animals could be infected. Consequently, the numbers of human cases may be exceedingly high. In the battle against this very important disease, Molecular and Cell Biology offer invaluable tools to understand the pathogen and, eventually, to combat it.

It may come as a surprise to many to learn that a disease that has been known for such a long time still poses several challenges from a diagnostic point of view. Most of the symptoms of animal and human brucellosis are not specific so that a definite diagnosis cannot be established without the help of laboratory tests, and although the value of immunological tests cannot be questioned, they leave some room for improvement. In some cases, they lack specificity due to cross-reactivity with other bacteria or to the interference of vaccination. In the human disease, they do not solve the problems of diagnosing relapses. In this context, molecular tests deserve particular attention, and Chapters 2 and 3 address the use of PCR-based assays.

The origin and evolution of pathogens may, to some people, seem to be mere academic questions with little bearing on the practical aspects of brucellosis but the fact is that the *Brucella* case proves the contrary. Infectious agents have plagued human beings since the very dawn of humanity. However, not all of the infectious diseases we suffer from nowadays have been constant companions of our kind. Back in Neolithic times, as humans learned to profit from captive animals and to tame the domestic breeds we use today, our ancestors came into contact with the infectious agents of livestock. Some pathogens, presumably through long co-evolution with the animal hosts, adapted to overcome common themes shared by the innate and adaptive immune systems of their primary and new hosts. Indeed, this is clearly the case of the *Brucellae,* for which we know the actual permanent hosts. In addition we know that secondary hosts, like humans, are inadequate for the perpetuation of the infection on a population basis. Although these facts have been obvious for years, they have not solved the question of where the actual roots of this animal disease were. Although soil, with its constantly changing microenvironments, is considered to be one of the “breeding grounds” of infectious diseases, it would not have been suspected that the evolution of several intracellular parasites, including *Brucella,* would have been influenced by it. Surprisingly, the evidence afforded by Molecular Biology demonstrates the position of the genus in close relationship to plant pathogens and endosymbionts suspected first in 1969 by Thiele and co-workers (Thiele *et al.,* 1968) on the basis of the lipid composition of the genus. In many bacteria, horizontal transmission of pathogenicity islands or virulence genes play a

very important role in their evolution towards pathogenicity. On the contrary, although there is at least one example of this horizontal transmission in *Brucella*, to understand the pathogenicity of this genus requires emphasis on its phylogeny. Indeed, the similarities between endosymbiosis and intracellular pathogenicity are very illuminating. Genomic, proteomic, structural and biological comparisons pervade many of the Chapters of this book, and Chapter 10 presents a specific example: how some regulatory elements are conserved in *Brucella* and in plant pathogens and endosymbionts.

It is remarkable that *Brucella* stands as one of the few genera for which we have the complete genomic sequence of several species. This allows interesting comparative analyses such as those described in Chapters 5 and 6. The possibilities offered by this kind of analysis in understanding the biology of the genus are augmented by the development of analytical tools such as that described in Chapter 4, and an application of this to the study of virulence is illustrated in Chapter 7. These comparative analyses have also bearing on other aspects of *Brucella* biology, including structure. Chapters 8 and 9 show how Molecular Biology can orientate structural, biosynthetic and biological analysis of two envelope molecules involved in virulence, the periplasmic glucans and the lipopolysaccharide.

After the classical work of and Robertson and McCullough (1968) and Sperry and Robertson (1975) on the glucose and erythritol metabolism by *Brucella*, there have been only fragmentary and indirect studies on the metabolism of this bacterium. This is an important aspect of the biology of any pathogen, and the new Molecular Biology tools enabled the return to some classical topics in *Brucella* metabolism. The relationship between erythritol and tissue tropism and virulence suggested by Keppie and Smith in 1965 has always drawn the interest of investigators and this topic is revisited in Chapter 11. Likewise, Chapter 12 reviews the metabolism of iron in *Brucella* following the track initiated by the Elberg group in 1955 (Waring et al., 1953).

At the beginning of the last century, Smith observed the characteristic intracellular location of *B. abortus* within trophoblasts (Smith, 1919). The remarkable pictures he published showed such a density of an intracellular population of bacteria that they pose immediate questions to cellular microbiologists and immunologists. However, only recently have the fine aspects of the intracellular behaviour of *Brucella* begun to be elucidated. The reader will find somewhat controversial views on the interaction between the bacteria and the cell membrane in Chapters 13 and 14. Moreover, Chapters 14 and 15 present the most conspicuous aspects of our present understanding of the development of the infectious process within the host cells, and how some bacterial elements including type IV secretion systems may direct this process. On the other hand, Chapter 16 develops the other side of the host-parasite relationship: the development of cell mediated immunity against this peculiar pathogen.

One of the ultimate goals of brucellosis research is to achieve its eradication or to prevent its expansion. To this end, repeated experience has shown that the stealthy spread of the disease in herds can only be prevented or reduced by the use of vaccines. Under natural conditions, a human vaccine may also be justified in some cases. Moreover, it is a sad but real fact that *Brucella* is a biological weapon candidate. In Chapter 17, the most recent studies on the development of a live attenuated human vaccine are presented. Although cell mediated immunity has been classically elicited by live attenuated vaccines, there are some recent developments on the controlled release of antigens and

drugs by synthetic carriers. These new adjuvants allow new approaches to vaccination and their application to brucellosis is discussed in Chapter 18.

It is a fundamental conviction of scientists, and a repeatedly proven fact, that basic research leads to practical results not necessarily sought as an immediate goal. *Brucella* and brucellosis provide an excellent example of this. The reader will find many chapters in this book showing that *Brucella* poses enticing scientific challenges. It may be less clear, but it is the common starting point for all the contributors, that brucellosis represents, first and above all, a call to the ethical commitments that have spurred science in the battle against human suffering.

Finally, it is our duty as Editors of this book to thank all co-authors for their willingness to share openly with the readers many interesting and challenging new ideas. Undoubtedly, these will have an impact not only in workers in the brucellosis field but also in those interested in microbial pathogenesis, vaccine development, bacterial structure and taxonomy to name a few. The timely initiative of the Publishers of the book deserves also our gratitude.

Ignacio López-Goñi and Ignacio Moriyón
Pamplona, June, 2004.

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Chapter 1
DNA Polymorphism and Taxonomy of *Brucella* Species

Axel Cloeckaert* and Nieves Vizcaíno

From: *Brucella:* Molecular and Cellular Biology. Edited by: Ignacio López-Goñi and Ignacio Moriyón

Abstract

Six species are currently recognized within the genus *Brucella: B. melitensis, B. abortus, B. suis, B. neotomae, B. ovis,* and *B. canis*. This classification is mainly based on differences in pathogenicity and in host preferences (sheep and goats, cattle, swine, desert rat, rams, and dogs respectively). Although the six species can be differentiated by conventional phenotypic tests, these species display a high degree of DNA homology in DNA-DNA hybridization assays (>90% identity). Therefore it has been proposed that the *Brucella* genus should comprise only one species i.e. *B. melitensis* and that the other species should be considered as biovars. However, several molecular genotyping methods has been used to show that *Brucella* species display significant DNA polymorphism allowing the species to be differentiated thereby justifying the current species classification. This is also true for the recent marine mammal *Brucella* isolates for which two new species names have been proposed, i.e. *B. pinnipediae* and *B. cetaceae,* according to the classical criteria of host preferentialism (pinnipeds and cetaceans respectively) and specific molecular markers.

1. Introduction

Brucellae are Gram-negative, facultative intracellular bacteria that can infect many species of animals and man. Six species are currently recognized within the genus *Brucella: B. melitensis, B. abortus, B. suis, B. neotomae, B. ovis,* and *B. canis* (Corbel and Brinley-Morgan, 1984). This classification is mainly based on differences in pathogenicity and in host preferences (Corbel and Brinley-Morgan, 1984). The main

pathogenic species worldwide are: *B. melitensis*, which is responsible for ovine and caprine brucellosis and producing the most severe infection in humans; *B. abortus*, the main etiologic agent of bovine brucellosis; and *B. suis*, responsible for swine brucellosis. These three *Brucella* species may cause abortion and subfertility in their hosts, which results in huge economic losses. *B. ovis* and *B. canis* are responsible for ram epididymitis and canine brucellosis, respectively. For *B. neotomae* only strains isolated from desert rats have been reported. The species *B. melitensis*, *B. abortus*, and *B. suis* contain several biovars. Species and biovars are currently distinguished by differential tests based on

* For correspondence email cloeckae@tours.inra.fr

phenotypic characterization by serotyping, phage typing, dye sensitivity, CO_2 requirement, H_2S production, and metabolic properties (Alton *et al.*, 1988).

Since the 1990s several *Brucella* strains have been isolated from a wide variety of marine mammal species, including seal, dolphin, porpoise, whale and otter species (Ewalt *et al.*, 1994; Ross *et al.*, 1994; 1996; Foster *et al.*, 1996; Garner *et al.*, 1997; Jahans *et al.*, 1997; Clavareau *et al.*, 1998; Foster *et al.*, 2002; González *et al.*, 2002; Watson *et al.*, 2003). These marine mammal *Brucella* isolates are distinct from the six currently recognized *Brucella* species in terms of their phenotypic and molecular characteristics (Jahans *et al.*, 1997; Clavareau *et al.*, 1998; Bricker *et al.*, 2000; Cloeckaert *et al.*, 2000a; Verger *et al.*, 2000; Cloeckaert *et al.*, 2001; 2003). Accordingly, the inclusion of new species in the genus *Brucella* has been proposed although official names have not yet been given for the marine mammal *Brucella* isolates.

Phylogenetically the genus *Brucella* belongs to the Rhizobiaceae group of bacteria (Yanagi and Yamasato, 1993). DNA-DNA hybridization studies have revealed a high degree of homology (>90%) between the six recognized *Brucella* species (Hoyer and McCullough, 1968a; 1968b; Verger *et al.*, 1985; 1987) and between the latter and the recently isolated marine mammal strains (Verger *et al.*, 2000). *Brucella* would thus constitute a monospecific genus, for which *B. melitensis* has been proposed as the sole representative species (Verger *et al.*, 1985, 1987). The other *Brucella* species would be considered as biovars of *B. melitensis*. However, the classical organization of the genus into six species is still preferred, as it is in accordance with the pathogenicity and host preference characteristics of each species. Molecular markers distinguishing the six *Brucella* species and some of their biovars have been described (reviewed by Vizcaíno *et al.*, 2000). Some of these, as discussed in the present review, might be related to host preferentialism although it has not yet been experimentally demonstrated.

2. Variability in the Genome Organization

Restriction fragment length polymorphism (RFLP) of the whole genome has been used to analyze the DNA variability in the genus *Brucella*. The first study was performed with restriction fragments of genomic DNA resolved by 0.8% agarose gel electrophoresis (O'Hara *et al.*, 1985). Different restriction patterns for *B. melitensis*, *B. abortus*, *B. canis*, and *B. ovis* were found when DNA was digested with *Hind*III (O'Hara *et al.*, 1985). However, conventional agarose gel electrophoresis is not a powerful method to separate

the whole genome restriction fragments. The use of pulsed-field gel electrophoresis (PFGE) to resolve DNA fragments obtained by digestion of whole DNA with low-cleavage-frequency restriction enzymes has greatly improved our knowledge about the DNA variability and the genomic organization in the genus *Brucella*. Thus Allardet-Servent *et al.* (1988) found five different fragment patterns following PFGE of whole DNA digested with *Xba*I; these corresponded to each of the five *Brucella* species tested (*B. neotomae* was not included in this study) but biovars within species were not differentiated. Additionally the chromosome size of *B. melitensis* 16M and *B. abortus* 544, the reference strains of these two species, was estimated to be about 2,600 kb (Allardet-Servent *et al.*, 1988).

Southern blot hybridization of *Spe*I, *Xho*I and *Xba*I fragments separated by PFGE allowed (i) the establishment of an initial physical map of the *B. melitensis* 16M chromosome, (ii) the identification of the presence of 3 rRNA operons and the localization of these in the chromosome map, and (iii) the recalculation of the *B. melitensis* 16M chromosome size, which had previously been estimated to be about 3,100 kb (Allardet-Servent *et al.*, 1991). However several problems, mainly resulting from the excessive number of fragments obtained with these restriction enzymes, were encountered with this technique. Further studies were performed by PFGE with DNA digested with *Pac*I, a restriction enzyme giving only eight fragments with the *B. melitensis* DNA (Michaux *et al.*, 1993). Southern blot hybridization of *Pac*I, *Spe*I, *Xho*I and *Xba*I fragments, partially confirmed the previous physical map of *B. melitensis* 16M but revealed the presence of two replicons of about 2,100 kb and 1,150 kb,. This was confirmed by PFGE analysis of intact *B. melitensis* 16M DNA (Michaux *et al.*, 1993). In a later study the *Pac*I and *Spe*I restriction maps and the physical maps of the six classical *Brucella* species reference strains were studied (Michaux-Charachon *et al.*, 1997). The PFGE profiles and the chromosome physical maps of the *Brucella* species were distinguishable, with the exception of those from the *B. suis* and *B. canis* reference strains, which were identical. The presence of two chromosomes was detected in the six *Brucella* species reference strains (Michaux-Charachon *et al.*, 1997). The differences between strains (as compared to the *B. melitensis* 16M physical map) included small insertions or deletions ranging from 1 to 34 kb (more frequently found in the small chromosome), a 21 kb fragment specific to *B. ovis* and a 640 kb inversion in the *B. abortus* 544 small chromosome (Michaux-Charachon *et al.*, 1997). This 640 kb inversion was detected in *B. abortus* biovars 1, 2, 3 and 4 but not in the other *B. abortus* biovars (Michaux-Charachon *et al.*, 1997). PFGE of DNA restriction fragments has also been used to study the DNA polymorphism of the *Brucella* strains isolated from marine mamals. Genomic DNA digested with *Xba*I, provided specific PFGE profiles for each of the six classical *Brucella* species and for the strains isolated from each species of marine mammals (Jensen *et al.* 1999).

The presence of two replicons has been demonstrated in the six classical *Brucella* species and their biovars, with the exception of *B. suis* biovar 3 which contains only one replicon of about 3,100 kb (Michaux *et al.*, 1993; Michaux-Charachon *et al.*, 1997; Jumas-Bilak *et al.*, 1998a). These two replicons have been suggested to be two independent chromosomes rather than one chromosome and one megaplasmid, as both replicons contain genes essential for survival of the bacterium i.e. two rRNA operons are located in the large replicon and another is located in the small operon, and the *dnaK* and

groE genes, important for the bacterial physiology, are located in the large and small replicon, respectively (Michaux *et al.*, 1993; Michaux-Charachon *et al.*, 1997). Although it is not a common feature, multiple chromosome arrangements have been found in other bacteria. Examples include *Rhodobacter sphaeroides* (Suwanto and Kaplan, 1989), *Burkholderia cepacia* (Cheng and Lessie, 1994), *Leptospira interrogans* (Zuerner *et al.*, 1993), some species of *Agrobacterium* (Jumas-Bilak *et al.*, 1998b) and *Ochrobactrum anthropi* (Jumas-Bilak *et al.*, 1998b). In the case of *Rhizobium*, three or four replicons, depending on the species, have been found and were considered as one chromosome and two or three plasmids. However although rRNA probes only hybridized with the largest replicon of the strains tested (Jumas-Bilak *et al.*, 1998b), genes essential for a good functioning of the bacterial cell, such as the multiple *groEL* chaperonin-encoding genes, have been found in the three replicons of *R. meliloti*. This has led to speculation that the genome of *Rhizobium* spp. consists of multiple chromosomes (Rusanganwa and Gupta, 1993).

The genus *Brucella* has been found to be closely related to members of the α-2 subgroup of the class *Proteobacteria* (De Ley *et al.*, 1987; Moreno *et al.*, 1990; Yanagi and Yamasato, 1993). Interestingly most of the bacteria containing more than one chromosome belong to the α-2 subclass including several microorganisms living in close association with animal (e.g., *Brucella*, *Ochrobactrum*) or plant (e.g., *Rhizobium*, *Agrobacterium*) eukaryotic cells. However the existance of a relationship between possessing a complex genomic organization and a common phylogeny is considered improbable, since this genome arrangement is not always shared by all the members of the same genus (e.g., *Rhodobacter* genus) or even by all the strains of the same species (e.g. *B. suis*) (Jumas-Bilak *et al.*, 1998b). It has been hypothesized, however, that there are structures in or functions of the genome of the α-2 subgroup of the class *Proteobacteria* that favour the presence of a multiple chromosome organization (Jumas-Bilak *et al.*, 1998b). In *Brucella* it has been shown that the presence of two chromosomes can be explained by rearrangements at the chromosomal regions containing the three rRNA operons (Jumas-Bilak *et al.*, 1998a) that, in *B. melitensis*, have been shown to be identical over a 6,271 bp region (Bricker, 2000).

The recent sequencing of the whole genomes of *B. melitensis* 16M (biovar 1) and *B. suis* 1330 (biovar 1) has allowed the determination of the exact sizes of the two chromosomes of each strain. The genome of *B. melitensis* 16M consists of two circular chromosomes of 2,117,144 bp (Chr I) and 1,177,787 bp (Chr II) (DelVecchio *et al.*, 2002) while *B. suis* 1330 contains two circular chromosomes of 2,107,792 bp and 1,207,381 bp, respectively (Paulsen *et al.*, 2002). Comparison of the two *Brucella* genomes reveals an extensive similarity. However, thirty-three DNA regions greater than 100 bp specific to either *B. melitensis* or *B. suis* have been identified (Paulsen *et al.*, 2002). These regions might account for the different pathogenicity and host preferences of these two *Brucella* species.

3. Insertion Sequences

A source of DNA polymorphism is provided by the distribution of insertion sequences (ISs) along the genome. An insertion sequence, named IS*711* or IS*6501*, has been

identified in the genus *Brucella* (Halling *et al.*, 1993; Ouahrani *et al.*, 1993). This IS was first detected in *B. ovis* strains where a copy was identified downstream of the gene coding for the BCSP31 protein (Halling and Zehr, 1990), an immunogenic protein detected in all the *Brucella* species with the exception of *B. ovis* (Bricker *et al.*, 1988). A *B. ovis* IS*711* probe hybridized with at least 24 *Hind*III fragments on the DNA of the *B. ovis* reference strain and with at least 6 *Hind*III fragments on the *B. abortus* reference strain DNA (Halling and Zehr, 1990). Moreover, different hybridization patterns were observed with the IS*711* probe depending on the *B. ovis* strain (Halling and Zehr, 1990). The IS*711* was also used as a probe in another study using EcoRI-digested DNA from all of the *Brucella* species and biovars, with the exception of *B. neotomae* DNA. Different hybridization patterns were obtained with each *Brucella* species and biovar, with variation found even within species, as found with *B. ovis* (Ouahrani *et al.*, 1993). The IS*711* copy number was determined to be 7 to 10 in *B. melitensis*, 6 to 8 in *B. abortus*, *B. suis* and *B. canis*, and more than 28 in *B. ovis* (Ouahrani *et al.*, 1993). The high variability obtained using hybridization with the IS*711* probe in the *Brucella* strains prevented use of this method for assignment of a particular strain to a species or biovar (Ouahrani *et al.*, 1993). In addition, this demonstrates the ability of mobile DNA elements, such as IS, to introduce important levels of DNA polymorphism in the bacterial genome.

Several PCR tests that take advantage of DNA polymorphism due to the IS*711* distribution in the chromosome have been developed. These can be used for the differentiation of the *Brucella* species and biovars. Four IS*711* units located at a specific locus in *B. ovis*, *B. suis* biovar 1, the three biovars of *B. melitensis* and the biovars 1, 2 and 4 of *B. abortus*, respectively, have been identified (Bricker and Halling, 1994). Sequencing of the DNA region downstream the four IS*711* units allowed the selection of specific primers for PCR hybridizing at different distances from each IS*711* copy. A cocktail of the four specific primers and a fifth primer hybridizing inside the IS*711* element were used to amplify PCR products of different sizes in the four *Brucella* groups (including the *Brucella* species and biovars typically isolated from cattle in the USA). Although the other *Brucella* species or biovars also contain copies of IS*711*, no fragments were PCR-amplified using these primers since their IS*711* units were not located in any of the loci used for the design of the PCR primers (Bricker and Halling, 1994). The primer cocktail was later modified by adding three new primers able to identify the *B. abortus* S19 and *B. abortus* RB51 vaccine strains (Bricker and Halling, 1995). It was found the *B. abortus* RB51 and its parental strain *B. abortus* 2308 possess two tandem copies of IS*711*, oriented in the same direction, in a specific locus. This fact allowed the identification of a primer inside the second IS*711* copy that together with the IS*711* primer included in the previous five primer cocktail was used to amplify a 364-bp fragment in *B. abortus* RB51 and 2308. This fragment was not observed in the other *Brucella* strains (Bricker and Halling, 1995). For identification of *B. abortus* S19, two additional primers were designed. These hybridized to the *ery* locus with one annealing to a region of the *ery* locus common to all the *Brucella* strains and the second annealing to a region of the *ery* locus that is deleted only in *B. abortus* S19 (Sangari *et al.*, 1994). Thus this two primer set will amplify a 178-bp fragment in all *Brucella* strains except in the *B. abortus* S19 vaccine strain (Bricker and Halling, 1995). Variability was also found between the *Brucella* species and biovars when PCR was performed with a primer

hybridizing to the IS*711* sequence and a second one chosen arbitrarily (Ouahrani-Bettache *et al.*, 1996). Differentiation was possible at the species level but some of the biovars could not be distinguished (Ouahrani-Bettache *et al.*, 1996).

An IS*711*-based RNA mismatch cleavage technique has also been used for molecular typing of the genus *Brucella* (Bricker, 1999). The DNA of an IS*711* element that is common to all the *Brucella* species and biovars was amplified by nested PCR. The primary PCR reaction was performed using primers hybridizing to both sides of the selected IS*711* copy. In the second reaction, the nested primers contained either the T7 or SP6 promoter sites adjacent to the borders of the IS element. The resulting nested PCR product of each *Brucella* species and biovar was transcribed using the SP6 RNA polymerase which transcribed the sense strand of the IS. Each sense strand was hybridized with the antisense strand of the *B. ovis* IS*711* that had been transcribed by the T7 RNA polymerase. The hybridization samples were digested with RNase and electrophoresed through an agarose gel to separate the DNA fragments generated by RNase cleavage at point mutations. This technique allowed the differentiation of all of the *Brucella* species, the three *B. melitensis* biovars and the five biovars of *B. suis*. Moreover the *B. abortus* biovars were subtyped into three groups: biovars 1, 2 and 4; biovars 3, 5 and 9; and biovar 6 (Bricker, 1999). However only one strain of each *Brucella* species and biovar was used in this work (Bricker, 1999) and further studies, including a wider number of strains, should be performed to validate these findings. Some difficulties were occasionally found due to a high background smear that was problematic when comparing nearly identical fingerprints and it was proposed that other useful target loci should be identified (Bricker, 1999).

Two short repeated palindromic DNA elements, with similar organization, have been detected in the genus *Brucella* by their occasional association with IS*711*. In *B. abortus* these elements, which have been designated Bru-RS1 and Bru-RS2, are 103- and 105-bp long respectively Each *Brucella* species contains at least 36 copies of these in the genome (Halling and Bricker, 1994; see also Chapter 5). Hybridization of HindIII-digested DNA with a probe designed from these repeated elements revealed variability in the genomic distribution of these elements providing a specific fingerprint for each *Brucella* species reference strain (Halling and Bricker, 1994).

IS*711* is also the basis of HOOF-Prints (Hypervariable Octameric Oligonucleotide Finger-Prints), another recently described DNA technique for typing of *Brucella* strains (Bricker *et al.*, 2003; see also Chapter 2). While searching the shotgun sequences of *Brucella abortus* 9–941 (biovar 1) for a subsequence of IS*711*, the authors found tandem repeats of an octameric sequence (AGGGCAGT) present at nine chromosomal loci that contained at least two copies of the repeated element. The nine loci were also identified in the sequenced genomes of *B. melitensis* and *B. suis*, but differences in the number of copies of the octameric repeat were found between species. PCR assays, using primers designed to anneal to the sequences immediatly upstream and downstream of each locus, were developed to determine the number of repeat units present in the nine loci of *Brucella* strains representative of all the six classical *Brucella* species and their biovars. The number of repeats at each locus was deduced from the size of the amplified DNA fragment. The technique differentiated the type strains of all *Brucella* species and biovars, from unrelated *B. abortus* biovar 1 field isolates in cattle, and from *B. abortus* strains isolated from bison and elk (Bricker *et al.*, 2003). Isolates from the same herd or

from short-term *in vitro* passage showed little or no variability in fingerprint pattern. Thus HOOF-Prints could be extremely useful for analysis of the epidemiology of brucellosis. However, prior identification of the bacteria should be performed using current PCR and bacteriological tests as no species- nor biovar-specific alleles were found (Bricker *et al.,* 2003).

DNA polymorphism involving IS*711* has been also studied in *Brucella* strains isolated from marine mammals. Southern blot hybridization of an IS*711* probe with *Eco*RI-digested DNA from a minke whale *Brucella* isolate was performed by Clavareau *et al.* (1998). The number of bands developed (higher than 25) was close to the number of bands detected in *B. ovis* but high polymorphism was revealed according to the band distribution profile of both strains (Clavareau *et al.,* 1998). A more extensive study analyzed the distribution of IS*711* in 23 *Brucella* strains isolated from marine mammals (Bricker *et al.,* 2000). More than 25 copies of the element were found in each strain. The Southern blot hybridization profiles of the 23 isolates were quite similar although 10 different fingerprints, that tended to group by marine host species, were found. The number of IS*711* copies was significantly higher in the marine mammal isolates than in the six classical terrestrial *Brucella* species, with the exception of *B. ovis*. However, the fingerprint pattern of marine mammal isolates differed extensively from that of *B. ovis*. Based on these results, it was postulated that several new *Brucella* species exist within the marine mammal isolates (Bricker *et al.,* 2000). A specific DNA marker, involving an IS*711* element, has been found for the marine mammal *Brucella* strains. This allows their differentiation from the six classical terrestrial mammal *Brucella* species (Cloeckaert *et al.* 2000a). A copy of IS*711* was detected downstream of the *bp26* gene, adjacent to a Bru-RS1 element in all of the 34 marine mammal *Brucella* isolates studied. This was absent in all of the 100 terrestrial mammal isolates analyzed, representative of the six classical species and their biovars (Cloeckaert *et al.* 2000a). More recently this specific marker was also used to identify the first human cases of brucellosis, especially neurobrucellosis cases, that were caused by marine mammal *Brucella* spp. (Sohn *et al.,* 2003).

Furthermore development of a method called infrequent restriction site-PCR (IRS-PCR, see also Chapter 2), taking into account the higher number of IS*711* elements in the genome of marine mammal isolates compared to terrestrial mammal *Brucella* species, allowed the classification of marine mammal isolates into two distinct clusters of strains that were also distinct from the clusters representing the current terrestrial mammal *Brucella* species (Cloeckaert *et al.,* 2003). These two clusters of strains correlated well with the host from which they had been isolated i.e. a cluster of cetacean isolates and a cluster of pinneped isolates. Therefore IRS-PCR confirmed the previous classification of marine mammal isolates, based on DNA polymorphism at the *omp*2 locus and their preferential host, into two new *Brucella* species, i.e. *B. cetaceae* for cetacean isolates and *B. pinnipediae* for pinniped isolates (Cloeckaert *et al.,* 2001). Four specific DNA fragments (named I, II, III, and IV) generated by IRS-PCR were sequenced to develop direct PCR identification tests. Fragment I was specific for pinniped isolates. Its nucleotide sequence did not reveal any homology with the nucleotide sequences of the recently released genomes of *B. melitensis* (DelVecchio *et al.,* 2002) or *B. suis* (Paulsen *et al.,* 2002) nor with other nucleotide sequences from databases. This sequence could thus be part of a genetic island specific of *B. pinnipediae*. Sequencing of the genomes of

B. melitensis and *B. suis* has revealed the existence of such genetic islands unique to both species (DelVecchio *et al.,* 2002; Paulsen *et al.,* 2002). The three other fragments were specific of cetacean isolates and contained portions of the IS*711* element. Fragment II consisted of the 5′ end of the D-α-hydroxybutyrate dehydrogenase gene, annotated as BMEI0268 in the *B. melitensis* 16M genome (GenBank accession number AE009614), followed downstream by part of an IS*711* element. Fragment III consisted of the cytochrome B561 gene, annotated as BMEII1073 in the *B. melitensis* 16M genome (GenBank accession number AE009739) with part of an IS*711* element located upstream of this gene. Adjacent to the IS*711,* a Bru-RS1 sequence was found; this was previously reported to be a hot spot for IS*711* insertion (Halling and Bricker, 1994). Fragment IV consisted of part of the penicillin-binding protein 1A gene, annotated as BMEI1351 in the *B. melitensis* 16M genome (GenBank accession number AE009573), followed by part of an IS*711* element. In fact in the latter case IS*711* interrupted the penicillin-binding protein 1A gene. it is possible that specific genetic islands also exist in *B. cetaceae* strains and this should also be investigated further. The specific PCRs developed could have several uses of diagnostic importance in the future, such as possibly tracing these marine mammal strains if they are transmitted to livestock or to humans (Sohn *et al.,* 2003).

4. DNA Polymorphism in Genes Encoding Surface Antigens

4.1. Genes Involved in the Biosynthesis of Lipopolysaccharide Antigens

B. melitensis, B. abortus, and *B. suis* strains may occur as either smooth (S) or rough (R) strains, expressing smooth lipopolysaccharide (S-LPS) containing O-chain, or rough lipopolysaccharide (R-LPS) lacking O-chain, as major surface antigen respectively. *B. ovis* and *B. canis* are two naturally R species thus expressing R-LPS as the major surface antigen. The antigenic determinants involved in serotyping of S strains with polyclonal sera are contained on the O-chain moiety of S-LPS. At present S *Brucella* strains are classified into three serotypes, i.e. A^+M^-, A^-M^+, and A^+M^+, according to slide agglutination with A and M monospecific polyclonal sera (Alton *et al.,* 1988). Serotyping is used to further classify S *Brucella* species into biovars. The antigenic profiles (A or M dominant) of the S *Brucella* strains are related to the structures of their respective O-chains (Meikle *et al.,* 1989).

Several genes involved in the biosynthesis of the *Brucella* O-chain have been identified and characterized (Allen *et al.,* 1998; Godfroid *et al.,* 1998; Vemulapalli *et al.,* 1999; 2000; Godfroid *et al.,* 2000; Monreal *et al.,* 2003). To date only seven genes of the *wbk* locus have been studied for DNA polymorphism among *Brucella* species and biovars (Cloeckaert *et al.,* 2000b; Godfroid *et al.,* 2000). The seven genes, i.e. *wbkA, gmd, per, wzm, wzt, wbkB,* and *wbkC,* code respectively for proteins homologous to mannosyltransferase, GDP-mannose 4,6 dehydratase, perosamine synthetase, ABC-type transporter (integral membrane protein), ABC-type transporter (ATPase domain), a hypothetical protein of unknown function, and a putative formyl transferase. The seven genes have a G+C content lower (around 48%) than that typical of *Brucella* spp. (58%) and thus may have been acquired from a species other than *Brucella*. The presence of IS

elements, termed IS*Bm1*, IS*Bm2*, and IS*Bm3*, at the *wbk* locus also supports the hypothesis that this locus was acquired by lateral gene transfer (Godfroid *et al.*, 2000). PCR-RFLP showed that the seven genes are highly conserved in *Brucella* species, including the naturally R species *B. ovis* and *B. canis*, suggesting that they may have been acquired before species differentiation. There were also no apparent deletions or insertions in the genes that could have explained the R characteristics of *B. ovis* and *B. canis* as well as those of R *B. abortus* and *B. melitensis* strains included in the study. Nevertheless, a few polymorphisms were observed and these consisted of the absence of or the addition of restriction sites. Such polymorphisms sometimes allowed differentiation at the species level (e.g. *B. ovis*) or at the biovar (e.g. some *B. suis* biovars) or strain level. Thus at present no molecular evidence has been provided to explain the R and S phenotype, and O-antigen A or M dominance, of *Brucella* species and biovars. Except in the case of the *B. abortus* vaccine strain RB51. The R phenotype of this strain was partly explained by disruption with an IS*711* element of the *wboA* gene, which encodes a putative glycosyltransferase, (Vemulapalli *et al.*, 1999, 2000). The *wboA* gene is not located at next to *wbk* on the chromosome. Sequencing of the *B. melitensis* 16M and *B. suis* 1330 has now revealed all chromosomal loci potentially involved in LPS biosynthesis. Futher analysis of these chromosomal loci for DNA polymorphism will help to understand LPS variations encountered in *Brucella* species and biovars.

4.2. Genes Coding for Outer Membrane Protein Antigens

In addition to LPS, the outer membrane of *Brucella* is mainly composed of the major (i.e. most abundant) outer membrane proteins (Omps). Interest in the *Brucella* Omps stemmed initially from their potential as protective antigens. This notion started in the early 1980s but since then interest in the major Omps has evolved (Cloeckaert *et al.*, 2002). Here, we present current knowledge about DNA polymorphism in the genes coding for the *Brucella* major Omps and its potential for the differentiation of *Brucella* species.

4.2.1. omp2a and omp2b

The genes encoding the major 36 to 38 kDa porin proteins, also referred as group 2 major Omps, of *B. abortus* strain 2308 (biovar 1) were the first major *omp* genes identified and reported by Ficht *et al.* (1988; 1989). Two closely related genes named *omp2a* and *omp2b* encode and potentially express the 36 to 38 kDa porin. The genes share about 85% DNA identity. They are separated by 900 bp on the chromosome and oriented in opposite directions. According to the respective nucleotide sequences, the *B. abortus* biovar 1 *omp2b* gene would encode a 36 kDa protein while *omp2a*, due to a deletion of 108 bp when compared to *omp2b*, would encode a protein of 33 kDa. However only the 36 kDa protein (encoded by *omp2b*) was detected which has led to the conclusion that the *omp2a* gene was not expressed, at least not in *B. abortus* biovar 1 (Ficht *et al.*, 1989). The 108 bp deletion in *omp2a* has also been detected in *B. abortus* biovars 2 and 4 (Ficht *et al.*, 1990; Cloeckaert *et al.*, 1995).

The gene arrangement at the *omp2* locus, studied by Southern blot hybridization of *omp2a* and *omp2b* specific probes with genomic DNA cut with different restriction enzymes to appeared to be conserved in type strains of all *Brucella* species (Ficht *et al.*,

1990). However, the *B. ovis* reference strain 63/ 290 had two genes closely related to *omp2a* instead of the one *omp2a* and one *omp2b* gene copy detected in the other *Brucella* species (Ficht *et al.*, 1990). The

Table 1. PCR-RFLP patterns of the genes coding for the major Omps in the six classical *Brucella* species and their biovars and in the two new *Brucella* species, grouping the marine mammal *Brucella* isolates, proposed recently

Brucella species[a]	biovar[a]	PCR-RFLP pattern of genes[b]:			
		omp2a[c]	*omp2b*[c]	*omp25*[c]	*omp31*[d]
*B. melitensis**	1	B, C	B, D, E	B	A
	2	B	E	B	A
	3	B	B, D, E	B	A
	R	B, C	B	B	A
*B. abortus**	2	A	A	A	–
	2	A	A	A	–
	3	B	B	A	–
	4*	A	B	A	–
	5	B	B	A	–
	6	B	B	A	–
	9	B	B	A	–
	R	A	A, C	A	–
*B. suis**	1*	D	B	A	A
	2*	E	F	A	B, C
	3	D	G	A	A
	4	D	G	A	A
	5*	F	B	A	A
*B. ovis**	–	G	H, I, J	C	D
*B. canis**	–	D	G	A	E, F
*B. neotomae**	–	H	K	A	A
*B. pinnipediae**	–	I	L, O, P	ND	ND
*B. cetaceae**	–	J, K	M, N	ND	ND

[a] *Brucella* species and biovars showing a specific PCR-RFLP overall pattern considering the four *omp* genes are marked with an asterisk. Several strains of each *Brucella* species and biovars were

studied. R, rough strains. ND, not determined
[b] The four genes were PCR amplified for each strain and digested with several restriction enzymes. Each capital letter reflects an overall pattern with all the restriction enzymes used for each gene. The same letter for an individual gene shows an identical restriction pattern but a same letter in two different genes do not indicate the same restriction pattern. Two or three different patterns have been found for several species or biovars depending on the strain tested.
[c] Patterns of *Brucella* strains described by Cloeckaert et al. in 1995 and 2001.
[d] Patterns of *Brucella* strains described by Vizcaíno et al. in 1997. *omp31* PCR-RFLP patterns of *B. abortus* strains are not shown as the gene is deleted in this species.

polymorphism of both porin genes has been further studied by PCR-RFLR This allowed the identification of 11 *omp2b* variants and 8 *omp2a* variants among *Brucella* strains isolated from terrestrial mammals (Ficht *et al.,* 1990; Cloeckaert *et al.,* 1995) (Table 1). PCR-RFLP showed (i) the diversity of both *omp2a* and *omp2b* among *B. melitensis* field strains; (ii) biovar-specific markers for three *B. suis* biovars; (iii) species-specific markers for *B. neotomae* and *B. ovis;* (iv) the intra-species diversity of *B. ovis* reference and field strains. In fact, the *omp2b* gene of *B. ovis* field strains appeared to contain more *omp2b*-specific motifs than that of reference strain 63/290 which was more similar to *omp2a* (Cloeckaert *et al.,* 1995). This observation was further confirmed by nucleotide sequencing which showed that the *B. ovis omp2b* from field strains actually consisted of an *omp2a* and *omp2b* chimeric gene (Paquet *et al.,* 2001).

The list of *omp2* variants have been recently extended and to this we can now include new ones encountered in isolates from marine mammals, where three new *omp2a* gene variants and five new *omp2b* gene variants have been identified (Cloeckaert *et al.,* 2001) (Table 1). Interestingly, PCR-RFLP and nucleotide sequencing showed that strains isolated from cetaceans, i.e. dolphin, porpoise, and whale marine mammal species, carry two similar (less than 10 bp divergence) *omp2b* gene copies instead of one *omp2a* and one *omp2b* gene copy or two similar *omp2a* gene copies reported in the currently recognized species (Cloeckaert *et al.,* 2001). The seal isolates were shown to carry one *omp2a* and one *omp2b* gene copy as encountered in isolates from terrestrial mammals. By PCR-RFLP of the *omp2b* gene, a specific marker was detected which could group the marine mammal *Brucella* isolates. Therefore, based on *omp2* DNA polymorphism and with respect to the current classification of brucellae according to the preferential host, two new species names were proposed for marine mammal *Brucella* isolates: *B. cetaceae* and *B. pinnipediae* for cetacean and pinniped isolates respectively (Cloeckaert *et al.,* 2001).

Thus, based on diversity of both genes, PCR-RFLP could distinguish between nearly all *Brucella* species, including the new ones proposed for marine mammal isolates, and some of their biovars. It could not be used to distinguish between *B. abortus* biovars 3, 5, 6, 9 and a number of strains of *B. melitensis* biovars 1 and 3, and between *B. canis* strains and *B. suis* biovars 3 and 4 (Cloeckaert *et al.,* 1995, 1996b; 2001; 2002) (Table 1).

DNA sequencing of the *omp2* loci of reference strains of the six *Brucella* species revealed variations in nucleotides at 183 positions in the 3,400 bp region representing 6% of the locus (Ficht *et al.,* 1996). The presence of a deletion of 138 nucleotides from the central region of the *omp2a* gene in *B. abortus* biovars 1, 2, and 4 was also confirmed. It was reported that the mechanism for removing this sequence may have involved the imperfect direct repeats of 26 bp to both deletion endpoints in the *omp2a* gene, which are

found in all *Brucella* taxa except *B. abortus* biovars 1, 2, and 4 (Ficht *et al.,* 1996). Specific oligonucleotide probes able to differentiate between the reference strains of the six classical *Brucella* species by hybridization with PCR-amplified *omp2a* fragments were designed (Ficht *et al.,* 1996).

The *omp2* polymorphism appears partly dependent on genetic conversion phenomena, leading to a homogenization of the two gene copy sequences (Ficht *et al.,* 1996; Vizcaíno *et al.,* 2000; Cloeckaert *et al.,* 2001; Paquet *et al.,* 2001; Cloeckaert *et al.,* 2002). We can consider the following scenario that may have occurred during evolution. The duplication of the porin gene, perhaps closely resembling that of *B. ovis* reference strain 63/290, resulted in the presence of two highly homologous genes oriented in opposite directions and separated by only 830 bp. Sequence divergences between both gene copies caused the appearance of distinct motifs, either typical of the *omp2a* (putative inactive) or *omp2b* (active) gene copy, at least detected at the *omp2* locus from strains isolated from terrestrial mammals. Nucleotide sequencing of some *omp2b* gene variants showed that these variants displayed a "mosaic" sequence regarding the presence of particular motifs, with some *omp2b* genes in several strains contained a typical *omp2a* motif in the middle of their sequence (Paquet *et al.,* 2001). This mosaic aspect of the *omp2* locus within brucellae could be explained by "gene conversion", i.e. the "correction" of a portion of the gene on the basis of another closely related sequence present elsewhere in the genome (Lewin, 1997). Supporting evidence for the occurrence of genetic conversion between the two versions of porin genes also comes from the study of *omp2* genes of the recent marine mammal *Brucella* isolates (Cloeckaert *et al.,* 2001). Few divergences (less than 10 bp) exist between the *omp2a* and *omp2b* gene copies from *Brucella* strains isolated from dolphins, porpoises, and a minke whale, although both copies, mostly similar to *omp2b,* contain specific motifs not encountered in terrestrial mammal *Brucella* isolates indicating that divergence occured at some point. Basically, these marine mammal isolates carry *omp2a* genes that are similar to *omp2b* of terrestrial mammal isolates, but both *omp2* genes in marine mammal isolates show a sequence typical of *omp2a* of terrestrial mammal isolates in their sequence, at the 3′ end between nucleotide positions 780 and 1080. The marine mammal isolate specific *omp2* motifs appear simultaneously in both *omp2* gene copies in the same strain and therefore it is most probable that the motif appeared in one of the genes which was subsequently used as template to convert the other gene. The lack of divergence observed between the two *omp2* gene copies in cetacean *Brucella* isolates could thus be partially explained by a high rate of genetic conversion which homogeneized their sequences. The seal isolates *omp2a* gene is noteworthy because it is similar to terrestrial mammal isolates *omp2a,* except for a small motif found at the 5′ end between nucleotide positions 150 and 240 which is typical of both *omp2a* and *omp2b* genes from most marine mammal *Brucella* isolates. The *omp2* locus from the seal isolates is thus intermediate in terms of sequence motifs between cetacean isolates and terrestrial mammal *Brucella* strains. The divergence between the *omp2a* and *omp2* gene copies in seal isolates involves about 92 nucleotide positions, much less than that found in *B. abortus, B. melitensis* or *B. suis* (around 130 nucleotide differences) and less than that found in *B. neotomae* (112 nucleotide differences). Seal isolates might thus represent an evolutionary link between cetacean *Brucella* isolates and terrestrial mammal *Brucella* species. The cetacean isolates might represent the "most evolved" *Brucella* species taking into consideration the high degree of homogeneization

between their *omp2* gene copies. From this evolutionary point of view it is interesting to note that marine mammals appeared on earth after terrestrial mammals and perhaps *Brucella* species may have coevolved with their particular host. Although these speculations on the origin of *omp2* gene diversity and the evolution of *Brucella* species are hypothetical, the genetic diversity observed certainly allows for an interesting classification of *Brucella* strains depending on their ecological niche i.e. strains isolated from cetaceans, which are permanently resident in the sea, presenting a very low level of divergence between the two *omp2* gene copies; while terrestrial mammal species presenting the highest level of divergence; and with isolates from seals, which live part of their time on land, presenting intermediate divergence.

4.2.2. omp25 *and* omp31

The *omp25* and *omp31* genes code for homologous proteins (34% identity) classified within the group 3 of major Omps (de Wergifosse *et al.,* 1995; Vizcaíno *et al.,* 1996). Their DNA polymorphism within the genus *Brucella* proved also to be useful for the molecular differentiation of *Brucella* species and biovars.

PCR-RFLP analysis of *omp25* suggested that this gene is highly conserved in *Brucella* species, biovars and strains (Cloeckaert *et al.,* 1995). Nevertheless, two species-specific markers were detected (Table 1) i.e. (i) the absence of the *Eco*RV site in *B. melitensis* strains and (ii) a short deletion at the 3′ end of the gene in *B. ovis* strains. The high conservation of *omp25* was further confirmed by nucleotide sequence determination of *omp25* of the six recognized *Brucella* species (reference strains) (Cloeckaert *et al.,* 1996b; 1996c). Variations in nucleotides were observed at only 12 positions of the 639 bp long *omp25*, which represents 1.9% of the gene. DNA sequencing confirmed also the presence of a short deletion of 36 nucleotides at the 3′ end of the *omp25* gene of *B. ovis*. This corresponds to a region containing two 8 bp direct repeats and two 4 bp inverted repeats found in other *Brucella* species. This region could have been involved in the genesis of the deletion by a mechanism called “slipped mispairing” (Allgood and Silhavy, 1988). The 4 bp inverted repeats were found within the deleted segment and close to the 8 bp direct repeats and could therefore be responsible for transient hairpin formation, bringing the deletion endpoints into close proximity at the moment of deletion formation. The 36 bp deletion caused an antigenic shift as shown by the differences in binding patterns of anti-Omp25 MAbs in ELISA at the surface of *E. coli* cells harboring the entire *B. melitensis* or the truncated *B. ovis omp25* gene and of cells of *B. ovis* and other *Brucella* species (Cloeckaert *et al.,* 1996b; 1996c).

Polymorphism at the *omp31* locus in *Brucella* spp. has been studied by Southern blot hybridization of *Hind*III digested genomic DNA and by PCR-RFLP analysis (Vizcaíno *et al.,* 1997). Southern blot hybridization showed that the *omp31* gene is present in all *Brucella* species except *B. abortus*. More recently, it has been shown that the reference strains of all the *B. abortus* biovars have a 25 kb deletion in its chromosome comprising *omp31* and a large gene cluster related to the synthesis of a polysaccharide (Vizcaíno *et al.,* 1999; 2001a). Two short direct repeats of 4 bp, detected in the *B. melitensis* 16M DNA flanking both sides of the fragment deleted from *B. abortus*, might have been involved in the process of deletion formation by a strand slippage mechanism during replication. Most of the proteins that would be encoded by the 22 genes deleted in *B.*

abortus show significant homology with proteins involved in the biosynthesis of polysaccharides from other bacteria, suggesting that they might be involved in the synthesis of a polysaccharide. Due to deletion of the genes, this polysaccharide can not be synthesized in *B. abortus* and represents a polysaccharide not yet identified in the genus *Brucella,* since all of the known polysaccharides are synthesized in all S *Brucella* species. Although the hypothetical polysaccharide might not be synthesized, and the genes detected in the 25 kb DNA fragment absent in *B. abortus* might be a remnant of the common ancestor of the alpha-2 subdivision of the class Proteobacteria, the impact of this deletion in pathogenesis and host preference should be further studied (Vizcaíno *et al.,* 2001a).

PCR-RFLP analysis of the *omp31* gene revealed additional species-specific markers for *B. canis* and *B. ovis* (Vizcaíno *et al.,* 1997) (Table 1). In addition, a specific restriction pattern was observed for *B. suis* biovar 2 (Table 1). By use of anti-Omp31 MAbs it was shown that Omp31 is produced in all *Brucella* species except, of course, in *B. abortus* (Vizcaíno *et al.,* 1997; 2001a). The *omp31* genes of all of the reference strains of *Brucella* species and biovars have been recently sequenced (Vizcaíno *et al.,* 2001b). Although the *omp31* genes appeared to be highly conserved in the genus *Brucella,* nine nucleotide substitutions were detected in the gene of *B. ovis* compared to that of *B. melitensis,* which result in seven amino acid differences between the encoded Omp31 proteins. As shown by differential binding properties of MAbs to the two *Brucella* species, these nucleotide substitutions were shown to result in different antigenic properties of Omp31. The antigenic differences were also evidenced when sera from *B. ovis* infected rams were tested by Western blotting with the recombinant *B. melitensis* or *B. ovis* Omp31 proteins (Vizcaíno *et al.,* 2001b).

Altogether, the PCR-RFLP patterns for the *Brucella* spp. genes coding for the major Omps enabled the identification of DNA markers specific for each of the six classical *Brucella* species (Vizcaíno *et al.,* 2000; Cloeckaert *et al.,* 2002) and for the two new proposed *Brucella* species that group the marine mammal *Brucella* isolates (Cloeckaert *et al.,* 2001; 2002) (Table 1). Moreover, four markers have been found at the biovar level (Table 1). Thus, *B. abortus* biovar 4 and *B. suis* biovars 1, 2 and 5 can be differentiated from each other and from the other *Brucella* species and biovars according to their PCR-RFLP profiles. The other *B. abortus* biovars can also be classified into two groups that separate *B. abortus* biovars 1 and 2 from *B. abortus* biovars 3, 5, 6 and 9 (Vizcaíno *et al.,* 2000) (Table 1).

The recent sequencing of the entire *B. melitensis* 16M and *B. suis* 1330 genomes has allowed the identification of five genes potentially encoding Omps with a high level of homology with Omp25 and Omp31. On the basis of amino acid sequence identity percentages, these proteins have been named Omp25b, Omp25c, Omp25d (about 40% of amino acid sequence identity with Omp25 and about 60% between eachother), Omp31b (67% amino acid sequence identity with Omp31) and Omp22 (Salhi *et al.,* 2003). The latter Omp, which has been also called Omp3b (Guzmán-Verri *et al.,* 2002), showed a lower calculated molecular weight and lower amino acid sequence identity levels with Omp25 and Omp31 (about 25%) than the four other new Omps (Salhi *et al.,* 2003). Taking into account the differences found in *omp25* and *omp31* within the genus *Brucella,* the study of the DNA polymorphism in these five new homologous genes might

also provide DNA markers for the differentiation between the *Brucella* species and their biovars.

4.2.3. Other Omp genes

The genes coding for three minor Omps, *omp10, omp16* and *omp19,* have also been used to find DNA polymorphisms in the genus *Brucella* (Verger *et al.,* 1998). The study was performed by using the three *omp* genes as probes for Southern blot hybridization with *Hind*III-digested DNA from 92 *Brucella* strains i.e. representatives of the six species and their biovars. Poor polymorphism was found with the three genes. The *omp19* probe gave the same pattern with the 18 reference strains of the *Brucella* species and biovars and was not tested with the remaining strains (Verger *et al.,* 1998). The gene *omp10* provided three different patterns depending on the strain but the bands revealed by Southern blot showed a similar size making difficult the differentiation. Finally, *omp16* provided only two patterns, one of them specific for *B. suis* biovar 2 (Verger *et al.,* 1998).

5. DNA Polymorphism in Genes Encoding Cytoplasmic and Periplasmic Protein Antigens

A few DNA polymorphism studies have been conducted on genes coding for cytoplasmic or periplasmic proteins that were of interest as diagnostic or protective antigens. Thus, *p39* and *br25* genes were used as probes for Southern blot hybridization with *Hind*III-digested DNA (Verger *et al.,* 1998). No polymorphism was revealed by the *p39* probe, in spite of the fact that expression of the protein was not detected in *B. abortus* biovars 5, 6 and 9, *B. ovis and B. neotomae* (Denoel *et al.,* 1997). The *br25* probe provided three different patterns that distinguish *B. ovis* and *B. melitensis* from each other and from the other *Brucella* species (Verger *et al.,* 1998). A gene located downstream of *p39* and identified as *39ugpa* revealed, by the same technique, two hybridization patterns, one of them specific for *B. ovis* strains (Verger *et al.,* 1998).

The *Brucella* gene coding for the cytoplasmic DnaK protein gave a DNA marker for *B. melitensis* strains by Southern blot hybridization with *Eco*RV-digested DNA or by PCR-RFLP (Cloeckaert *et al.,* 1996a). DNA polymorphism of the *bp26* gene, coding for a periplasmic protein of diagnostic importance, was assessed by nucleotide sequencing of the gene in reference strains of *B. abortus, B. melitensis, B. suis,* and *B. ovis* (Seco-Mediavilla *et al.,* 2003). Only minor differences were found consisting of only one nucleotide substitution in *B. suis* and another one in *B. ovis*.

Thus, in contrast to some *omp* genes, genes coding for internal proteins appear rather conserved in *Brucella* species and biovars. The greater diversity in surface antigens among *Brucella* species may appear logical since they are the most likely to participate in host interaction and preferentialism.

6. Random DNA Polymorphism

Evidence of the variability in the *Brucella* genome has also been found in several studies based on non-characterized DNA fragments. Thus random *Brucella* DNA fragments were cloned and used as probes for hybridization with BamHI-digested DNA from 112 *Brucella* strains, including the reference and field strains of all of the *Brucella* species and biovars (Grimont *et al.,* 1992). The combination of the fingerprints obtained with seven probes provided DNA markers for each of the six *Brucella* species and another one was able to identify *B. suis* biovar 2. In several biovars different, strain dependent, hybridization patterns were observed with some probes. In some cases a specific fingerprint was obtained for strains with atypical phenotypic characteristics and particular geographic origin (i.e., two *B. melitensis* biovar 1 strains with atypical antibiotic and dye characteristics isolated in Israel, four biochemically atypical *B. abortus* biovar 3 strains isolated from African cattle and a *B. suis* biovar 3 strain isolated from a wild rodent in Australia) (Grimont *et al.,* 1992).

Random amplified polymorphic DNA (RAPD) technique has proved to be a powerful method to demonstrate DNA variability among the strains of the genus *Brucella*. This technique could serve as an interesting epidemiological tool provided that one takes into account that a high standardization is needed to obtain reproducible results. RAPD markers able to differentiate the 25 *Brucella* strains tested have been found with 5 arbitrarily chosen primers, used alone or in pairs (Fekete *et al.,* 1992). Similarity of RAPD profiles was examined for the different biovars of *B. melitensis, B. abortus* and *B. suis*. Although the results must be interpreted cautiously, because of the limited number of strains of each biovar that were tested, it was found that biovars within a species are more closely related to each other than to the biovars of the other species (Fekete *et al.,* 1992) and the usefulness of the technique in establishing genetic relationships was proposed. Tcherneva *et al.* in 2000 also demonstrated the usefulness of RAPD for epidemiological studies in brucellosis, although they also stated the need for highly accurate conditions to ensure the reproductibility of results.

Two extragenic repetitive elements: repetitive extragenic palindromic (REP) sequences and enterobacterial repetitive intergenic consensus (ERIC) sequences, have been detected in a wide number of gram-negative and some gram-positive bacteria. PCR amplification of these elements (REP-PCR and ERIC-PCR) has been used to find DNA polymorphism in the *Brucella* spp. genome (Mercier *et al.,* 1996; Tcherneva *et al.,* 1996). A combination of both REP-PCR and ERIC-PCR identified most of the *Brucella* isolates tested, including all of the *Brucella* species and biovar reference strains and field strains of *B. melitensis* biovar 3 and *B. abortus* biovars 3 and 9. However it could not be used to identify some strains of *B. abortus* biovars 3 and 9 (Mercier *et al.,* 1996). With the primers and amplification conditions used, the authors found ERIC-PCR more discriminative than REP-PCR and reproducible results were obtained in different amplifications using either the same DNA sample or a different DNA preparations from the same strain (Mercier *et al.,* 1996). Different results were described by Tcherneva *et al.* in 1996 with the same techniques (Tcherneva *et al.,* 1996). In this case, less polymorphism was detected among the genus *Brucella* and the authors found that REP-

PCR differentiated, at least at the species level, and was more discriminative than ERIC-PCR (which differentiated at the genus level and occasionally discriminated between individual strains). In both studies, REP-PCR and ERIC-PCR have revealed important levels of DNA polymorphism in the highly homogeneous genus *Brucella*. However, the different results obtained in both studies emphasize the fact that PCR techniques using non-specific primers must be carefully optimized to obtain reproducible results. The distinct amplification parameters and a slight difference in the sequence of one of the ERIC-PCR primers, might be the reason for the different results obtained in these two studies.

7. Conclusions and Future Directions

In recent years, an important controversy has developed concerning the taxonomy of the genus *Brucella*. Doubts about the classical six species definition of the genus, mainly based on differences in pathogenicity and host preference between species, arose from studies of DNA-DNA hybridization showing a high degree of DNA homology between the *Brucella* species. Accordingly, a monospecific genus with the sole species *B. melitensis*, the other classical species being considered as biovars of *B. melitensis*, was proposed, which has been accepted in some data banks and culture collections. However, most of the laboratories working on brucellosis research still continue to use the six species classification for practical reasons. Therefore, an unified and well-reasoned criterion should be adopted to avoid confusion.

In spite of the high degree of DNA homology between the brucellae, it must be remembered that DNA-DNA hybridization only superficially assesses genome similarities and differences (van Belkum *et al.*, 2001). On the other hand, the results from the many studies concerning DNA polymorphism within the genus *Brucella* (reviewed above) provide supporting information that justifies the classification of the genus *Brucella* according to the host preferentialism criterion. Moreover the *Brucella* strains recently isolated from marine mammals also show phenotypic and DNA characteristics that clearly differentiate them from the terrestrial mammal *Brucella* species. These marine mammal *Brucella* strains may even be differentiated, at the DNA level, into two groups that correlate to the marine mammal host (cetacean or pinniped) and two new species, *B. cetaceae* and *B. pinnipediae*, have been proposed to enlarge the genus *Brucella*. Additionally, a 25 Kb DNA fragment has been shown to be absent from the genome of all *B. abortus* biovars, but present in the other classical *Brucella* species, and the recent sequencing of the entire genomes of *B. melitensis* 16M and *B. suis* 1330 has revealed the existence of thirty-three DNA regions greater than 100 bp specific to each strain. The discovery of these *B. melitensis* 16M or *B. suis* 1330 specific DNA regions suggests that other specific DNA regions may also be present, or absent, in the other brucellae. Whether these regions are only strain specific, species-specific or whether they are present in some other *Brucella* species must now be investigated. Study of these regions in a high number of strains representative of all the *Brucella* species and biovars would provide new information about the DNA polymorphism within the genus *Brucella*. Moreover, the identification of species-specific DNA regions that might be involved in the differences in pathogenicity and host preference observed between the *Brucella*

species would also support the classification of the brucellae on the basis of host preferentialism.

Even minor DNA differences between the brucellae, mainly in genes coding for outer membrane molecules, might also contribute to explain their differences in the interaction with the animal host. As reviewed above, minor nucleotide differences in *omp25* and *omp31* of *B. ovis*, when compared to the respective genes of the other *Brucella* species, lead to antigenic differences in the encoded proteins. These Omp antigenic differences between *Brucella* species suggest that immunogenic differences, with a protential role in the interaction with the animal host, might also exist between species. Additionally, Omp25 has been shown to be involved in virulence (Edmonds *et al.*, 2001; 2002a; 2002b) and Omp31 is underexpressed, when compared to *B. melitensis* virulent strain 16M, in *B. melitensis* attenuated vaccine strain Rev.1 (Eschenbrenner *et al.*, 2002). Five other Omps homologous to Omp25 and Omp31, all of which have been detected in at least one *Brucella* species, have been recently identified. Omp25 and one of these new Omps, Omp22 (also named Omp3b) (Guzmán-Verri *et al.*, 2002; Salhi *et al.*, 2003), were reported to be absent in mutants lacking the two-component regulatory system BvrR/BvrS (Guzmán-Verri *et al.*, 2002) shown to be essential for virulence of *B. abortus* (reviewed by López-Goñi *et al.*, 2002; see also Chapter 10). Accordingly, the study of DNA polymorphism in these new *omp* genes would be of interest, not only searching for species- or biovar- specific markers, but also because differences in these surface protein genes between the brucellae might be related to the differences in the interaction with the animal host. Study of polymorphism in the genes involved in the synthesis of LPS would also provide valuable information in this sense.

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Chapter 2
Molecular Diagnostics of Animal Brucellosis: A Review of PCR-Based Assays and Approaches

Betsy J.Bricker*

From: *Brucella:* Molecular and Cellular Biology. Edited by: Ignacio López-Goñi and Ignacio Moriyón

Abstract

Most of the countries that are faced with the economic losses and public health issues caused by animal brucellosis have governmental programs for the eradication or control of the disease. Accurate diagnostic procedures are critical for the success of these programs. This review examines PCR-based diagnostics for animal brucellosis in three contexts: 1) for screening large populations of livestock to identify infected herds, 2) for confirming the presence of infection in herds and identifying the species involved so that appropriate regulatory actions are taken, and 3) for identifying epizootic strains to help epidemiologists trace-back infections to their sources. Emphasis is placed on the diversity of methods that have been developed thus far, how the tests are used, and which tests are currently being tested and used in the field.

1. Introduction

Brucellosis is a disease caused by gram-negative bacteria of the genus *Brucella*, a member of the α-Proteobacteria class. Various species of *Brucella* cause disease in livestock with world-wide economic impact. Currently, six species of *Brucella* are formally recognized (Corbel and Brinley-Morgan, 1984): *Brucella melitensis*, infecting goats and sheep; *B. abortus*, infecting cattle and bison; *B. suis*, infecting primarily swine, but also hares, rodents and reindeer/caribou; *B. ovis*, infecting sheep; *B. canis*, infecting dogs; and *B. neotomae*, infecting wood rats. However, not all *Brucella* species are strictly host specific and some species will cross host-species barriers naturally. Within the past

decade, discovery of *Brucella* bacteria in marine mammals has led to the proposal of two additional species (Cloeckaert, *et al.,* 2001): *B. cetaceae,* infecting cetaceans, and *B. pinnipediae,* infecting pinnipeds. Species of *Brucella* are typically pathogenic in their natural hosts, resulting in reproductive failure and/or infertility in the infected animal (Alton *et al.,* 1975; Foster *et al.,* 2002).

Definitive identification of *Brucella* is key to the success of surveillance and eradication efforts. For brucellosis, two diagnostic approaches are currently used: 1) the serological screening of potential hosts as an indirect indicator of infection (e.g. agglutination tests), and 2) the isolation and identification of the pathogen

* For correspondence email bbricker@nadc.ars.usda.gov

from potentially infected hosts. Since a serologically positive response can occur in convalescent hosts or from antigenically cross-reactive bacteria, characterization of cultured bacteria remains the “gold standard” upon which official regulatory action is typically based.

For *Brucella*, the identification of cultured organisms relies on an array of approximately 25 phenotypic traits, including serological typing for the A and M antigens, phage typing, requirement for elevated CO_2 atmosphere, and metabolic processes. However, there are some problems associated with these tests including the following: 1) time: it takes approximately 10–14 days to culture the bacteria and complete the tests; 2) biosafety: live organisms are required for testing, exposing the laboratory personnel to possible infection; 3) training: the differential tests used are complex and require skilled technicians; 4) limited subtypes: epidemiologists rely on unique strain markers for trace-back, but for *Brucella* species only a few subtypes are defined, and often a single subtype will dominate a geographic area; and 5) ambiguous results: identification depends on the characterization of numerous traits, many of them defined in relative terms such as the rate of urease activity (Alton *et al.,* 1975). Atypical strains can exhibit a collection of traits that do not fit the description of any one species or subtype.

As with any disease, control of brucellosis would benefit from new and improved diagnostic tests that address some of the problems encountered with current methods. Recently, concern about intentional release of pathogens by bioterrorists and agri-terrorists has fostered an additional sense of urgency for faster and more precise methods (Firmani and Broussard, 2003). Diagnostic procedures need to address several features associated with the establishment and spread of the infectious agent. Rarely can a single test provide all the necessary information. Therefore, several assays may be needed in an eradication or surveillance program. The first aspect to consider is a screening test that could be used to detect a small number of affected animals within a larger population. In this capacity, sensitivity is slightly more important than specificity since it is better to examine a few false-positive reactors than to risk missing some infected animals. Ideally, an effective screening test should be quick, simple, inexpensive, sensitive, and robust. It should be impervious to uncontrollable conditions inherent in the sample, in the environment, or in the testing parameters. Specificity should also be designed into screening assays. The initial screening test should, at a minimum, definitively identify the genus involved. In the case of brucellosis, this role is currently performed by serological

tests that are genus but not species specific. However, issues regarding specificity and time are prompting investigators to consider alternatives such as PCR.

The second level of the diagnostic process is definitive confirmation of the disease agent. In most countries, the government response to brucellosis is dictated by policies specific for the species of *Brucella* involved. Therefore, the confirmatory diagnostic test must be able to differentiate *Brucella* strains at the species level so that the correct action is taken. It is also necessary to differentiate vaccine strains from wild type field strains. PCR technology is well equipped to meet these needs and several promising PCR tests have already been developed.

In the third phase of the diagnostic process, the specific field strain needs to be characterised for epidemiological application. Once an outbreak has been identified and confirmed, the causative agent must be traced back to its original source and all potential opportunities for transmission between the point of origin and the epizootic location have to be identified to prevent additional spread of the disease. The epidemiology effort can be facilitated by methods which can positively link the epizootic strain to its point of origin via type-specific markers. For years, finding unique, strain-specific markers has been the most challenging aspect of *Brucella* diagnostics. The remarkable genetic homogeneity within the genus (Verger *et al.,* 1985; 1987; Gandara *et al.,* 2001) has made it difficult to find polymorphic genetic targets. Recently, progress in this facet of diagnostics has been advanced through PCR-based technologies.

PCR technology is transforming the diagnostic field. PCR-based assays are sensitive, specific, quick to complete, inexpensive to perform, simple to design and carry out, and often can be automated to accommodate minimal labour and/or high throughput. Since this revolutionary technique was first reported (Mullis and Faloona, 1987), many quality PCR tests for *Brucella* have been developed. The following is a review of the major assays that have been published, what each assay was designed to identify, and the advantages and disadvantages of each method.

2. Screening Livestock for Brucellosis

2.1. Simple Genus-specific PCR Assays Potentially Amenable for Preliminary Screening

The minimal requirements of a diagnostic test for large scale screening of animals for brucellosis include: 1) simple and easy to perform by personnel with limited training; 2) sensitive enough to detect the low levels of target that would be expected both early and late in the infection cycle; 3) specific for *Brucella;* 4) robust under a variety of testing conditions; 5) rapid so that the results can be assessed with little or no delay to the owner; and 6) inexpensive. Early PCR assays exploited single unique genetic targets that provided genus-specific identification of *Brucella*. This was driven by the small number of *Brucella* genes that had been cloned and sequenced. Fekete *et al.* (1990) published the first PCR-based test for *Brucella*. The assay targeted a gene encoding a 43-kDa outer membrane protein from *B. abortus* strain S19. Based on assay results, the target sequence was conserved in 25 strains representing all of the known *Brucella* species and biovars. Amplification of the target sequence did not occur with 16 other bovine pathogens

(including 3 viruses, 12 bacteria and 1 fungus); nor was the target sequence detected in bovine genomic DNA. The authors reported a sensitivity of 0.1 pg DNA, less than 100 bacteria. When the assay was tested on aborted foetuses and maternal tissues (Fekete *et al.,* 1992b), the authors reported 98% sensitivity and 96% specificity for the assay. Nevertheless, the exact assay protocol was patented and never published, limiting application by other laboratories.

The first *Brucella* gene target to become widely used was the 16S rDNA gene. Herman and De Ridder (1992) were the first investigators to exploit this locus. Based on the sequence published by Dorsch *et al.* (1989), the *Brucella* 16S rDNA sequence was aligned against the homologous sequences from five other bacteria including that of the taxonomically related bacteria, *Agrobacterium tumefaciens*. Assay performance was evaluated against 10 strains of *Brucella,* representing all known *Brucella* species and several biovars, and against 17 other bacteria that are either closely related to *Brucella* genetically, or commonly found in cattle. Among the negative control DNAs, only *Ochrobactrum anthropi*, the closest known genetic relative to *Brucella,* produced a positive result. When the assay was field tested (Cetinkaya *et al.,* 1999) with the stomach contents from 16 aborted sheep foetuses of unknown etiology, the PCR results were similar to the corresponding culture results for detecting *Brucella*. *Brucella* was cultured from five samples of which four were PCR-positive, while one sample that was culture negative for *Brucella* was PCR-positive.

Other investigators have also targeted the rRNA operon for *Brucella*-specific PCR-based assays. In 1995, Romero *et al.* (1995a) designed six primers for the rDNA locus (three forward primers and three reverse primers) which were analysed in nine primer combinations to find the optimal combination. They tested a panel of bacterial strains composed of 42 *Brucella* strains (type strains, laboratory strains, a vaccine strain, and field isolates) and 51 *non-Brucella* control strains. Similar to the findings of Herman and De Ridder (1992), all of the *Brucella* strains and none of the *non-Brucella* control strains produced positive assay results with the exception of *Ochrobactrum anthropi* biotype D. Two other strains of *O. anthropi* produce negative results. The same year, the investigators field tested the assay with milk samples from 56 dairy cows that were culture positive for *Brucella* and 37 cows that were culture negative and serologically negative (Romero *et al.,* 1995b), attaining 100% specificity and 85.5% sensitivity. The limit of detection was later improved by optimising sample preparation (Romero and López-Goñi, 1999).

Along the same line, Barry *et al.* reported in 1991 that the specificity of bacterial PCR-ribotyping can be improved by targeting the 16S-23S rDNA intergenic spacer region. Because of the greater level of sequence diversity among bacteria in this region, PCR assays anchored in this region tend to be more genus-specific than those based on the 16S gene sequence, and in some cases species-specific assays are possible. Rijpens *et al.* (1996) were the first investigators to develop a 16S-23S intergenic spacer PCR-ribotyping assay for *Brucella*. The 4-primer nested PCR assay developed for this study was validated with 18 strains of *Brucella,* including all species and many biovars, as well as 56 *non-Brucella* control strains. This study confirmed that higher specificity can be attained by targeting the 16S-23S intergenic spacer region of *Brucella* than by targeting the 16S rDNA sequence. Whereas the 16S rDNA PCR assays reported false-positive results with certain strains of *O. anthropi,* Rijpens *et al.* had no false-positive results from

nine strains of *O. anthropi* included in their panel of negative control bacteria. A similar study was conducted by Fox *et al.* (1998). They also amplified the 16S-23S intergenic spacer region but with a different set of primers. Their results were comparable to those reported by Rijpens *et al.*

At about the same time that Herman and De Ridder were developing their PCR assay utilising the 16S rDNA gene, Baily *et al.* (1992) published a new genus-specific assay that targets the gene encoding BCSP31, the first *Brucella* gene to be cloned and characterised (Mayfield *et al.*, 1988; Bricker *et al.*, 1988). The PCR assay was simple, robust, and sensitive, although the report was very limited in scope. Only two species of *Brucella, B. abortus* and *B. melitensis*, and seven *non-Brucella* bacteria were examined. In 1996, Da Costa *et al.* extended the investigation by including all the *Brucella* species and biovars as well as 98 non-*Brucella* bacteria. The assay correctly identified all *Brucella* strains and properly excluded all non-*Brucella* strains except one isolate of *O. anthropi*. This assay has received wide acceptance by other laboratories. The assay performed well (reported sensitivity of 2 CFU/ml) with aborted bovine foetal tissues and stomach contents (Cortez *et al.*, 2001). Gallien *et al.* (1998) established 100% assay sensitivity with six different tissues from naturally infected cattle. The assay has also been extensively used for diagnosing brucellosis in humans (see also Chapter 3).

The BCSP31 gene was also targeted by Serpe *et al.* (1999) in a similar but slightly different PCR assay. The emphasis of this work was the application of PCR to soft cheeses that might be contaminated from unpasteurized milk, rather than for use in the diagnosis of animal brucellosis. However, in a later study, Guarino *et al.* (2000) used this assay to detect *Brucella* in the blood of seropositive buffalo in Italy. They reported that PCR was significantly less sensitive than either ELISA or complement fixation assays.

In 1989, Ficht *et al.* discovered a *Brucella* locus containing two very similar genes, *omp2a* and *omp2b*. The two genes share 85% sequence homology. This locus was the basis for the creation of a genus-specific PCR assay (Leal-Klevezas *et al.*, 1995b) which uses primers that are homologous to both copies of the gene, providing twice the number of targets per bacterium. In this study, the technique was applied to blood and milk from infected goats and cattle and found to be more sensitive than serology or culture.

Another commonly used target for PCR-based diagnostics is the genetic element IS*711* (Halling *et al.*, 1993), also known as IS*6501* (Ouahrani *et al.*, 1993). In the work by Ouahrani-Bettache *et al.* (1996), primers designed to amplify a portion of the element (600 bp) identified all *Brucella* species, but the product was not amplified from seven other pathogenic bacteria that were tested at the same time. The advantage of using the IS*711*/IS*6501* target sequence lies in the natural amplification of the target sequence since all *Brucella* species contain at least 5 and as many as 35 copies of the element distributed throughout their genomes (Ouahrani *et al.*, 1993; Bricker and Halling, 1994; Clavareau *et al.*, 1998; Bricker *et al.*, 2000b; see also Chapter 1). This method was later validated for detection of *Brucella* in semen from naturally infected rams (Manterola *et al.*, 2003). In an extensive study of 192 semen samples collected from 101 rams in confirmed *Brucella*-infected herds, 35 rams from brucellosis-free herds and 14 experimentally infected rams, the investigators demonstrated high specificity (100% when including samples from *Brucella*-free rams) and high sensitivity (0.91 proportion of agreement with culture results from the same samples).

As described above, a number of genus-specific PCR assays have been developed for detection and identification of *Brucella*. One thing that these assays have in common is the simplicity of a single, highly conserved target sequence. This simplicity contributes to the robust nature of these assays, a trait that makes each of these assay amenable to use with field samples consisting of blood, milk, or abortion tissues. The strong hybridisation of the conserved primers to the target sequences allows these assays to withstand minor changes in assay conditions caused by sample contaminants, sample extraction methods, or fluctuations in assay conditions.

2.2. Simultaneous Testing for Multiple Disease Agents Including *Brucella*

As effective screening tests, PCR-based assays can have added value by multiplexing primer pairs to differentially identify additional pathogenic genera that might be found in the same test population. Testing for multiple diseases simultaneously is advantageous because it decreases the overall cost of a correct diagnosis. Recently, several laboratories have been engaged in developing multi-agent PCR assays. Sreevatsan *et al.* (2000) designed a multiplex assay targeting the BCSP31 gene of *Brucella* and the *hsp65* locus of *Mycobacterium bovis*. They successfully applied the assay to various sample types including nasal swabs, lymph nodes, and milk from infected cattle.

The BCSP31 gene locus was also targeted by Richtzenhain *et al.* (2002) in a multiplex PCR assay for the detection and differentiation of *Brucella* and *Leptospira*. The *Brucella* specific primers were the same used by Baily *et al.* (1992), while the *Leptospira* primers targeted the 16S rDNA as described by Merien *et al.* (1992). When applied to clinical samples of aborted bovine foetuses or experimentally infected hamsters, the assay showed 100% sensitivity for both *Brucella* and *Leptospira* infections as well as 92–93% specificity (dependent on the methods used to extract the DNA) when compared to positive culture results.

A more complex assay was reported by McDonald *et al.* (2001) to identify and differentiate four different bacterial pathogens: *Coxiella burnetti*, *Brucella melitensis*, *Bacillus anthracis*, and *Yersinia pestis*. Because this research has military significance, most of the details, including the primer sequences, were not provided. This multiplex assay was designed to identify and differentiate biowarfare agents; however, the strategy is equally applicable to agricultural pathogens.

Recently, the perosamine synthetase gene was targeted by Lubeck *et al.* (2003) in a multiplex PCR assay that distinguishes *Brucella* from *Yersinia enterocolitica* serotype O:9, a bacterium with LPS antigenically cross-reactive with *Brucella* LPS. *Y. enterocolitica* O:9 is responsible for a significant portion of false-positive serological results during routine screening of market cattle for brucellosis.

Cross-reactivity to LPS from non-*Brucella* bacteria is increasingly problematic as the incidence of brucellosis in livestock decreases. As demonstrated in Belgium, France, and other countries, the majority of serological reactors “post-eradication” are due to false-positive serological reactions (Weynants *et al.*, 1996; Gourdon *et al.*, 1999; Emmerzaal *et al.*, 2002). In addition to *Y. enterocolitica*, a number of other bacteria cross-react with *Brucella* in standard agglutination tests. These include: *E. coli* strains O:157 and O:116, *Salmonella* spp. with Kauffmann-White group N serotypes, and *Pseudomonas*

maltophilia, (Corbel *et al.*, 1984); *Francisella tularensis* (Behan and Klein, 1982); and *Vibrio cholerae* O:1 (Ito and Yokota, 1988). Multiplex PCR assays that differentiate several pathogens simultaneously would be a cost-effective way to continue surveillance measures following disease eradication.

3. PCR-Based Identification and Differentiation of *Brucella* at the Species/Biovar Level

As a result of adequate vaccines and diagnostic tests, some countries have successfully eradicated some or all *Brucella* species from their livestock. Most other countries have government-supported eradication or control programs. For a number of reasons, such as differences in host preference, in the husbandry of host species, in modes of transmission, in pathogenicity to humans, in geographic distribution, and in the behaviour of reservoir hosts, the governmental regulatory policies for *Brucella* are usually species-specific. Thus, the correct identification of the species involved is essential for the initiation of appropriate action.

Currently, species identification is part of the confirmation process initiated after finding a suspected case during the screening phase. Confirmation depends on isolation of organisms from host tissues or fluids followed by characterization of the organism through classical bacteriological techniques. Based on the sum of characteristics the organism can be identified by genus, species, and sometimes subspecies (biovars). This phase of diagnosis can be quite lengthy, typically 10–14 days, making PCR-based tests appealing since results could be available in 24 to 48 hours. The process can be further streamlined by preparing and storing reaction mixes in advance.

With PCR-based assays, discrimination is accomplished by exploiting species-specific sequence variations. The simplest assays are based on a single genetic locus that contains a sequence polymorphism that separates one *Brucella* species from all the rest. However, these assays are limited to the definitive identification of only one species and may necessitate additional testing and consequently additional expense.

Discrimination of multiple species simultaneously is more complicated and typically utilises one of two approaches. The first approach is to design complex reaction mixtures containing multiple primer pairs that each targets a unique species-specific DNA sequence polymorphism. These assays are usually difficult to design because a large amount of sequence data must be known in advance to find polymorphic sequences for each species to be identified. Recently, this approach has progressed with the sequencing of the complete genomes from several *Brucella* species (DelVecchio *et al.*, 2002; Paulsen *et al.*, 2002; Halling *et al.*, 2003). Multiplex assays containing large numbers of primers are frequently more fastidious than the classical single reactions. This is because the assay conditions are empirically selected based on the best overall performance with all primer pairs, which may not promote the optimal performance of individual primer pairs. The second approach uses a single primer pair to amplify a DNA sequence containing internal species-specific polymorphisms. The internal polymorphism must then be characterised by some method of downstream processing. With this strategy, the PCR assay itself is fairly simple, it isn’t necessary to know the exact polymorphic sequence of

every species, but it may be difficult to find a downstream process that can exploit and differentiate the sequence polymorphisms.

3.1. One-step Single PCR Assays That Can Differentiate One *Brucella* Species From All the Others

Several investigators have successfully targeted unique genetic mutations to identify a specific *Brucella* species and differentiate that species from the other *Brucella* species. In 1996, Ficht *et al.* reported a 115-bp deletion in the *omp*2a genes of *B. abortus* biovars 1, 2, and 4. Leal-Klevezas *et al.* (1995a) targeted this polymorphism in the development of a PCR assay that differentiates *B. abortus* biovars 1, 2 and 4 from other *Brucella* species. This assay was later successfully field tested with milk and blood samples from infected goats (Leal-Klevezas *et al.,* 2000). The results showed that PCR was significantly more sensitive than serology or blood culture in detecting *Brucella* infections.

In 2000, the first PCR assay specific for *Brucella* from marine mammals was reported (Bricker *et al.,* 2000b). The assay was based on a locus that contains a copy of the genetic element IS*711* (Halling *et al.,* 1993), also known as IS*6501* (Ouahrani *et al.,* 1993). The IS*711* element was found at the specific locus in 23 strains originating from marine mammal hosts including dolphins and seals; IS*711* did not reside at this locus in any of the classical *Brucella* species from terrestrial hosts except *B. ovis* which weakly amplifies a similar product.

A few months later, Cloeckaert *et al.* (2000a) also published a PCR assay specific for *Brucella* from marine mammal hosts (see also Chapter 1). The new assay was also based on the insertion of an IS*711* element into a unique chromosomal locus. In this case, the locus was immediately downstream of the *bp*26 gene (also known as the *omp*28 gene). The primers were designed to enclose the complete *bp*26 gene and flanking DNA, and in the case of marine mammal isolates, they enclose the IS*711* element as well. A product was amplified from all *Brucella,* but the amplicons from marine isolates are 850 bp larger than the amplicons from the classical terrestrial *Brucella*. This gives the newer assay the advantage that in a single assay all *Brucella* species are detected and the marine mammal isolates can be distinguished on the basis of amplicon size.

Even more recently, a PCR-based assay that could differentiate *B. suis* biovar 1 from *B. abortus* biovar 1 was published (Fayazi *et al.,* 2002). The primer pair designed for this assay was derived from a cloned fragment selected from a *B. suis Mbo*I library. The assay was reported to amplify different sized products from an unnamed *B. suis* biovar 1 field isolate and an unnamed *B. abortus* biovar 1 field isolate, respectively. In an attempt to map the polymorphism to the published *B. suis* biovar 1 strain 1330 genome (Paulsen *et al.,* 2002), our laboratory compared the published primer sequences to the published *B. suis* genome. We were surprised to find the primer sequence homologues resided on different chromosomes. Further examination of the cloned fragment sequence (GenBank accession number AF387316) showed that the clone contained four *Mbo*I fragments that, when compared to the *B. suis* genome, were positioned on unlinked loci from both chromosomes. It is unclear how the authors were able to amplify products from native *Brucella* DNA with the reported primers. *Brucella suis* biovar 3 has been previously shown to contain a single chromosome with genomic rearrangements relative to other *Brucella* species (Jumas-Bilak *et al.,* 1998); however, Fayazi *et al.* (2002) verified their

assay with an unnamed field strain identified as *B. suis* biovar 1 and with a *B. abortus* biovar 1 isolate.

3.2. Multiplex PCR Assays for the Identification and Differentiation of *Brucella* Species and/or Biovars

This strategy combines multiple primer pairs in a single reaction whereby each primer pair targets a species-specific locus. Discrimination is achieved either by the number or pattern of targets amplified or by characterization of the amplicon(s). Amplicon characterization is most commonly based on species-specific, differential product sizes designed into the assay. One of the challenges with utilising this approach for the identification of *Brucella* species is the high degree of genetic homology among species (Verger *et al.*, 1985; Bricker, 2000a; Gandara *et al.*, 2001). As a result, species-specific targets are relatively rare.

The first species-specific multiplex PCR assay was the AMOS-PCR Assay developed in our laboratory (Bricker and Halling, 1994). The assay was called the "AMOS Assay" as an acronym for the *Brucella* species it can identify and differentiate (*B. abortus* biovars 1, 2 and 4; *B. melitensis*, *B. ovis;* and *B. suis* biovar 1). The species-specific polymorphisms targeted in this assay result from unique genomic translocations of the insertion sequence element IS*711* described above. These translocations are rare and very stable.

The AMOS PCR Assay consists of five primers; one common primer that is anchored in the IS element and four species-specific primers that anneal to the unique sequence flanking the insertion site. Thus, each amplicon is a hybrid of IS*711* sequence and flanking sequence. Species are identified by the observation of unique-sized amplicons delineated by species-specific primers positioned at varying distances from the common primer. Following the publications of sequence polymorphisms discovered by Sangari *et al.* (1994) and Vemulapalli *et al.* (1999), additional strain-specific primers were incorporated into the primer cocktail for identification of two *B. abortus* vaccine strains, S19 and RB51 (Bricker and Halling, 1995). Modifications of the assay have been introduced over time to improve performance. Also, an abbreviated version of the assay, named the BaSS-PCR Assay (an acronym for *Brucella abortus* Strain Specific PCR Assay) was created to specifically identify and distinguish *B. abortus* field strains from the two vaccine strains (Ewalt and Bricker, 2002; Bricker *et al.*, 2003a). The assay also detects other *Brucella* species but it does not identify the individual species. Discrimination between vaccinated and field infected animals is essential for making a correct diagnosis; the AMOS-PCR and BaSS-PCR Assays continue to be the only differential PCR assays to specifically identify both *B. abortus* vaccine strains. The AMOS-PCR and BaSS-PCR Assays have been used by the U.S. national diagnostic laboratory (The National Veterinary Services Laboratories, APHIS, USDA, Ames, IA [Ewalt and Bricker, 2000]). The assay has also been used to identify RB51 vaccinates from field infected cattle in Italy (Adone *et al.*, 2001), to identify *B. abortus* and *B. melitensis* in milk from naturally infected cattle, sheep, goats and camels (Hamdy and Amin, 2002), and to identify *B. melitensis* from aborted sheep foetuses in Turkey (Leyla *et al.*, 2003). When the *B. melitensis* primers from the AMOS-PCR assay were used by Amin and colleagues (2001) to detect the presence of *B. melitensis* in semen from

serologically positive bulls and rams, they reported that PCR was more sensitive than culture at 100 CFU/ml of semen, well within the range of *Brucella* typically found in infected semen. The authors point out that while brucellosis is not usually transmitted by natural insemination, it is passed through artificial insemination.

3.3. PCR-RFLP Assays for the Identification and Differentiation of *Brucella* Species and/or Biovars

In this strategy, a single primer pair anneals to conserved DNA sequences bracketing a region exhibiting a higher than normal mutation rate. Although the mutation rate is elevated, the resulting sequence polymorphisms are stable enough to serve as species-specific markers. The advantage of this approach is that it isn't necessary to know the exact polymorphic sequence of every species. However, the challenge is developing a post amplification method to detect and differentiate the sequence variations quickly and reproducibly. A few *Brucella* genes exhibit greater than normal sequence diversity. This is true for many of the *omp* (outer membrane protein) genes (for a review on *Brucella omp* genes and corresponding proteins, see Cloeckaert *et al.*, 2002b). Omps are constantly exposed to variable environmental conditions as the bacteria go through the various phases of their lifecycle. The bacteria must contend with variations in temperature, osmolarity, pH, toxins, and other stresses, when outside the host, and also be able to cope with immunological attack and intracellular survival when inside the host. Thus, *omp* genes experience greater selective pressure than most other *Brucella* genes. At the same time, Omps have to maintain their functions, so DNA sequence changes must be limited and must either be silent or be localised to regions of the protein that are not directly or indirectly necessary for proper function.

PCR-RFLP is the most commonly used differential technique for assessing minor DNA sequence divergence. Gene-specific primers are designed from highly conserved regions within the gene or adjacent sequence. The amplified product is processed with one or more restriction endonucleases to track nucleotide changes within the respective enzyme recognition sites.

This technology has been adopted very successfully for species identification of *Brucella*. Currently, the PCR-RFLP assays with the greatest discriminatory power target the *omp*2 locus. As previously mentioned (see also Chapter 1), the *Brucella omp*2 locus contains two very similar genes, *omp*2a and *omp*2b; they are arranged head-to-head on opposite strands. Each gene contains an ORF for a 36-kDa outer membrane protein. The two genes share 85% sequence homology but within the proposed ORFs, the predicted protein sequences are 96% identical. Ficht *et al.* (1990) used Southern hybridisation RFLP of the *omp*2a and *omp*2b to type all six known species of *Brucella*. Cloeckaert *et al.* (1995) extended Ficht's work by developing a PCR assay based on the *omp*2a and *omp*2b genes. Digestion of the resulting amplicons with a variety of restriction enzymes, revealed differential RFLP patterns caused by nucleotide polymorphisms within the enzyme recognition site. PCR-RFLP of the *omp*2 loci distinguished most *Brucella* species and some biovars. However, it was not possible to distinguish *B. canis* isolates from *B. suis* biovar 3 and 4 isolates, nor was it possible to differentiate all *B abortus* strains from all *B. melitensis* strains.

Investigators have evaluated other *Brucella* genes by PCR-RFLP with a wide range of restriction endonucleases. In the same study described above, amplification of the *omp*25 gene revealed that *B. melitensis* amplicons lack a conserved *Eco*RV site and *B. ovis* amplicons have a 50-bp deletion at the 3′ end that was apparent with all restriction enzymes (Cloeckaert *et al.*, 1995). The following year, Cloeckaert *et al.* (1996) discovered that the *dna*K gene is a valuable marker for *B. melitensis* due to the loss of a conserved *Eco*RV recognition site. Vizcaino and colleagues (1997) were the first investigators to successfully differentiate *B. canis* from *B. suis* by PCR-RFLP with the discovery of a polymorphism in the *omp*31 locus.

However, many of the *Brucella* loci examined have shown little or no diagnostic value. A 14-kb segment containing seven genes associated with LPSO antigen synthesis was analysed by PCR-RFLP with a large array of restriction enzymes (Cloeckaert *et al.*, 2000b). Overall, the genes were highly conserved and no polymorphisms could be detected in two of the genes. The other five genes were found to contain minor polymorphisms in certain species or biovars, typically observed as the loss of a single restriction site. These differences could be useful for supplemental identification or the verification of identifications made by other tests, but they do not appear extensive enough to serve as exclusive assay targets. Unproductive loci include: 16S rDNA, BCSP31, *gro*EL, *dna*J and *htr*A (Da Costa *et al.*, 1996). It is likely that many other non-productive loci have been found but not reported.

As previously mentioned, the ability to differentiate vaccine strains from field strains is an important component of a diagnostic test. Cloeckaert *et al.* (1995) noted that a few *B. melitensis* strains, including the vaccine strain Rev-1, lacked a *Pst*I recognition site in *omp*2a. The polymorphism could differentiate Rev-1 from *B. melitensis* field isolates originating from Israel and several other European and Middle Eastern counties. It could not differentiate Rev-1 from New World isolates and a few Old World isolates. This work was extended to additional *B. melitensis* field isolates by Bardenstein *et al.* (2002) with the same result. Thus, it was a significant development when recently the first definitive PCR-based method for distinguishing the vaccine strain, Rev-1, from all field isolates was reported. Cloeckaert *et al.* (2002a) discovered a unique mutation in the *rps*L gene of Rev-1 that eliminates a *Nci*I restriction site, and thus, could be detected by PCR-RFLP.

Predictably, PCR-RFLP has become a preferred method in many laboratories for differential identification of *Brucella* species or for characterising new species or strains (Miller *et al.*, 1996; Clavareau *et al.*, 1998; Garin-Bastuji *et al.*, 1998; Brew *et al.*, 1999; Cloeckaert *et al.*, 2001). All *Brucella* species can be differentiated by PCR-RFLP with a combination of the *omp*2a, *omp*2b, *omp*25, and *omp*31 loci and differentiation of *B. melitensis* Rev-1 can be included by the addition of the *rps*L locus.

4. Differentiation of *Brucella* Strains and Isolates by PCR for Epidemiological Trace-back

Epidemiological trace-back is an important component of any disease eradication or reduction program. During an epizootic event, finding the source of infection and identifying possible points of transmission are key elements in preventing further spread

of the disease. In the case of *Brucella,* strain identification is challenging because of the genetic homogeneity among species of the genus. Classical bacteriology allows for the identification of only a small number of subtypes (biovars) below the species level. Furthermore, certain subtypes may dominate a geographic area. For example, when bovine brucellosis had a significant presence in the USA, about 85% of infections were caused by *B. abortus* biovar 1. Trace-back of brucellosis typically requires detective work without the benefit of direct scientific evidence for support. At this time, molecular technology appears to hold the greatest potential for field strain/isolate typing since this technology can often identify small regions of hypervariability including ones that have no phenotypic effect. Several molecular approaches have been tried in recent years to find and exploit regions of hypervariability for strain identification.

Many of the earliest attempts to develop epidemiological markers for *Brucella* strains and isolates utilised molecular biology methods that had been successful for subtyping other groups of bacteria. Restriction mapping (McGillivery *et al.*, 1988), pulsed field gel electrophoresis (Allardet-Servent *et al.*, 1988; Jensen *et al.*, 1995), ribotyping (Rijpens *et al.*, 1996; Fox *et al.*, 1998), and IS-RFLP typing (Halling *et al.*, 1993; Ouahrani *et al.*, 1993; Bricker and Halling, 1994:1995; Bricker *et al.*, 2000), were all successful in identifying genetic variation in *Brucella* to some degree, but none of these techniques identified an adequate number of strain-specific markers to be suitable for routine epidemiological use.

4.1. Arbitrary-Primed PCR

The first PCR-based method for epidemiology subtyping successfully applied to *Brucella* was arbitrary-primed PCR (AP-PCR) or random amplified polymorphic DNA PCR (RAPD-PCR) by Fekete and her colleagues (Fekete *et al.*, 1992b). Based on short, arbitrarily designed primers, no specific DNA sequence information is needed for this type of assay, a major advantage in 1992 when very little *Brucella* sequence was known. Strain-specific fingerprints are developed from the collection of amplicons produced when the primers bind to the numerous loci that are homologous to the short arbitrary sequences of the primers. Fekete demonstrated that AP-PCR could discriminate among species, biovars and strains. However, reproducibility between laboratories has consistently been a problem for AP- and RAPD-PCR typing (Gao *et al.*, 1996). Because very permissive assay conditions are needed to guarantee annealing by these very short primers, hybridisation is weak and often brief. Slight changes in factors such as salt concentration, pH, temperature fluctuations, and sample concentration can affect the annealing efficiency and radically modify the results by changing the numbers and yields of amplicons. In an attempt to address the issues regarding reproducibility, Tchernava *et al.* (2000) methodically optimised assay parameters. However, the technology has not been widely adopted for brucellosis epidemiology.

4.2. Repetitive Element PCR

The use of semi-conserved primers can considerably improve sensitivity and specificity. One technique that applies this approach is rep-PCR (repetitive element PCR; De Bruijn, 1992), which targets naturally occurring, multicopy, genetic elements dispersed

throughout many bacterial genomes. These greatly conserved elements are small (around 35–150 bp) and usually palindromic. Genomic rearrangements often occur around the elements increasing localised genetic diversity. ERIC (enterobacterial repetitive intergenic consensus) and REP (repetitive extragenic) elements are the two most commonly used targets for rep-PCR, since they occur in high copy numbers in most Gram-negative bacteria. In rep-PCR, primers homologous to the element sequence are used to amplify the sequences between closely positioned elements. The specific products amplified reflect the number of elements clustered together, their relative orientations, and their distances apart. It is not necessary to know a great deal of genomic sequence data to make use of this technique.

Rep-PCR has been utilised for *Brucella* strain identification by two laboratories, Mercier *et al.* (1996) and Tcherneva *et al.* (1996). They independently compared the discriminating power of ERIC-PCR versus REP-PCR, and although they worked with the same primer pairs for amplification, they had different results for the same *Brucella* strains studied. ERIC-PCR was more discriminating for Mercier and colleagues, while REP-PCR produced greater differentiation for Tcherneva's group. Individual fingerprint patterns obtained by the two laboratories for the same *Brucella* type strains were remarkably different. The differences encountered by the two laboratories highlight a problem inherent in the technique. The respective sequences of ERIC and REP elements are conserved but they are none-the-less polymorphic, consequently REP and ERIC primers are designed from their respective consensus sequences. To facilitate annealing, REP primers are degenerate while ERIC primers are long (by the standards of the time that the work was published). Because ERIC and REP primers are often imperfectly matched to the target sequence, target amplification can be affected by even small variations in the assay conditions, though less severely than seen with the random primers used for RAPD-PCR. It has been reported that the commonly used assay parameters for ERIC-PCR will also amplify arbitrary loci in addition to the loci that contain authentic ERIC elements (Gillings and Holley, 1997). Consequently, although these methods clearly have merit, the difficulties with laboratory-to-laboratory reproducibility have limited their use by colleagues.

In a similar approach, Ouahrani-Bettache *et al.* (1996) introduced IS-anchored arbitrary PCR. In this case, the repeated DNA that is the basis for the assay is the element IS*6501* (also known as IS*711*). However, the numbers of IS*6501*/IS*711* elements in the genomes of most *Brucella* species are substantially fewer than the rep-PCR targets. Since the elements are most likely too far apart to permit amplification of the intervening sequence by PCR, the authors chose to pair a random primer with an "anchor" primer that is homologous to the terminal sequence of the IS*6501*/IS*711* element. While the investigators were successful at differentiating *Brucella* strains, the technique suffers from the same drawback encumbering REP-PCR and ERIC-PCR. Reproducibility among laboratories can be difficult to achieve because the tenuous binding of the arbitrary primer to short, semi-homologous sequences may be disrupted by minor changes in sample and assay conditions.

4.3. Infrequent Restriction Site-PCR

Employing a different tactic, Cloeckaert *et al.* (2003) used an elegant strategy for typing *Brucella* species and subspecies based on infrequent restriction site-PCR (IRS-PCR). This technique is a modification of the Amplified Fragment Length Polymorphism (AFLP; Vos *et al.*, 1995) procedure in which genomic DNA is cut

Table 1. *Brucella* species and biovars

	Species	Biovar	Strain	Source (host)	Source (location)
1	*B. abortus*	1	544	Bovine	England
2	*B. abortus*	2	86/8/59	Bovine	England
3	*B. abortus*	3	Tulya	Human	Uganda
4	*B. abortus*	4	292	Bovine	England
5	*B. abortus*	5	B3196	Bovine	England
6	*B. abortus*	6	870	Bovine	Africa
7	*B. abortus*	9	C68	Bovine	England
8	*B. melitensis*	1	16M	Goat	USA
9	*B. melitensis*	2	63/9	Goat	Turkey
10	*B. melitensis*	3	Ether	Goat	Italy
11	*B. canis*	na	RM-6/66	Dog	USA
12	*B. neotomae*	na	5K33	Wood Rat	USA
13	*B. ovis*	na	63/290	Sheep	Africa
14	*B. suis*	1	1330	Swine	USA
15	*B. suis*	2	Thomsen	Swine	Denmark
16	*B. suis*	3	686	Swine	USA
17	*B. suis*	4	40	Reindeer	Former Soviet Union
18	*B. suis*	5	513	Mouse	Former Soviet Union
19	*B. abortus*	1	fs2018	Bovine	Chino, CA
20	*B. abortus*	1	fs2019	Bovine	Chino, CA
21	*B. abortus*	1	fs2046	Bovine	Marathon, FL
22	*B. abortus*	1	fs2047	Bovine	Marathon, FL
23	*B. abortus*	1	fs2075	Bovine	Chino, CA
24	*B. abortus*	1	fs2079	Bovine	Chino, CA
25	*B. abortus*	1	fs2089	Bovine	Chino, CA

26	*B. abortus*	1	fs2167	Bovine	Chino, CA
27	*B. abortus*	1	fs2168	Bovine	Chino, CA
28	*B. abortus*	1	fs2236	Bovine	Chino, CA
29	*B. abortus*	1	fs2146	Bovine	Okeechobee, FL
30	*B. abortus*	1	fs2147	Bovine	Okeechobee, FL
31	*B. abortus*	1	fs2148	Bovine	Leesburg, FL
32	*B. abortus*	1	fs2340	Bovine	Chino, CA
33	*B. abortus*	1	fs2344	Bovine	Chino, CA
34	*B. abortus*	1	fs2355	Bovine	Chino, CA
35	*B. abortus*	1	fs2372	Bovine	Chino, CA
36	*B. abortus*	2	fs2381	Bovine	Canal Pt., FL
37	*B. abortus*	2	fs2382	Bovine	Canal Pt., FL
38	*B. abortus*	1	fs2421	Bovine	San Jacinto, CA
39	*B. abortus*	1	fs2422	Bovine	San Jacinto, CA
40	*B. abortus*	1	fs2423	Bovine	Arkansas
41	*B. abortus*	1	fs2428	Bovine	Huntsville, AL
42	*B. abortus*	1	fs2429	Bovine	Huntsville, AL
43	*B. abortus*	1	fs2430	Bovine	Huntsville, AL
44	*B. abortus*	1	fs2431	Bovine	Huntsville, AL
45	*B. abortus*	1	fs2432	Bovine	Weir, KS
46	*B. abortus*	2	fs2444	Bovine	Waurika, OK
47	*B. abortus*	2	fs2475	Bovine	Wauchula, FL
48	*B. abortus*	2	fs2476	Bovine	Wauchula, FL
49	*B. abortus*	1	fs 8–1097	Elk	Madison Co., MT
50	*B. abortus*	1	fs 7–1493	Elk	Laramie, WY
51	*B. abortus*	1	fs 2–0993	Bison	Park Co. MT
52	*B. abortus*	1	fs 8–0999	Bison	Stanley Co., SD

with two restriction enzymes, one with a high frequency recognition sequence and one with an infrequent recognition sequence. Two primer-size oligonucleotide adapters are synthesised that match the cohesive ends formed by the restriction enzymes, and the adapters are ligated to the ends of the fragments. A subgroup of the fragment pool is selectively amplified with primers that are homologous to the adapter sequence and that also contain a randomly chosen 1 to 3 nucleotide tail at the 3′ end. The subgroup composition is determined by hybridisation of the 3′ primer tail with only those

fragments that have complementary nucleotides immediately flanking the adapter. This technology eliminates the reproducibility concerns typically associated with most of the other fingerprinting strategies because the fragment adapters are conserved and long enough to permit annealing under the most stringent conditions for highly specific amplification.

With IRS-PCR, Cloeckaert and colleagues were able to identify and differentiate all species of *Brucella* except *B. suis* and *B. canis* and they were also able to identify and differentiate many biovars and several new subspecies. When the technique was applied to *Brucella* isolates collected from marine mammals, the results strongly supported the proposed division of isolates into two species, *B. pinnipediae* and *B. cetaceae* (Cloeckaert *et al.,* 2001), and also revealed subspecies within both species. Based on the polymorphic loci they discovered, the authors were able to develop species-specific primers for *B. cetaceae* and *B. pinnipediae* (see also Chapter 1).

4.4. Multi-locus VNTR Analysis

Recently our laboratory has used a different tactic for typing *Brucella* strains for epidemiological trace-back (Bricker *et al.,* 2003b). This strategy exploits the observation that most organisms (prokaryotic and eukaryotic) contain strings of tandem repeat sequences distributed throughout their genomes (Bennett, 2000). Repeat sequences, sometimes called mini- or microsatellites, are found in both intragenic and extragenic loci and in some cases may affect protein expression or structure. Investigators noticed that many tandem repeats, especially short tandem repeats containing repeat units that are 1 to 10-bp in length, mutate at a higher rate (up to 1,000 times higher) than average for the rest of the genome (Jeffreys *et al.,* 1988). Unequal crossing-over and slip-strand mispairing (Levinson and Gutman, 1987) promote the insertion or deletion of repeat units at an accelerated rate, especially within strings containing large numbers of repeat units. These rapidly mutating series of repeats are also known as variable number tandem repeats (VNTRs). Multi-locus VNTR analysis (MLVA) has become a powerful tool for distinguishing among closely related strains of organisms because VNTR loci typically mutate independently of one another.

Strain typing based on MLVA is replacing several other molecular typing techniques because of a number of advantages associated with this technology. First, it is simple and rapid. It is not necessary to purify genomic DNA and no sample modifications are required (e.g. compared to AFLP or PFGE). Second, the assay is discriminating, yet robust, because primers can hybridise tightly to long stretches of highly conserved flanking sequence. Third, the process contains limited steps and is amenable to high throughput. With applications to human disease and forensic science, automated instrumentation is widely available and software has been developed to automatically identify alleles based on customised parameters. Finally, assays can be multiplexed to analyse three or four loci simultaneously either by agarose gel electrophoresis or with instrumentation that is capable of detecting four to five different fluorescent dyes.

Several loci containing tandem repeats were found in the *B. abortus* genome sequence (Halling *et al.,* 2003). Comparison of the DNA sequences of the repeat loci in *B. abortus* with the homologous sequences from the published genomes of *B. melitensis* and *B. suis*, led to the discovery of several variable repeat loci. Eight loci containing tandem repeats

of the 8-bp sequence "AGGGCAGT" have been studied in depth (Bricker *et al.*, 2003b). PCR assays were designed to amplify the complete repeat sequence and a small amount of flanking DNA from each of the eight loci. The exact number of repeat units can then be deduced from the sizes of the amplified products. The assay, named HOOF-Prints as an acronym of Hypervariable Octameric Oligonucleotide Finger-Prints, generates fingerprint patterns based on the number of repeat units at each of the eight loci. For each locus, the alleles are named according to the number of repeat units at that locus, so that Allele-1 contains 1 repeat unit, Allele-2 contains 2 repeat units, etc…. The large size of the repeat sequence, 8-bp, allows easy assignment of alleles due to the clear resolution of repeat units either by gel electrophoresis or capillary electrophoresis.

We have examined *Brucella* isolates representing each of the type strains for all known *Brucella* species and biovars, and for some common laboratory strains (Table 1). The resulting fingerprint data are summarised in Figure 1 as a graphic display of the alleles at each of the eight loci. These fingerprint patterns were originally reported in the paper by Bricker *et al.* (2003b). It is apparent from the diagram that the fingerprints are highly diverse, especially among unrelated strains

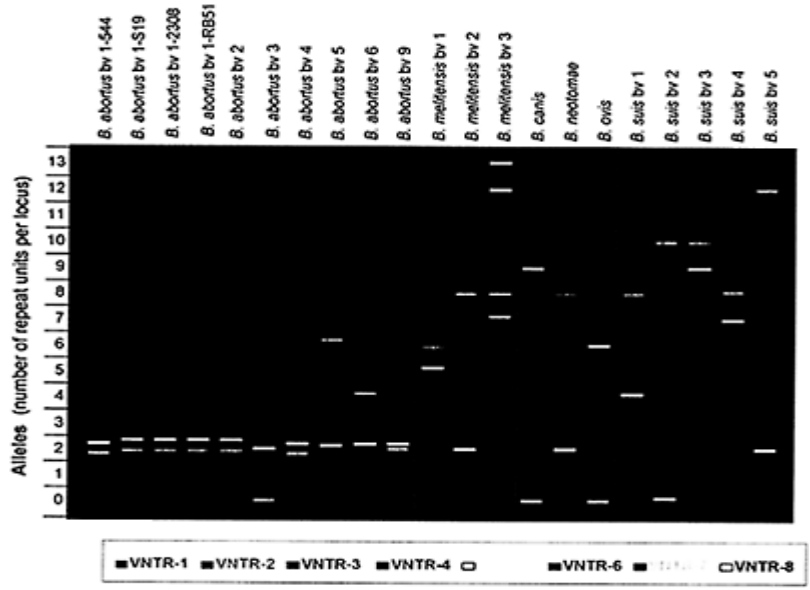

Figure 1. Multilocus allele analysis of*Brucella* species and biovar type strains. Graphical representation of the alleles generated by fluorescent tagged-PCR amplification of tandem repeat loci from the genomic DNA of *Brucella* species and biovars. The allele number reflects the total number

of repeat units calculated for each locus. Each lane is the compilation of data from the independent amplification of each of the eight VNTR loci. Loci are colour-coded for direct comparisons. A star next to the VNTR Locus-4 allele from some samples idicates a match with an alternative allele ladder created by an additional 11-bp of sequence in the non-repeat, flanking DNA of certain *Brucella* species.

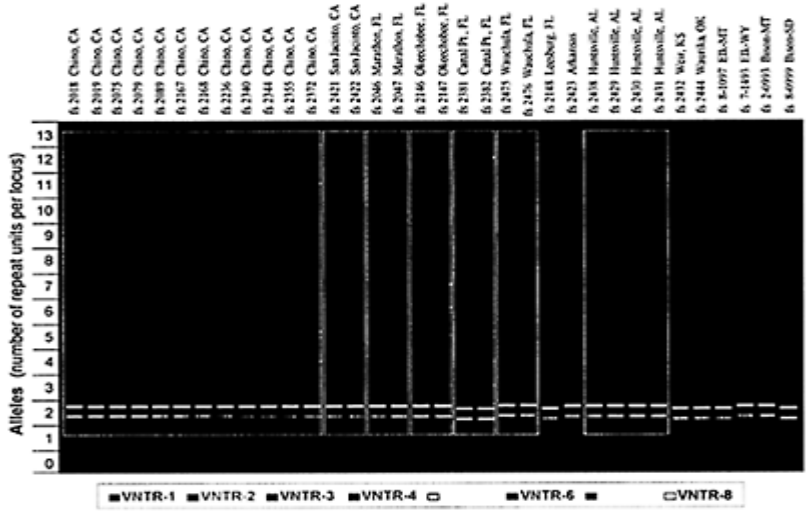

Figure 2. Multilocus allele analysis of *B. abortus* field strains. Graphical representation of the alleles generated by fluorescent tagged-PCR amplification of tandem repeat loci from the genomic DNA of *B. abortus* field isolates. Each lane is the compilation of data from the independent amplification of each of the eight VNTR loci. Loci are colour-coded for direct comparisons. Each

sample is labeled with the isolate number and the CIty and state of origin. Isolates from the same herd are boxed. Each isolate consists of a pool of *B. abortus* colonies derived from a single animal.

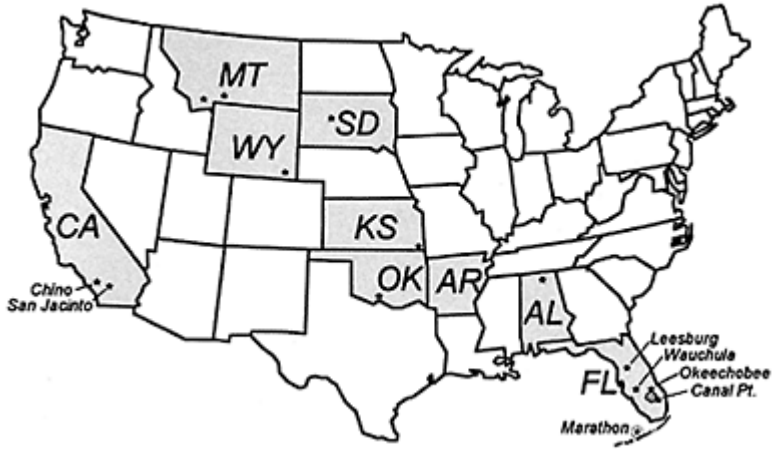

Figure 3. Distribution of *B. abortus* infected herds. The distribution of infected herds from which the *B. abortus* field isolates where obtained are indicated by asterisks on the U.S. map. In California and Florida, multiple cattle herds are distinguished by the city nearest to the herd (see Table 1). The specific location of the herd in Arkansas is no longer available.

and different species. Note that some strains amplified more than one product from a single locus (e.g. *B. abortus* biovar 9, VNTR-4). This is because in most cases the strains, including those obtained from commercial repositories such as the American Type Culture Collection (ATCC), are not clonally selected but rather consist of a population of organisms present in the initial isolation.

One of the biggest challenges for epidemiologists using classical methodology is that *Brucella* species are limited to a small number of subtypes (biovars), and in some geographic regions a single biovar may be dominant. To see if the HOOF-Print Assay

could discriminate among isolates of the same species, we tested 34 field isolates of *B. abortus* collected in 1991 from 11 cattle herds and 4 wildlife reservoirs residing throughout the U.S., at a time when brucellosis was still widely found there (Table 1). The resulting fingerprint patterns are shown in Figure 2 (note that some fingerprints were previously reported in Bricker *et al.,* 2003b). Isolates that originate from the same infected herd are boxed in white, although not all isolates from a herd were collected at the same time. Based on allele composition, the collection of isolates exhibit over 20 different fingerprint patterns, however the fingerprints obtained from animals within the same herd are identical or very similar (e.g. the isolates from Chino, CA). By visual inspection, fingerprint pattern similarities can also occur among herds. The geographic distribution of the herds used in this survey are shown on the map in Figure 3. The *B. abortus* isolates from the Chino and San Jacinto herds have similar fingerprint patterns while the herds are clustered geographically as well.

It is interesting that all of the *B. abortus* field isolates share Allele-2 (2 repeat units) at Locus 5 and at Locus 8 (Figure 2). In fact, all *B. abortus* biovars have Allele-2 at Locus 8 (Figure 1). Most of the field isolates also have Allele-2 at Locus 6. Because of the mechanisms involved in generating VNTR variability, loci containing few repeats are less likely to mutate. Therefore, small alleles can become fixed in the population. Nevertheless, with the limited amount of data available, the fingerprint patterns cannot be used to identify the species or biovar of an isolate, and therefore, the assay cannot be used as a primary diagnostic tool. However, it should be valuable as a supplemental tool for epidemiological trace-back.

5. Accelerated Identification with Real-time PCR

Recent improvements in PCR technology have made it possible to amplify and detect DNA targets simultaneously by real-time PCR. Concurrently, several companies have developed real-time PCR machines that are rugged and designed to be used in the field under harsh conditions. The appeal of nearly instant results has encouraged the development of real-time assays for the detection and identification of pathogenic and potential biowarfare agents. For *Brucella,* real-time PCR could be used for simultaneously screening animal populations and definitively identifying the genus and species of pathogens, eliminating the time and expense of multiple tests.

Two real-time assays have been published for the recognition of *Brucella*. Both assays target the junction between unique copies of IS*711* and flanking DNA. The first assay was developed by Redkar *et al.* (2001) for identification of three principal species of *Brucella: B. abortus, B. melitensis,* and *B. suis* biovar 1. Based on the same principle as the AMOS-PCR assay, this real-time assay consists of three separate PCR reactions, one for each of the species identified. Each reaction contains primers specific for one *Brucella* species, such that only one of the reactions can amplify the target sequence. The determination is based on fluorescence generated by the simultaneous hybridisation of paired FRET (fluorescence resonance energy transfer) probes to the amplified products in the selected reaction.

The second real-time PCR assay was developed specifically for identification of *B. abortus* (Newby *et al.,* 2003). This assay targets the same locus used for the *B. abortus*

component of the standard AMOS-PCR assay and the real-time assay of Redkar. The authors compared three detection methods: SYBR Green I dye binding; a 5′-exonuclease probe (*Taq*man-like), and a pair of FRET probes. The FRET-probe method provided the highest specificity.

6. Future Challenges

Perhaps the biggest challenge regarding the use of PCR-based assays for brucellosis diagnostics will be establishing the best sample material to use and the best method of preparing the sample for optimal sensitivity and specificity. To gain acceptance, sampling procedures must be simple to perform and free of cross-contamination. Currently, serological screening of blood at market satisfies this requirement. However, as countries approach and gain eradication status, false-positive serology caused by cross-reacting LPS from other bacteria becomes an increasingly significant problem. The high specificity exhibited by PCR-based assays may promote these tests to the first choice in the diagnostic toolbox.

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Chapter 3
Polymerase Chain Reaction: A Powerful New Approach for the Diagnosis of Human Brucellosis

Juan D.Colmenero*, María I.Queipo-Ortuño and Pilar Morata

From: *Brucella:* Molecular and Cellular Biology. Edited by: Ignacio López-Goñi and Ignacio Moriyón

Abstract

Brucellosis is a zoonosis transmittable to humans that shows a high degree of morbidity. More than 500,000 new cases of human brucellosis are reported each year, and according to the World Health Organisation, this figure underestimates the magnitude of the problem. Owing to the heterogeneous and poorly specific clinical symptomatology of the disease, its diagnosis always requires laboratory confirmation. The conventional microbiological methods used for the diagnosis of human brucellosis have important limitations. Although blood culture provides the best results in microbiological diagnosis, its sensitivity is considerably reduced in patients with long-term clinical courses or with focal complications. Furthermore, blood cultures are time-consuming and handling of the microorganisms represents a high risk for laboratory personnel, since *Brucella* spp. are Class III pathogens. Serological diagnosis lacks specificity in areas where the disease is endemic and in those persons exposed professionally to *Brucella*. Moreover, cross-reactions with other bacteria can also occur. In order to overcome some of the limitations of these conventional techniques, assays based on the polymerase chain reaction (PCR) have been proposed as a very useful tool for the diagnosis of human of brucellosis. Our group has recently reported that PCR methods applied to blood samples provide better results than conventional culture techniques for the diagnosis of both primary infection and relapses, as well as for focal complications of the disease. In the present Chapter, we review the current status and future contribution of PCR techniques for the diagnosis and follow-up of human brucellosis.

1. Introduction

1.1. Epidemiology of Human Brucellosis

Brucellosis is a zoonosis distributed worldwide. *Brucella* species are Gram-negative, facultative intracellular bacteria that infect humans and animals. The high degree of associated morbidity, both for animals and humans, is an important cause of financial loss and represents a serious public health problem in many

* For correspondence email jdcolmenero@telefonica.net

developing countries (Corbel, 1997). In developed countries, the animal disease has been brought under control, which has led to a subsequent decrease in the number of human cases. The occurrence of the disease in humans is largely dependent on the animal reservoir. Where brucellosis exists in sheep and goats, its incidence in humans is greatest. Brucellosis is transmitted through contaminated and untreated milk and dairy products and by direct contact with infected animals (cattle, sheep, goats, pigs, camels, buffaloes, wild ruminants and, very recently, seals), animal carcasses, and abortion materials. Millions of individuals are at risk throughout the world, especially in developing countries where the infection in animals has not been brought under control, procedures for the heat treatment of milk (e.g. pasteurisation) are not routinely applied, and nutritional habits such as consumption of raw milk and poor hygienic conditions favour human infection. Under such conditions, transmission to humans occurs frequently (Anonymous WHO, 1997). Although the current incidence of brucellosis in developed countries is low, it occurs sporadically in occupationally exposed groups, including farmers, veterinarians, and laboratory and slaughterhouse workers. The growing phenomena of international tourism and migration, together with the variable incubation period of the disease, may cause symptoms to develop late, far from where the infection was actually contracted (Arnow *et al.*, 1984; Chomel *et al.*, 1994). Although human brucellosis is a notifiable disease in many countries, official figures do not fully reflect the number of people infected each year and reported cases largely underestimate the size of the problem. Cases very often remain unrecognised because of inaccurate diagnosis, and are thus mistakenly treated as if they were other infectious diseases (Anonymous WHO, 1997).

1.2. Diagnosis of Human Brucellosis

Owing to its heterogeneous and poorly specific clinical symptomatology, the diagnosis of brucellosis always requires laboratory confirmation, either by isolating the pathogen or by demonstration of high titres of specific antibodies or seroconversion. However, all these methods have serious limitations (Kiel *et al.*, 1987).

Peripheral blood is the clinical sample most commonly used for the isolation of *Brucella* spp. In acute forms produced by *B. melitensis*, the yield of blood cultures is usually high, reaching 70–80% of cases (Ariza *et al.*, 1995). However, this figure is notably reduced in cases of long evolution, in those patients with focal complications (e.g. meningitis, endocarditis, spondylitis and orchiepididymitis), and in infections caused

by *B. abortus* and *B. suis*, where the percentage of culture positive results rarely surpasses 30–50% (Colmenero *et al.*, 1996). Additional shortcomings of the method are that blood culture is a time-consuming procedure requiring prolonged incubation (Yagupsky, 1999), and that *Brucella* spp. are Class III pathogens, the handling of which poses a considerable threat to laboratory personnel (Yagupsky, 2000).

A large number of tests have been used or proposed for the serological diagnosis of brucellosis, thus demonstrating the lack of an ideal technique. The sensitivity of these serological tests ranges from 65 to 95%, but their specificity in endemic areas is low because of the high prevalence of antibodies in the healthy population. Moreover,

Table 1. Target gene and primers used to detect *Brucella* DNA

Gene target	Primers	Sequence	Reference
31 KDa *B. abortus* protein	B4/B5	TGGCTCGGTTGCCAATATCAA/ CGCGCTTGCCTTTCAGGTCTG	Baily *et al.*, 1992
Outer membrane protein *B. abortus* (*omp*2)	JPF/JPR	GCGCTCAGGCTGCCGACGCAA/ ACCAGCCATTGCGGTCGGTA	Leal-Klevezas *et al.*, 1995
B abortus 16S RNAr	Ba148–167F/ Ba928–948R	TGCTAATACCGTATGTGCTT/ TAACCGCGACCGGGATGTCAA	Herman and De Ridder, 1992
B. abortus 16S RNAr	F4/R2	TCGAGCGCCCGCAAGGGG/ AACCATAGTGTCTCCACTAA	Romero *et al.*, 1995
Outer membrane protein *B. abortus* 43 kDa	NP[a]	NP[a]	Fekete *et al.*, 1990

[a]NP: Not published

Table 2. PCR results with DNA from different *Brucella* strains and from bacteria antigenically or genetically related to *Brucella* spp. (Casañas *et al.*, 2001)

Bacterium (n° of isolates)	Strain	Origin[1]	PCR
B. melitensis biovar 1	16 M	FMV	+
B. melitensis biovar 1	Rev1	CAJA	+
B. melitensis biovar 2	63/9	FMV	+
B. melitensis (5) biovar 2		FMV(clinical strain)	+
B. melitensis biovar 3	Ether	FMV	+
B. melitensis (6) biovar 3		FMV(clinical strain)	+
B. abortus (2) biovar 1		FMV(clinical strain)	+

B. abortus biovar 1	B19		CAJA	+
B. abortus biovar 2	86/8/59		FMV	+
B. abortus biovar 3	Tulya		FMV	+
B. abortus biovar 4	292		FMV	+
B. abortus biovar 5	B3196		FMV	+
B. abortus biovar 6	870		FMV	+
B. abortus biovar 7	63/75		FMV	+
B. abortus biovar 9	C/68		FMV	+
B. suis biovar 1	10036		FMV	+
B. suis biovar 2	10510		FMV	+
B. suis biovar 3	10511		FMV	+
B. suis biovar 4	40		FMV	+
B. suis biovar 5	10980		FMV	+
B. neotomae	10084		FMV	+
B. ovis	Reo198		FMV	+
B. canis	10854		FMV	+
Escherichia coli	O:157 H:7		CECT	−
Francisella tularensis			FMV	−
Pasteurella multocida			CECT	−
Salmonella urbana			CECT	−
Yersinia enterocolitica	O:9		FFG	−
Agrobacterium radiobacter			CECT	−
Agrobacterium tumefaciens			CECT	−
Agrobacterium vitis			CECT	−
Bartonella bacilliformis			IP	−
Ochrobactrum intermedium		3301	FMN	+
Ochrobactrum anthropi		3331	FMN	+
Ochrobactrum anthropi			CECT	+
Ochrobactrum anthropi			HCH	−
Phyllobacterium myrsincearum			CECT	−
Phyllobacterium rubiacearum			CECT	−
Vibrio cholerae			CECT	−

[1] FMV, Facultad de Medicina, Universidad de Valladolid, Spain; CAJA, Consejeria de Agricultura, Junta de Andalucia, Sevilla, Spain; CECT, Colección Española de Cultivos Tipo, Valencia, Spain; FFG, Facultad de Farmacia, Universidad de Granada, Spain; FMN, Facultad de Medicina, Universidad de Navarra, Spain; IP, Institut Pasteur, Paris, France; HCH, Hospital "Carlos Haya", Málaga, Spain.

most serological tests are sensitive to cross-reactions with other bacteria, and suffer also important limitations in the early phases of the disease. They are also difficult to interpret in persons exposed professionally to *Brucella*, in patients with a recent history of brucellosis, and in patients with relapses (Kiel *et al.*, 1987; Young, 1991; Ariza *et al.*, 1992).

2. Molecular Diagnosis of Human Brucellosis

Amplification of DNA by PCR is used routinely in the clinical laboratory for the diagnosis of several infectious diseases caused by fastidious or slowly growing bacteria or fungi (Bogard *et al.*, 2001; Kami *et al.*, 2001; Kuoppa *et al.*, 2002). Although there are reports concerning the use of PCR techniques to diagnose animal brucellosis (Fekete *et al.*, 1990; Leal-Klevezas *et al.*, 1995; Romero *et al.*, 1995) there is very little information concerning the use of this diagnostic method in human brucellosis. In the last decade, some reports have communicated good results in the application of molecular methods to human brucellosis diagnosis. However, the protocols for sample preparation and the method of detection have not been standardised. Matar *et al.* (1996) first reported the high sensitivity of a PCR method for the diagnosis of human brucellosis. Their study, however, had several limitations: the number of patients included was small, the clinical information was scarce, brucellosis was confirmed bacteriologically in only one patient, and the control group did not include any patient from the groups usually posing problems in the interpretation of the diagnostic tests (i.e., persons exposed professionally, with a recent history of brucellosis, or with anti-*Brucella* antibodies but no evidence of active disease).

2.1. Targets and Primers

As with any other microorganism, PCR methods to detect *Brucella* DNA can use different target primer pairs, and varying PCR conditions and extraction procedures (Romero *et al.*, 1995; Da Costa *et al.*, 1996; Gallien *et al.*, 1998; Bricker, 2002; Navarro *et al.*, 2002). Since identification of *Brucella* at the species level is not necessary to initiate therapy in human brucellosis, it is possible to use a genus-specific PCR assay. In all our studies, we have used as a target the gene coding for BCSP31, an immunogenic membrane protein of 31 kDa of *B. abortus*. This gene is specific to the *Brucella* genus and is present in all biovars. It was first described and cloned by Mayfield *et al.* (1998), and its cognate B4 and B5 primers have been described by Baily *et al.* (1992). Other target sequences and primer pairs have been used for the amplification of *Brucella* DNA (Table 1), but the clinical experience with them is still reduced.

To evaluate the specificity of the B4 and B5 primers, we carried out a study with DNA from all *Brucella* classical species and biotypes plus the two vaccine strains currently used. Moreover, we also included DNA from a broad panel of micro-organisms that are serologically, phylogenetically or clinically related to *Brucella* (Table 2). All the strains of the *Brucella* spp. showed clear amplification. With the exception of *Ochrobactrum intermedium*, no similar amplification products were detected with any of the micro-organisms included in Table 2, all related genetically or antigenically to *Brucella* (Casañas *et al.*, 2001). These results are similar to those reported previously by Romero *et al.* (1995) and Da Costa *et al.* (1996). Moreover, there were no cross-reactions with a wider panel of other micro-organisms, including some intracellular bacteria that show a clear tendency to cause bacteriemia, and others able to produce clinical pictures involved in the differential diagnosis of brucellosis. The existence of a cross-reaction in our PCR assay with *Ochrobactrum* spp. is not surprising when we consider that these bacteria are the closest known relatives of *Brucellae*. However, this cross-reaction has very little clinical relevance. *Ochrobactrum* spp., formerly in CDC group Vd, comprise a group of very ubiquitous micro-organisms, which appear to be distributed world-wide. Although the ecological niche of this micro-organism is not well known, it has been isolated from soil, water, multiple hospital materials, and different clinical specimens, and may be part of the normal microbiota of the large intestine. Thus, it seems to occupy a microbial niche similar to that of *Pseudomonas aeruginosa*, although its pathogenic role remains poorly understood. Since the first infection by *Ochrobactrum* was reported in the form of a pancreatic abscess in 1980, only forty-five cases have been described in the literature. Almost all of these cases have occurred in severely immunosuppressed patients or in those with debilitating illnesses, and the infections reported have been nosocomial, in patients with catheters or other foreign bodies (Gransden *et al.*, 1992; Ezzedine *et al.*, 1994). Thus, from a clinical point of view, this is a scenario very different from that of brucellosis which, with the exception of infections in laboratory personnel, is always a community-acquired infection, generally affecting immunocompetent individuals.

Whole blood and several blood fractions, including, mononuclear leukocytes and serum can be used to identify *Brucella* DNA in brucellosis patients. As the members of the genus *Brucella* are facultative intracellular pathogens and the inoculum found in brucellosis patients is normally very small, we used whole blood samples in our initial studies. This sample, although made technically more difficult by the presence of inhibitors in blood, has the advantage of providing the maximal amount possible of bacterial DNA.

2.2. Conventional PCR Methods

In an open study, we analysed the performance of a single-step peripheral-blood-based PCR assay in 47 patients representing 50 episodes of brucellosis. As controls, we used a group of 60 individuals composed of patients with febrile syndromes of aetiologies other than brucellosis, asymptomatic subjects that were seropositive for *Brucella* and healthy subjects. The sensitivity was 100%, since the PCR assay correctly identified all 50 episodes of brucellosis, independently from the duration of the disease, the result of the blood culture or the presence of focal forms. The specificity of the assay was 98.3% and

the only false-positive was a patient who had had brucellosis two months before and had possibly a self-limiting relapse (Queipo-Ortuño *et al.*, 1997). Clear visualisation of PCR-amplified fragments was possible upon electrophoresis in agarose gel. These results are especially important if we consider that 25.5% of those brucellosis cases presented focal forms, and that 22% had clinical pictures corresponding to more than one month evolution, two clinical situations associated with a lower number of circulating *brucellae*.

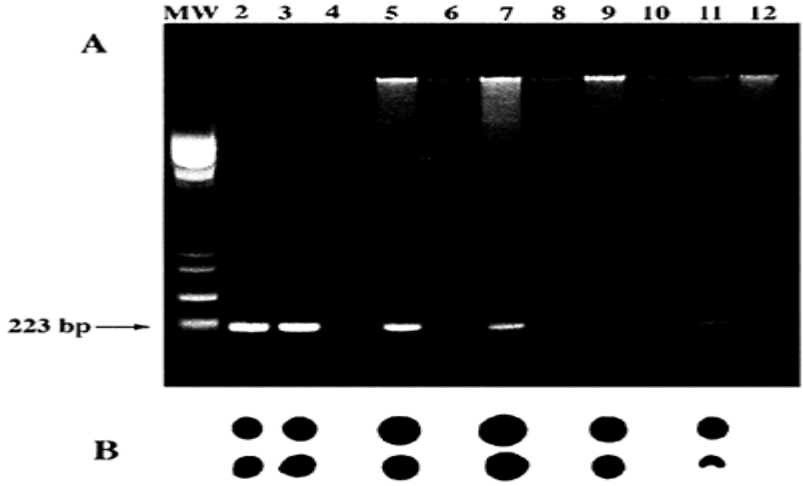

Figure 1. (A) Agarose gel electrophoresis and ethidium bromide staining of PCR products. Lanes 2 and 3: positive controls with *B. abortus* B-19 and *B. melitensis* Rev1 DNAs, respectively. Lane 4: negative control with no DNA. Lanes 5 and 7: DNAs from two patients with brucellosis and positive blood culture. Lane 6: DNA from a healthy subject. Lane 8: DNA from a patient with a bacteremia due to *E. coli*. Lanes 9 and 11: DNAs from two patients with active brucellosis but negative blood culture. Lane 10: DNA from a patient with psittacosis. Lane 12: DNA from a patient with past brucellosis, without evidence of active disease and high serological titers of *Brucella* antibodies. MW=DNA ladder

molecular marker. (B) Dot-Blot hybridisation. The PCR products from positive controls and patients with brucellosis hybridize to fluorescein-labeled probe BR-1, demonstrating that these samples contained DNA from *Brucella*. No hybridisation was observed in any patient from the control group. Duplicated samples were used in all cases. From Queipo-Ortuño *et al.,* 1997.

The high sensitivity of the assay probably relates to two facts. First, the analytical sensitivity, since this method was able to detect 10 fg of bacterial DNA. This amount of DNA is equivalent to two bacterial cells, the number possibly present in one ml of peripheral blood from patients with clinical brucellosis. Second, this was an open study with control groups and, therefore, those results that were doubtful could be repeated and confirmed by dot-blot hybridisation (Figure 1). An excess of host DNA and heme compounds can inhibit the PCR signal. Accordingly, we optimised the assay by decreasing the amount of total DNA to 2 to 4 µg and simplifying the washing phase in the process of DNA extraction by using H_2O_2 (Morata *et al.,* 1998; Queipo-Ortuño *et al.,* 1999). In a blind study performed with the improved protocol and in which 52 new patients with brucellosis were included, we confirmed that the sensitivity of our conventional one-step PCR assay was 92.3%. This sensitivity was higher than that of blood cultures, which were positive in only 69.2% of cases.

Almost one third of all cases of brucellosis present focal complications, either initially or during the course of the disease. The focal complications can affect any organ or system. This explains why these patients are not always seen by specialists in infectious diseases, so that many other medical and surgical specialists are involved in the care of these patients (Samra *et al.,* 1993; Young, 1995; Colmenero *et al.,* 1996). The diagnosis of focal forms of brucellosis is sometimes difficult, as the yield of conventional cultures in non-blood samples is as low as 10–40% of all cases (Lifeso *et al.,* 1985; Khateeb *et al.,* 1990). Moreover, as *Brucella* spp. are slow-growing pathogens, culture requires prolonged incubation, which can at times lead to excessive delays in diagnosis. Serological diagnosis lacks adequate specificity in areas where the disease is endemic and its results are difficult to interpret in some slowly evolving focal forms (Ariza *et al.,* 1992).

In order to evaluate of our PCR assay in the diagnosis of focal complications of brucellosis, we studied by both PCR and conventional microbiological techniques 34 non-blood samples from 32 patients with different focal forms of brucellosis. These samples were synovial fluid (8 cases), pus from abscesses (5 cases), urine (5 cases), cerebrospinal fluid (5 cases), vertebral or other bone tissue (4 cases), sputum (2 cases), renal cyst fluid (2 cases), and pleural fluid, renal tissue and thyroid tissue (1 case of each). The controls were an equal number of samples from the same materials or anatomical locations but with a different aetiology. Owing to their heterogeneous nature,

the volume extracted and the DNA extraction process was different according to the type of sample studied. Thirty-three of the 34 non-blood samples from the patients with brucellosis had a positive PCR result (97%), whereas *Brucella* spp. were only isolated in the cultures from the same samples in 29.4% of cases. A clear visualisation of PCR-amplified fragments was possible in positive cases (Figure 2) (Morata *et al.*, 2001). The explanation for this low yield of cultures appears to relate to the lack of a suspicion of brucellosis and the wide use of antimicrobial therapy in patients with no etiological diagnosis rather than to the difficulty of isolating *Brucella* spp. in clinical samples. The high sensitivity of the PCR assay, even in those patients with prior antibiotic therapy, seems to relate to the high detection capacity of the technique. Moreover, PCR is able to amplify intramacrophagic pathogens, as well as pathogens which are damaged or non-viable as a result of previous treatment and which would be impossible to isolate in conventional cultures.

Besides the high performance of the PCR assay in the diagnosis of focal complications in brucellosis, other aspects make it especially attractive for this type of cases. First, PCR is fast to perform, providing results in 24 hours, which is a time much shorter than that required in conventional methods to rescue fastidious micro-organisms such as *Brucella*. Second, the technique almost completely obviates the need for a direct handling of the pathogen, thus drastically reducing the risk of infection in laboratory personnel. Third, the sample can be stored at −20°C until processing, thereby enabling it to be collected by any physician and processed immediately or else stored and safely sent to another laboratory if required.

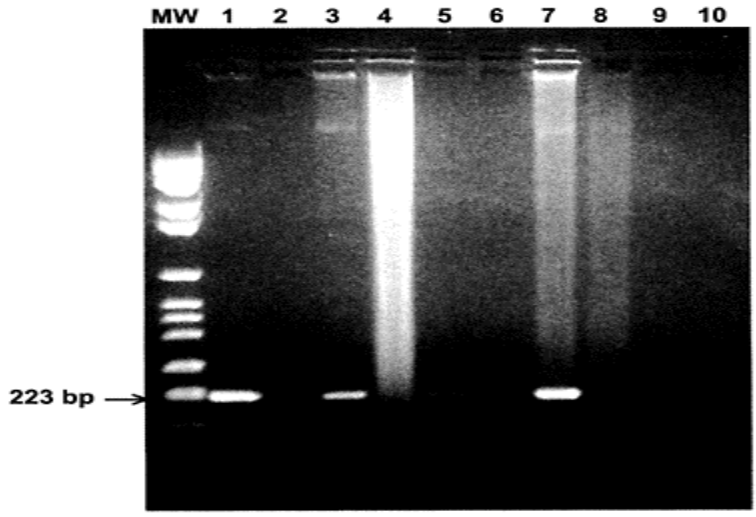

Figure 2. Agarose gel electrophoresis and ethidium bromide staining of PCR products. Lane 1: positive control with *B. abortus* B-19 DNA. Lane 2:

negative control with no DNA. Lane 3: synovial fluid from a brucellosis patient with knee arthritis. Lane 4: synovial fluid from a patient with knee arthritis due to *S. aureus*. Lane 5: urine sample from a patient with orchiepididymitis due to *B. melitensis*. Lane 6: urine sample from a patient with *E. coli* pyelonephritis. Lane 7: sample of pus from a liver abscess due to *B. melitensis*. Lane 8: sample of pus from a liver abscess due to *E. coli*. Lane 9: CSF from a brucellosis patient with meningitis. Lane 10: CSF from a patient with meningitis due to *M. tuberculosis*. MW=DNA ladder molecular marker. From Morata *et al.*, 2000.

One of the main characteristics of brucellosis is its marked tendency to relapse after treatment. This problem is related to the ability of *Brucella* spp. to elude some of the basic mechanisms of the host immune system (Orduña *et al.*, 1991; Ocon *et al.*, 1994). Even after a correct treatment, the incidence of relapses in brucellosis remains high, ranging from 4 to 22% of cases in the largest series reported to date (Ariza *et al.*, 1995; Solera *et al.*, 1998). Since almost 90% of relapses occur during the six months following conclusion of treatment, strict follow-up is necessary during this period to detect any relapse as soon as possible and instigate adequate therapy. However, a high proportion of brucellosis patients report non-specific symptoms after conclusion of their treatment and since there are no clear-cut criteria to establish complete recovery from brucellosis, it is often difficult to decide whether these patients are really cured. In general clinical practice, the post-treatment follow-up of patients with brucellosis includes the appropriate clinical examination together with blood cultures and serological tests.

Clinical manifestations of relapsed brucellosis are milder, overlapping, and non-specific, with the haematological and biochemical changes being even subtler than in the initial infection. The diagnosis in relapses is, therefore, generally difficult, and microbiological techniques are often required for confirmation. However, the yield of blood cultures in relapses is no higher than 60–70% (Ariza *et al.*, 1995; Solera *et al.*, 1998), and the value of serological diagnosis in this situation is limited (Pellicer *et al.*, 1988). Because of the preceding reasons, we evaluated the usefulness of a PCR-based assay in the post-treatment follow-up and the early diagnosis of relapses in brucellosis patients. To this end, we studied a cohort of 30 patients diagnosed of brucellosis and treated with antibiotics accordingly. These patients were examined at the end of treatment and after two, four, and six months, as well as at intermediate times when a relapse was

suspected. At each evaluation, the following tests were carried out: blood cultures, rose Bengal test, Wright's seroagglutination test, Coombs' test and PCR assay. At the time of diagnosis, the PCR assay was positive in 29 of the 30 patients (96.6%). On concluding the treatment, this assay was negative in 28 of the 29 patients (96.5%) whose PCR was initially positive. The only patient in whom the PCR remained positive at the end of treatment was asymptomatic and the blood cultures were negative. The patient received no additional treatment and remained asymptomatic, the PCR becoming negative in the following revision and remaining so in all the subsequent controls performed during the follow-up period. Six patients (20%) had symptoms suggestive of relapse during the follow-up period. Of these, two (6.6%) had a confirmed relapse 2 and 5 months after concluding the treatment, and in the other four (13.3%) relapse was eventually ruled out. Both patients with relapses had a positive PCR, but only one had positive blood cultures again. PCR was negative in all four patients in which a relapse was discarded. The PCR in the two patients with relapses became negative again after concluding the treatment for the relapse and, as with 27 of the remaining 28 patients (96.4%), remained persistently negative during the whole follow-up period (Morata *et al.*, 1999). These results show that, although a few patients may have a positive result at the end of treatment, PCR is more sensitive than conventional microbiological methods, not only for the diagnosis of a first episode of infection but also for post-therapy follow-up of the disease and the early detection of relapses. It seems, however, necessary to be cautious in interpreting its results in individuals that are permanently exposed.

2.3. ELISA-PCR Assay

Despite its high diagnostic yield, interpretation of the results of conventional PCR assays is somewhat subjective. It requires normally agarose gel electrophoresis, as well as handling of toxic products such as ethidium bromide, and sometimes Dot or Southern-Blot analysis for interpretation of uncertain results. These steps are often time-consuming and poorly suited for use in general diagnostic laboratories (Fredricks *et al.*, 1999). In an attempt to circumvent these difficulties, we developed a microplate PCR-hybridisation assay (PCR-ELISA) and evaluated its diagnostic performance in peripheral blood samples from patients with brucellosis.

In this PCR-ELISA, the amplification target sequence, primers and DNA extraction procedure from whole blood samples were the same as in our previous studies with conventional PCR. For the detection of PCR products by PCR-ELISA, we used commercial streptavidin-coated plates prepared in large lots with minimal well-to-well variations, provided stabilised and dry-packed to avoid day-to-day variations. After an incubation period of 30 min at 37°C, the plates were washed twice. Then, an anti-digoxigenin Fab/peroxidase conjugate diluted 1:3,500 in a peroxidase stabiliser buffer was added to each well and plates incubated for 30 min at 37°C. Colour was developed by the addition of tetramethylbenzidine, and the A450 nm was measured in an ELISA reader. Positive and negative controls were included in each assay, and the result was taken as positive if the mean optical density value was more than three standard deviations above the mean value for the healthy controls.

The reproducibility of this PCR-ELISA was very good and its analytical sensitivity was as good as the combined use of conventional PCR and Southern hybridisation. In

fact, correct detection of 10 fg of bacterial DNA was possible in spite of the presence of high concentrations of leukocyte DNA. In 59 peripheral blood samples from 57 patients with active brucellosis and 113 control samples, this PCR-ELISA was 94.9% sensitive and 96.5% specific, whereas the sensitivity of the blood culture was only 70.1% (Morata *et al.*, 2003).

In this assay, detection of the PCR product is rapid, easy and objective, and does not require electrophoresis apparatus, UV transiluminator or darkroom. Moreover, the technique allows simultaneous handling of a large number of samples and automatisation. Like conventional PCR, it yields a few false negative results in patients with positive blood cultures. However, this is hardly surprising since the amount of blood in each blood culture bottle is 8–10 ml, a much greater volume than the 0.5 ml sample used in the amplification. Sample size is a major limitation for PCR based assays, so, the use of very small samples from patients with low concentrations of circulating brucellae could result in the absence of amplicon in the sample to be studied (Zambardi *et al.*, 1995).

2.4. Real-time PCR Methods

Despite the apparent advantages that PCR-based methods have over conventional microbiological tests for the diagnosis of human brucellosis, their application in clinical practice remains very limited. There are several possible reasons for this. Interpretation of the results of conventional PCR assays is to some extent subjective and sometimes requires Dot or Southern-Blot analysis to clarify uncertain results.

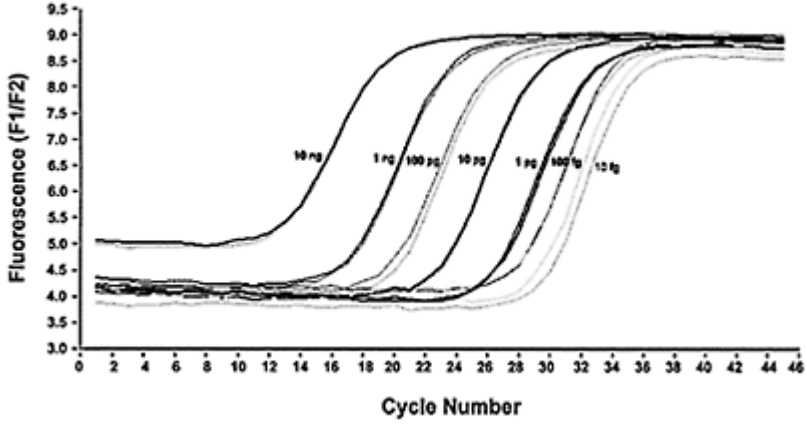

Figure 3. Real-time PCR assay analytical sensitivity. The graphic shows the evolution of the fluorescent signal related to cycle number on a panel with ten fold serial dilutions between 10 ng and 10 fg of *B. abortus* B-19 DNA

Although PCR-ELISA assays could avoid these inconveniences, their processing continues to pose a certain degree of technical difficulty. Furthermore, these assays are often time-consuming and may thus be poorly suited for routine use in clinical laboratories.

Real-time PCR assay technology has recently become available. This assay is more rapid, sensitive and accurate than conventional PCR or PCR-ELISA assays. It is also less burdensome because it uses fluorescent materials, which avoids the handling of the PCR products (Stevens *et al.,* 2002; Leruez-Ville *et al.,* 2003; Reischl *et al.,* 2003). Real-time PCR is an automated amplification technique that quantitatively monitors PCR products as they accumulate during thermal cycling. Currently, different real-time PCR strategies can be used. SYBR Green 1 Double Stranded DNA Binding Dye Assay is a lower-cost alternative when high specificity is not required, and it is specially suited for screening assays. Fluorescence-labeled probes with TaqMan, Molecular Beacons, Fluorescence Resonance Energy Transfer (FRET) and Scorpions can be used when higher specificity is required. Real-time PCR based methods have proved more sensitive than conventional PCR and may achieve a level of assay sensitivity similar to nested-PCR methods without the associated inconveniences of the latter (Pusterla *et al.,* 1999; Lin *et al.,* 2000).

The circulating bacterial inoculum in patients with brucellosis is usually low (Gamazo *et al.,* 1993). Because of this, we always used whole-blood samples in our previous studies with both conventional PCR assay and with PCR-ELISA. Theoretically, the bacterial concentration in this type of sample should be higher than in plasma or serum samples. Thus, in spite of the inconvenience of the presence of *Taq*-polymerase inhibitors, test sensitivity should be better in this type of sample. However, in a study performed with a methodology very similar to ours on 40 brucellosis patients, Zerva *et al.* (2001), reported recently that the diagnostic yield in serum was higher than in whole blood samples. Based on these results and on the high sensitivity of real-time PCR technology, we are working on the development and optimisation of a real-time PCR assay in serum samples. If successful, the method would simplify the molecular diagnosis of human brucellosis and make it more accessible to any clinical laboratory. Preliminary results demonstrate a real-time PCR assay based on the SYBR Green I DNA dye-binding fluorophore is able to amplify 10 fg de *Brucella* DNA in serum artificially spiked with *B. abortus* B 19 DNA (Figure 3). Such small amounts of DNA can expected to be present in serum samples from patients with active brucellosis. Likewise, our initial studies with clinical samples, show that real-time PCR assays correctly amplify *Brucella* DNA in serum samples from 8 patients with brucellosis demonstrated by isolation of *B. melitensis* in blood cultures.

Since real-time PCR assays are performed in a closed tube system, the risk of contamination by product carryover is drastically reduced. Moreover, this type of assay allows simultaneous handling of a large number of samples and it can be automatised, making it very suitable for use in any clinical laboratory. Although these results are preliminary and further studies are necessary, real-time PCR could prove to be a very useful tool thus opening a new era in the diagnosis of human brucellosis.

3. Acknowledgements

These works received financial support from F.I.S. (grant PI020957 and programme Redes Temáticas de Investigación Cooperativa—Promoción de las Regiones, objetivo 2, 2000–2006, Red Temática de Investigación en Brucellosis, G03/204).

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Chapter 4
A *Brucella melitensis* Genomic Database

Xavier De Bolle*, Christophe Lambert, Eric Depiereux and Jean-Jacques Letesson

From: *Brucella:* Molecular and Cellular Biology. Edited by: Ignacio López-Goñi and Ignacio Moriyón

Abstract

The availability of genomic sequence information for the *B. melitensis* 16M and *B. suis* 1330 strains opened the way to a variety of predictions, ranging from the coding sequences delimitation to predictions of function and comparative genomics. In this chapter we briefly summarise the functional predictions made from the putative coding sequences of *B. melitensis* as obtained using homology search algorithms. Comparisons were made to information stored in a database, which also contains predictions of subcellular localisation and tridimensional structure. The conservation of each putative coding sequence in other α Proteobacteria genomes is also reported, allowing the first steps into comparative genomics within this diversified family. The database described here also allows simple genome-wide investigations, and some examples of such analyses are described. The database is user-friendly and would benefit from a close interaction with experimental work.

1. Introduction

There are several genomes currently sequenced for the *Brucella* genus, two of which were published in 2002: the *B. melitensis* 16M and the *B. suis* 1330 strains (DelVecchio *et al.,* 2002; Paulsen *et al.,* 2002). This was a great opportunity for laboratories working with transpositional mutants to quickly locate the transposon insertion site in the mutants displaying an interesting phenotype (Kohler *et al.,* 2002; Lestrate *et al.,* 2003). The availability of complete genomes opened the door to looking at *Brucella* from new perspectives, such as predictions about metabolic pathways or regulatory networks (see also Chapter 7), as well as genomewide analyses. In this chapter, we propose to briefly

review some bioinformatics tools currently available for the *Brucella* genomes focusing mostly on *B. melitensis*.

One of the major predictions to be made from a genomic sequence is the functional annotation of the deduced products of predicted coding sequences (pCDS). The vast majority of functional annotations are made using homologies found between a putative protein sequence and a given database. The detection of a significant homology is an excellent argument to validate the prediction of a CDS in a particular region of the genome. Evolutionary pressure selects for conservation

* For correspondence email xavier.debolle@fundp.ac.be

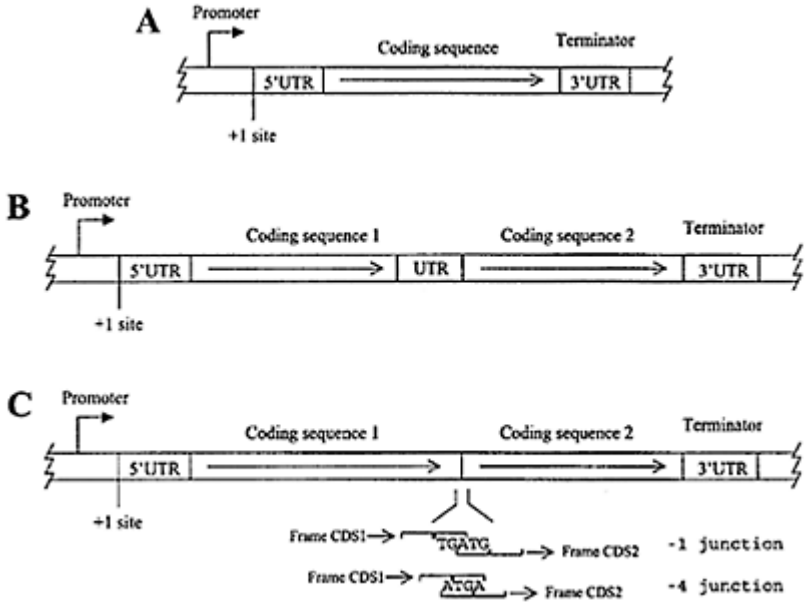

Figure 1. Definition of gene. (A) Description of a bacterial gene containing promoter, +1 site, 5′UTR, coding sequence, 3′UTR and terminator region. (B) Example of operon with two CDS separated by a UTR. (C) Frequent operon configuration with −4 or −1 CDS junction, without UTR between the CDS.

of protein sequences among different species. However, adaptation to different niches selects for modifications of the proteome through the process of evolution, and therefore

proteins may be largely divergent, even when compared between closely related species. This very simple fact is sometimes underestimated, by researchers and leads to incorrect predictions of function. A simple example would be the homology found between a putative protein and a transcriptional regulator; in this example the similarity between these two proteins does not extend along the whole length of the transcriptional regulator with the DNA binding region missing in the putative protein. A simple algorithm will predict that the putative protein is a transcriptional regulator, and of course it is probably not.

In this chapter, we will first concentrate on gene delimitation, and we will describe the database available for the *B. melitensis* 16M genome, with the predictions currently available and the tools allowing searches in this database. Some genomewide analyses will be presented and perspectives relative to the predictions and (post-)genomic data management.

2. Genes Delimitation

Starting with a rough nucleotide sequence, genome annotation is the first step, probably the most important step, in the analysis process. In the genomic annotation of a bacterial genome, there may be multiple problems, the first being that the annotation process is made using data that are not accessible to the scientific community, even after publication. Therefore, some or numerous annotations may not be justified for the expert user. Moreover, the start codon prediction is likely to be incorrect in approximately 25% of the cases. This is of course a major problem for undertaking genomic based approaches such as ORFeome building, the ORFeome being a collection of pCDS cloned into vectors allowing directional cloning using recombinases (Reboul *et al.,* 2003). A large-scale study involving five European laboratories was necessary to correct the predicted start codons in the *B. melitensis* 16M genome.

"What's a gene?" is probably the first question that should be addressed when considering the gene delimitation problem. Therefore let's start with some definitions (Figure 1). A gene may be defined as a part of the genome having a function that can be changed by mutation. If one considers a known protein-coding gene that is not in operon, four successive regions may be predicted as follows: the promoter, the 5′ untranslated region (UTR), the coding sequence (CDS), the 3′ UTR and the terminator region. If a mutation affects promoter function, it also affects gene function, therefore the promoter is part of the gene. A gene is not only a CDS, and a CDS is not a gene. In operons, the CDS may be separated by an UTR region (Figure 1B), but this UTR is often absent (Figure 1C). There are two kinds of frequent junctions between CDS in operons, the −4 junctions and the −1 junctions (Salgado *et al.,* 2000). As depicted in Figure 1C, the sequence of the stop codon of the previous CDS is fused to the start codon of the next CDS.

Knowing the definition of a gene, how can we predict its delimitation? Currently, the prediction of promoter regions and particularly +1 sites is not reliable. The prediction is therefore limited to CDS, giving predicted CDS (pCDS). Here again, a clear distinction must be made between a CDS and an open reading frame (ORF). Conceptually, an ORF is a reading frame that is not interrupted by a stop codon, while a CDS is coding for a protein, and thus begins with a start codon and ends at a stop codon. The pCDS detection

is made by the delimitation of ORFs, and then by the positioning of a putative start codon. Usually, programs like Glimmer 2.0 (Salzberg *et al.,* 1998) are first trained with a collection of the longest ORFs, truncated to the first possible start codon. The underlying theory is that long ORFs are unlikely to occur by chance, and therefore long ORFs constitute a reliable training set. This program was validated with genomes having a normal GC content, like *Escherichia coli* K-12. The problem with genomes with high GC content such as the *Brucella* genomes, is that when a coding sequence is present in one frame, there is usually an ORF on the reverse frame, e.g. if there is a CDS in frame +1, there is an ORF in frame −1 (see Figure 2 for examples extracted from the *B. melitensis* 16M genome). This phenomenon is due to the fact that a high GC content is linked to a particular codon bias, in which the third position of the codon is very frequently a G or a C, if possible. Therefore the first position of the codon in the "reverse" frame (e.g. frame −1 if frame +1 is a coding sequence) is rarely a A or a T. Since all three stop codons have a T as the first base, it is logical that in high GC genomes, the proportion of stop codon is low in the reverse frames relative to predicted CDS. If long ORF are used as a training set of the hidden Markov models (HMM) analyses, there may be a strong bias and the final result must be treated

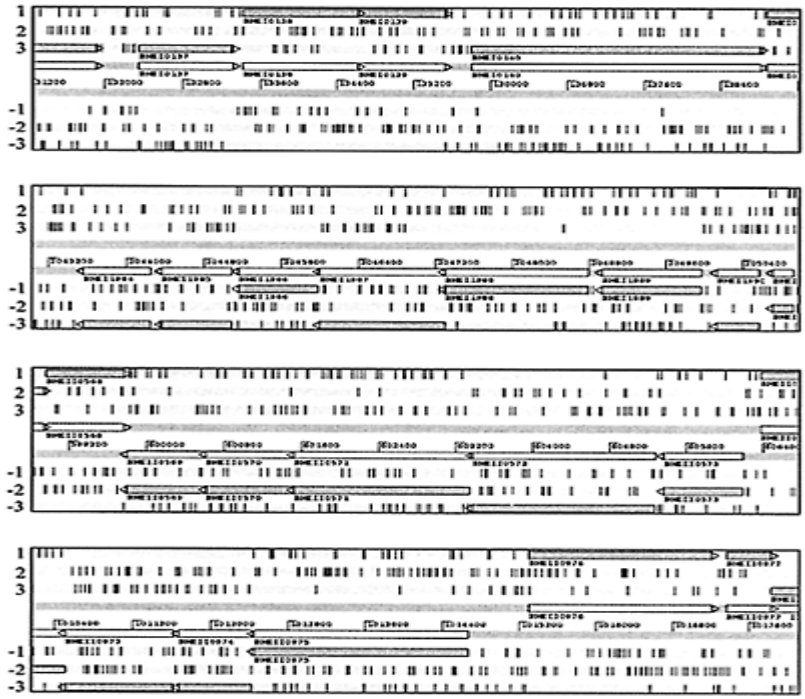

Figure 2. Example of the problem when assigning coding sequences in *B. melitensis:* when there is a pCDS in frame +x, there is generally an ORF in

frame −x (with x ranging from 1 to 3). The pCDS are indicated as grey boxes in the 3 forward (up) and 3 reverse (down) frames. The small vertical lines in each reading frame are stop codons.

carefully. If a HMM is trained with pCDS displaying strong homologies with pCDS described elsewhere or if a HMM is trained on pCDS of a close homologue (*Sinorhizobium meliloti* for example), then the Markov models may be used to identify frames in which a pCDS may be found.

Assigning the position of the correct start codon is often the subject of discussion. In the absence of experimental data, statistical data may be helpful in the prediction of the most likely start codon. The first criterion is the presence of a ribosome binding site (RBS), whose sequence is close to AGGAGGU (usually

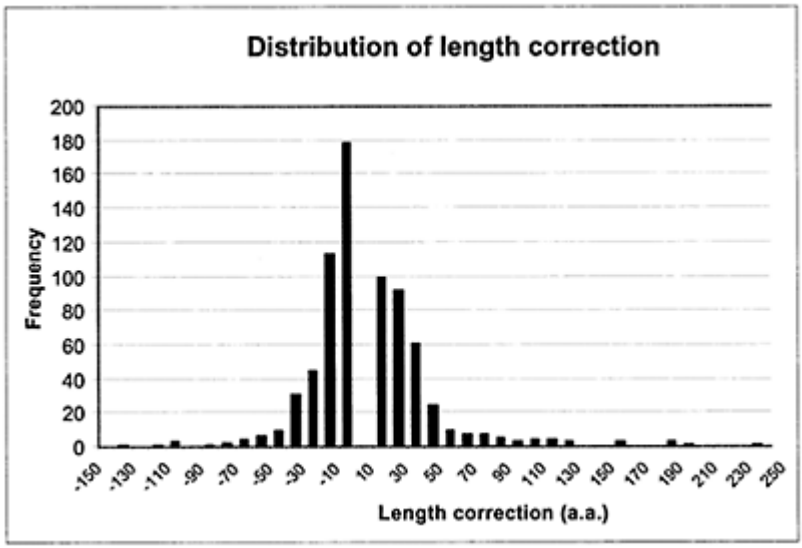

Figure 3. Frequency distribution of the length corrections when modifying the start position for the 908 corrected pCDS of *B. melitensis;* 565 pCDS were shortened and 334 have been lengthened.

AGGA, GGAG or GAGG). If the pCDS is part of an operon, the −1 and −4 junctions are very frequent, and the length between two subsequent pCDS is not expected to exceed ~40 nt (Salgado *et al.*, 2000). The presence of homologues systematically having a larger

size is also an argument for trying to find a more likely start site. One of the difficulties with predicting the most likely start site is linked to the nature of the start codon0, which may be AUG, GUG, UUG or, extremely rarely, CUG. The AUG is more frequent than GUG, which is more frequent than UUG. Given the complexity of the problem, it is not surprising that simple automatic algorithms do fail to correctly predict the most likely translational start sites. A consortium of 5 European laboratories reviewed the predicted translational start sites in the *B. melitensis* 16M genome. A total of 730 corrections were introduced in the database that will be described later in this chapter; this represents 23% of the pCDS of the *B. melitensis* 16M genome. This first round of corrections was not perfect, but it yields a new version of the pCDS collection that is worth working with. The majority of the pCDS length changes upon correction were less than 50 bp (Figure 3), but some corrections were expanded some pCDS by 300 nt.

3. Database Presentation

The database is available at the http://serine.urbm.fundp.ac.be/~seqbruce/GENOMES/ web site. It is actually a collection of pCDS. For each pCDS, a web page giving the prediction currently made for this putative protein is displayed. The data derived from comparison with databases such as *nr*, *Swiss-Prot* or *Pfam* are updated on a weekly basis. The pCDS pages may be searched using two search engines, the *basic* search and the *advanced* search.

3.1. The pCDS Pages

In the next paragraphs, we will detail each of the various fields of a pCDS page. The first option for the user is to navigate from one pCDS to the previous or to the next pCDS in the chromosome. It is particularly interesting to do this while having the genome open, e.g. using the Artemis free software (Rutherford *et al.*, 2000) available at the Sanger Centre web page (http://www.sanger.ac.uk/).

The first field is the **Function** field. It is divided in four parts, GenBank annotation, Swiss-Prot similarity, Pfam summary and Observed function. The **GenBank annotation** is the functional annotation given in the GenBank files (NC_003317.gbk and NC_003318.gbk files) available at the mailto:ftp://ftp.ncbi.nih.gov/genomes/Bacteria/Brucella_melitensis/ web site. The user must be very careful while using these annotations since, unfortunately, some may be wrong. Below the functional annotation given in GenBank (Benson *et al.*, 2003), a link is available with the result of a blastp search against the *nr* database of peptidic sequences. This link is particularly useful since it gives the blastp result in a much shorter time than the one required by a search at the NCBI blastp site. Analysis of this blast output gives the opportunity to check the GenBank annotation. For example, the BMEI1340 pCDS is annotated as “Phage Host Specificity Protein”, but blastp analysis does not reveal any homologue having such a function.

The major problem associated with the analysis of a blastp search against *nr* is the abundance of homologues, most of them having an uncontrolled annotation. It is therefore interesting to turn to the **Swiss-Prot similarity** field, in which the pCDS

peptidic sequence is compared to the Swiss-Prot database (Bairoch *et al.*, 1999), which is a protein database in which all entries have been reviewed. This means that functional annotations have been reviewed by experts, and therefore the functional annotations are reliable. The major drawback of this search is the gap between the publication of functional data about a given protein, and the annotation of that information in the Swiss-Prot database. Therefore, a lot of proteins with described function are not included in the Swiss-Prot database. The user must be particularly careful while analysing the blastp results. Indeed, the expected value (e-value, the number of expected matches for a given protein in a given database) must be critically evaluated for each sequence comparison available, and it is generally admitted that similarities with e-value greater than 10^{-3} may not be interpreted as real homologues. It is also important to examine the alignment between the sequence deduced from a pCDS (query sequence) and a sequence extracted from a database (subject sequence), since the subject sequence may be homologue only for a part of its length to the query sequence. Therefore the functional annotation cannot be transferred directly from the subject sequence to the query sequence without a careful examination.

Another interesting field for the prediction of function is the **Pfam summary**. Each pCDS of the *B. melitensis* database is compared to the Pfam database (Bateman *et al.*, 2002). This Pfam database contains a collection of common protein domains, usually with known or predicted function. These domains are deduced from multiple alignments and HMM analyses. Each pCDS-deduced peptidic sequence is searched against the Pfam database, and the similarity between the query sequence and a given domain is quantified by a score and an e-value similar to the one produced by blastp, as described in the previous section. In the Pfam summary field, the domains having a low e-value are listed, with their score, their e-value and the number of occurrences (N) of this domain in the query sequence. The Pfam summary sometimes gives the opportunity to propose a putative function for a sequence that does not share similarity with a protein of known function. It does also allow searches to identify all proteins able to perform a given function, or belonging to a particular family.

The **Observed function** field is a space in which users may add their experimental data, which could help to propose a function for that pCDS. It is typically in this field that data read in the literature may be added.

In the **Metabolic and regulatory pathway from KEGG** field, a link is available towards the entry of a given pCDS at the KEGG (Kyoto Encyclopedia of Genes and Genomes) database. This allows the user to locate the putative product of that pCDS in a metabolic pathway. For example, the BMEI1301 pCDS, annotated as a dihydrodipicolinate synthase (DapA), may be located in the KEGG metabolic pathway, and the enzymes that are represented in green are those who have a homologue on the basis of the GenBank *B. melitensis* 16M genome annotations. In the case of DapA, it is clear that *B. melitensis* does have the potential enzymatic pathways to synthesise diaminopimelate and L-lysine from L-aspartate. Again, these data must be viewed as a good starting point to generate hypotheses, but it is strongly recommended that the user check the predicted homologies before performing experimental work on this basis.

The **Similarity in close organisms** field allow the user to have a quick overview of the distribution of homologues in the genomes of a Proteobacteria and *E. coli*, as the Gram-negative model. The e-value associated with the best blastp hit in the indicated

genome is presented. A large fraction of the pCDS have a closer homologue (with a low e-value) in the a Proteobacteria genomes (especially in *Mesorhizobium and Agrobacterium*). Some proteins have a homologue only in a Proteobacteria genomes, and this field is a first screen to detect them.

In the two next fields, **Comment** and **Free comment,** there is space for the user to add comments regarding function prediction, e.g. possible errors in any of the other fields, addition of unpublished data, etc… The Comment field has been used while correcting the start codon prediction. These fields will essentially depend on the attitude of users in front of the database. This database needs to be interactively set up, in such a way that it could become a useful tool for the whole *Brucella* community.

The **Cellular localisation** field is divided in **Observed** and **Predicted** parts. Obviously, the cellular localisation is unknown for the vast majority of predicted proteins. The prediction of cellular localisation is performed using the Psort (Nakai *et al.*, 1999) program (http://psort.nibb.ac.jp/). This program is an expert system, analysing the sequence using various programs predicting signal sequence, transmembrane segments, etc… Using a set of "if-then-else" rules, the program produces a prediction of subcellular localisation (essentially inner membrane, outer membrane, periplasmic or cytoplasmic). The compatibility between predicted subcellular localisation and predicted function may be checked for the pCDS of interest.

A short set of simple predictions are made and stored in the **Position and chromosome, GC content** and **Theoretical physico-chemical properties** fields. These data help the user to build statistics as those presented in paragraph 4 of this chapter. The **Cross-references** field allows the user to be redirected towards KEGG and TrEMBL general databases. In the **References** field, users are invited to add bibliographic references that are related to the pCDS treated in this entry. Ideally, each group publishing a paper should introduce the relevant data into the database, for example in the Comment section, with a complete reference in the Reference field.

In the next sections, predictions regarding primary, secondary and tertiary structures are made. In the **Predicted secondary structure** field, the putative sequence is depicted in various colours, black being loops and turns, red being predicted a helices and blue being predicted β strands. These secondary structure elements are predicted using the PSI-PRED2 (McGuffin *et al.*, 2000) method. The transmembrane helices are predicted using the TMHMM2 (Krogh *et al.*, 2001) method (http://www.cbs.dtu.dk/services/TMHMM/), and are indicated in bold underlined characters. It is noticeable that N-terminal transmembrane segments may be signal sequences. In the **3D structure prediction** field, a homology modelling procedure is reported if a protein with detectable similarity has been found in the Protein Data Bank (PDB). The method used for these modelling procedures is ESyPred3D, a method developed by Lambert *et al.* (Lambert *et al.*, 2002). This method is producing a multiple alignment of the query sequence with several sequences from the PDB, and builds a consensus of high reliability. This reliable alignment is subsequently used for the homology model building. The final model may be downloaded on your computer using the "Download 3D model" link. The model may be examined using a freeware such as SwissPDBViewer program freely available from the Expasy web site (http://www.expasy.org/). The 3D structure may also be displayed using a plug in (Chime) available elsewhere. The template (protein of known 3D structure) used for the

alignment and model building is also indicated, and a link is available to have information and co-ordinates of the template structure at the PDB web site. The percentage of sequence actually modelled is indicated. Using this information, the user may know if the complete structure is modelled, or only some domains of the protein.

For each pCDS, the **AA sequence** and the **DNA sequence** are given in a fasta format, allowing the user to quickly use the sequences without reformatting for other prediction tools that would not be available on the *B. melitensis* database website. For users interested in promoter and terminator regions, or those simply interested in looking at the sequences surrounding the pCDS in the genome, a tool is available at the bottom of each pCDS page. It provides an opportunity to display the sequence of *x* nt upstream and *y* nt downstream the pCDS.

3.2. Searching the pCDS Database

There are several ways to search the *B. melitensis* pCDS database. Basically, two different web pages allow searches. The first page is a basic search page, while the second is an advanced search page. The advanced search page may be viewed by using a link available on the basic search page.

In the **basic search** page, the pCDS may be searched according to their number, e.g. BMEI0423. These numbers come from the GenBank file containing the *B. melitensis* 16M genome. The pCDS numbers containing "BMEI" at the beginning are located on the large chromosome (I), while those with "BMEII" are located on the small chromosome (II). The pCDS database may also be searched using a string (i.e. text) search. Various fields of the pCDS pages may be the target of that search: the GenBank annotations, the output files of blastp against *nr* database, the best blastp hits in the Swiss-Prot database, the output files of blastp against Swiss-Prot database, the observed functions, the comments, the predicted or observed cellular localisations, the Pfam summaries or complete results, or the templates used in the 3D modelling procedure. This search tool is very useful, particularly for the generation of pCDS collections sharing the same type of function. For example, if a user is interested in a pCDS potentially able to synthesise cyclic diguanylate (c-diGMP), the name of the domain responsible for that function may be found in the Pfam database (http://pfam.wustl.edu/), the GGDEF domain in this particular case, and the "GGDEF" term may be used to search the pCDS database in the Pfam summary fields. The main advantage of that kind of search is that it is independent of errors or ambiguities in the GenBank annotation process, since the information available in the Pfam summary field originates from a similarity search against the Pfam database.

The next type of search available from the basic search web page is similarity comparison using the Blast programs. A peptidic or a nucleotidic sequence may be compared to the whole genome (either peptidic sequences deduced from pCDS or genomic sequence). Using e-values as a screen to identify close homologues, it is possible to identify closely homologous proteins characterised in bacteria such as *S. meliloti*, *M. loti*, *A. tumefaciens* or *C. crescentus*. This is particularly interesting if the user wants to discriminate between a close homologue (a potential orthologue, i.e. a homologue with the same function in a closely related bacterium) and homologues belonging to the same family. For example, if one searches for a homologue of CtrA in

the *B. melitensis* genome, using the *C. crescentus* CtrA protein as a query sequence, 17 homologues are found using a blastp search against the pCDS peptidic sequences, but the first hit is BMEI0423 with an e-value of 10^{-92}, the other hits being response regulators having e-values larger than 10^{-27}. This tool is therefore also very useful to identify putative orthologs, and in the case of CtrA, experimental data confirm this prediction (Bellefontaine *et al.*, 2002). In other applications such as the identification of transposon insertion site, the sequence of an iPCR product may be used as a query to search the pCDS database or the genomic sequence using Blastn. This tool is also useful to find paralogs (i.e. homologues found in the bacterium, that have presumably arisen by duplication of an ancestral gene).

The last tool of the basic search window is the motif searching in the pCDS. If the user is interested in a particular motif within peptidic or nucleotidic sequences, the motif may be entered and will be searched against all pCDS sequences. For example, if one is searching for putative C-terminal tags of proteolysis, the motif "(L|I|V)AA$" may be searched, following the instructions given at the bottom of the web page. The "$" means that the pattern is searched at the C-terminus of the deduced peptidic sequence of the pCDS. The "(L|I|V)" means that at this position, there may be a leucine, an isoleucine or a valine. This search yields 18 predicted proteins, including a putative histidine protein kinase (BMEI0372) and a probable monodomain response regulator (BMEII0050, incorrectly annotated as "sensory transduction histidine kinase") located close to the genes homologous to *nodVW*

Table 1. Number of pCDS for the *B. melitensis* 16M genome having a close homologue in other α Proteobacteria genomes (conserved genes) and pCDS displaying no detectable homology in these genomes (absent genes)

Bacterial species	**Conserved genes (*e-value* $<10^{-50}$)**	**Absent genes (*e-value* $>10^{-3}$)**
Brucella suis	2,789	28
Brucella abortus	2,779	15
Mesorhizobium loti	1,652	535
Agrobacterium tumefaciens	1,633	567
Sinorhizobium meliloti	1,631	585
Rhizobium leguminasorum	1,432	627
Rhodopseudomonas palustris	1,141	858
Caulobacter crescentus	864	1,112
Escherichia coli	666	1,240

from *Bradyrhizobium japonicum* (Gottfert *et al.,* 1990). It may be suggested that these two proteins are subject to targeted proteolysis.

In the **advanced search** page, it is possible to perform searches involving several criteria. It is, for example, possible to select pCDS predicted to code for inner membrane proteins belonging to the ABC family of transporters. This search is performed by asking "ABC-tran" in the **Pfam summary** field and "inner" in the **predicted cellular localisation** field. Such combined search may help to select a subset of pCDS for further analysis. It is also possible to include quantitative data in the search, by giving an interval of predicted pI, molecular weight, position in the genome, number of transmembrane segments, GC percentage of the pCDS, nucleotidic or peptidic length, percentage of identity with the modelling template or the percentage of the deduced sequence modelled using the ESyPred3D system. All "and' and "or" combinations can be used. The output pCDS are given according to their BME number, and a second term chosen by the user is added. This allow easy import into Microsoft Excel tables using a saved version of the output in a text format, and therefore statistics on a search including all pCDS sequences or a subset of them is possible.

A last search available in this web page is one that allows the identification of pCDS that have a homologue in one or several bacteria but not in others. The target genome and the cut-off e-value may be chosen. For example, it is possible to search for pCDS that, during a blastp search, have a match in *M. loti* and *S. meliloti,* but not in *C. crescentus*. This search may be combined with a criterion defined in the upper part of the web page. Using this advanced search page, it is possible to quickly identify proteins that are very well conserved among α Proteobacteria but absent from the *E. coli* K12 genome.

The tools proposed at the *B. melitensis* database are constructed to facilitate the generation of experimentally testable hypotheses. It is obvious that additional tools are necessary, such as a library of sequence alignments and the data from other genomes such as *B. suis* 1330 and *B. abortus,* but the improvement of the database will depend on the interactions with the users.

4. Predictions From the *B. melitensis* 16M Genome

The availability of an annotated genomic sequence allows simple predictions to be made. In this part of the chapter, we will focus on some of them.

4.1. Comparison With Other α-Proteobacteria

In order to compare the *B. melitensis* 16M genome to the other a Proteobacteria genomes available, we performed tblastn searches, and we considered as conserved genes those sharing an e-value lower than 10^{-50} (Table 1). This analysis confirms that *B. melitensis* 16M, *B. suis* 1330 and *B. abortus* 2308 are extremely similar, since only 28 genes are absent from the *B. suis* genome and 15 genes are absent from the *B. abortus* genome, compared to the *B. melitensis* genome. The pCDS of *B. melitensis* are close to those from a group of three species, *M. loti, A. tumefaciens* and *S. meliloti,* since the *B. melitensis* 16M genome shares ~1,600 highly homologous genes with each of these three species.

As indicated by Paulsen *et al.* (2002), there is also extensive synteny between *Brucella* genomes and *M. loti*.

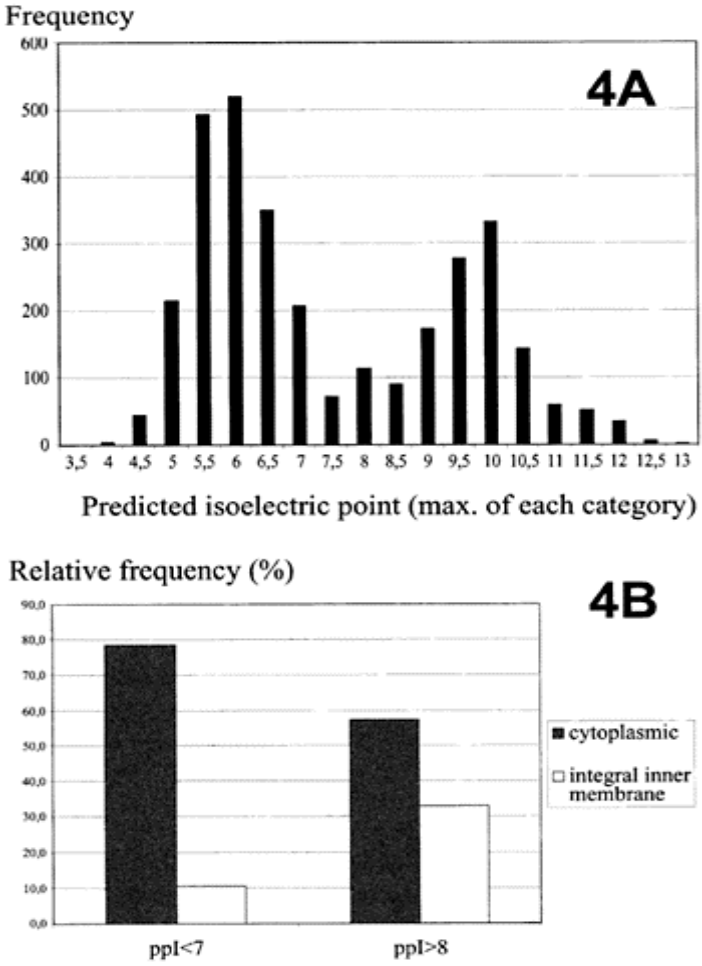

Figure 4. (A) Frequency distribution of predicted pI (ppI), computed for each pCDS of the database. The bimodal distribution was observed for all bacterial genomes tested. (B) Predicted cellular localisation of proteins with ppI greater than 8 and lower than 7. Only 10.6% of proteins having predicted pI lower than 7 are

proposed to be integral inner membrane proteins. On the contrary, 32.9% of proteins with a predicted pI greater than 8 are probable integral membrane proteins.

4.2. Predicted Isoelectric Points

In Figure 4A, we present a frequency distribution of the predicted pI (ppI), computed for each pCDS of the database. It is noticeable that this distribution is bimodal, suggesting the involvement of some evolutionary selective pressure. The interpretation of this phenomenon is unclear at the moment, but it was observed in all bacterial genomes tested to date (C.Lambert and X.De Bolle, unpublished). Thanks to the advanced search tool, we observed that there is a correlation between the ppI category and the predicted localisation. From the distribution presented in Figure 4A, we divided the pCDS into three categories: those with a ppI lower than 7 (1,446 pCDS), those ppI comprised between 7 and 8 (130 pCDS), and those with a ppI higher than 8 (677 pCDS). In the first category, we observed that 78.7% of the pCDS are predicted to localise into the cytoplasm, while 10.6% were predicted to be integral inner membrane proteins. On the contrary, in the third category (ppI>8) 57.5% of proteins are predicted to be in the cytoplasm, and 32.9% are probable inner membrane proteins (Figure 4B). A simple χ^2 test using these proportions demonstrates that this difference is highly significant ($p<0.001$). The correlation between subcellular localisation and bimodal distribution of ppI has also been observed in a previous study (Schwartz *et al.*, 2001). Even if this is statistically significant, it is still not easy to find a simple interpretation for this observation. The charge distribution in the integral inner membrane proteins should be further investigated considering the membrane potential, since they are three times more abundant in the class with ppI higher than 8.

4.3. Hypothetical Proteins

In the *B. melitensis* 16M genome, 688 pCDS are annotated as “hypothetical”. We analysed the conservation of these hypothetical proteins across the other bacterial genomes. We considered as “orthologs” the pCDS that were found with an e-value lower than 10^{-10} in another genome. Among the 688 hypothetical proteins, we found 286 orthologs in *M. loti*, 254 orthologs in *A. tumefaciens*, 249 orthologs in *S. meliloti*, 93 orthologs in *C. crescentus* and only 24 in *E. coli*. This supports a closer evolutionary relationship between *Brucella* and *M. loti*. Interestingly, 295 pCDS among the 688 hypothetical proteins do not have homologues (at an e-value cut-off of 0.001) in the *M. loti*, *A. tumefaciens*, *S. meliloti*, *C. crescentus* and *E. coli* genomes. These 295 hypothetical proteins therefore seem unique to *Brucella*. Note that from the 3,198 pCDS of the *B. melitensis* 16M, only 332 have no ortholog in the genomes listed above, with the same cut-off value, which indicates that most of the pCDS unique to *Brucella* are hypothetical proteins. Analysis of the length of

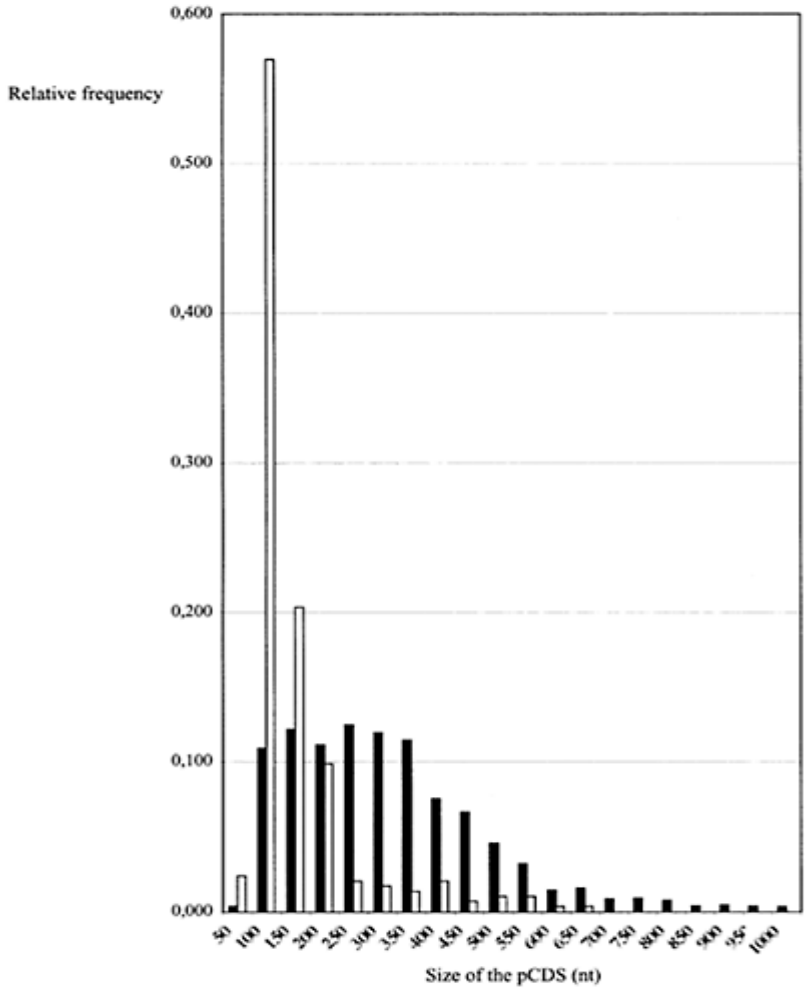

Figure 5. Comparison of the length distribution of all *B. melitensis* pCDS (grey rectangles) and the 295 hypothetical proteins unique to *B. melitensis* (blank rectangles).

these 295 pCDS reveals that their size distribution is very different from the size distribution obtained for the 3,198 pCDS (Figure 5). Actually, ~50% of the smaller pCDS (predicted size <100 aa) of the genome are unique to *Brucella*, according to these data. Two hypotheses have been proposed to explain this observation. First, since these pCDS are shorter, they have not been found in the screens of mutants libraries, and a set of hypothetical pCDS is therefore enriched with small pCDS. A second hypothesis would be

that these pCDS are actually not coding for any protein, and would be false predictions. However, 15 of the 295 pCDS have a hit in the Pfam database. In this set of pCDS, some predicted phage proteins are found (which is in agreement with the absence of a homologue in other related bacteria), as well as proteins putatively involved in virulence, such as a phospholipase (BMEI0401) and a protein homologous to a portion of the Toll-like receptors (BMEI1674).

5. Conclusion and Perspectives

The genomic database for *Brucella* may be viewed as an opportunity for scientists of experimental and bioinformatics fields to interact. The database was constructed according to the needs of the researchers actively experimenting on the molecular biology of *Brucella*. This explains the immature structure of the database, which is rather a collection of files than a structured database. The intensive use of the database will raise new questions, and therefore the generation of new tools to solve these. For example, it is conceivable that experimental data should be added to the system, such as results of microarray experiments or proteomic analyses. Also, the generation of the ORFeome could also be linked to this database, giving information about the availability of entry clones and destination clones for various applications such as high-throughput protein purification and interactomics.

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Chapter 5
Comparative Genomics of *Brucella melitensis, B. suis,* and *B. abortus*

Shirley M.Halling*, Cynthia Gibas and Stephen Boyle

From: *Brucella:* Molecular and Cellular Biology. Edited by: Ignacio López-Goñi and Ignacio Moriyón

Abstract

The genomes of the classical *Brucella* species and their biovars have two chromosomes with the exception of *B. suis* biovar 3 strain 686 which has a single chromosome. The larger chromosome has approximately 2.1 Mbp and has a bacterial origin of replication. The smaller chromosome has approximately 1.2 Mbp and has plasmid replication functions. There is a large inversion within the small chromosome of *B. abortus* biovars 1, 2, and 4. There is a single large unique genetic island among the genomic sequences of *B. melitensis, B. suis,* and *B. abortus*. This island resides in the small genome of *B. suis* and encodes homologs of transfer functions and phage related genes. Given the high similarity among the genomic sequences of brucellae, differences among them with regards to host preference, virulence and infectious cycle could be due to subtle variations in the conserved DNA and differential expression of conserved genes, rather than due to unique genomic DNA fragments. Detailed comparative sequence analysis identified common and unique regions and diverged regions within conserved genes, and suggests sequence targets to be used in a comparative approach to functional genomics experiments.

1. Introduction

Brucellosis is a widespread disease of agriculturally important animals and is the most prevalent bacterial zoonosis. In the host animal, it often manifests itself by abortion. There are six classical species of *Brucella,* named for their host preference. Most of the infections caused by *Brucella* are due to four of the six classical *Brucella* species; *B. melitensis, B. abortus, B. suis* and *B. ovis*. These species of *Brucella* preferentially infect

caprine, bovine, swine and ovine, respectively. *B. canis*, which is found primarily in dogs, is recognised as an emerging disease agent. *B. neotomae*, the sixth recognised classical species, was isolated from the desert rat. *Brucella* have recently been isolated from a wide variety of sea mammals (Foster *et al.*, 2002). Though the *Brucella* are closely related and proposed to constitute biovars of a single species in the bacterial genus *B. melitensis* (Verger *et al.*, 1985), the classical names are still in use. There is an

* For correspondence email shalling@nadc.ars.usda.gov

ongoing discourse among taxonomists on what constitutes a species (Moreno *et al*,, 2002; see also Chapter 1). There is an added dimension to the discussion when the bacteria are intracellular pathogens like *Brucella* that have undergone selective pressure during host adaptation and in isolation from other bacteria.

Taxonomically, the genus *Brucella* is in the class Proteobacteria, subdivision α-2. This subdivision also includes rickettsiae, agrobacteria, and rhizobiae (Moreno *et al.* 1990). These bacteria, like brucellae, have a close association with either plant or animal cells. Some of the bacteria in this subdivision are obligate intracellular pathogens while others are facultative intracellular bacteria, either pathogens or symbionts. The structures of the genomes among the α-2 subdivision vary. There may be more than one chromosome and the presence of large plasmids.

2. Sequence and Characteristics of the *Brucella* Genomes

The whole genomic sequences of *B. melitensis* (Acc. NC_00317 and NC_00318, DelVecchio *et al.*, 2002) and *B. suis* (Acc. NC_004310 and NC_004311, Paulsen *et al.*, 2002) have been determined. The draft sequence of *B. abortus* 9–941, a field strain isolated from naturally infected bovine, has been completed through a collaboration of National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture and the University of Minnesota and is in draft form. TIGR in a collaboration with Virginia Tech, National Animal Disease Center, and Walter Reed Army Institute of Research, has nearly completed the whole genomic sequence of *B. ovis*. Of the four *Brucella* genomes that have or are being sequenced, only *B. ovis* is not pathogenic for man. As there are differences in host preference and pathogenicity of these four *Brucella* species, their genomic sequences provide a basis for designing experiments to determine the genetic basis of virulence, pathogenicity, evolution, and host-pathogen relationships.

Whole genomic sequence comparisons were made between *Brucella* and other α-2 Proteobacteria (Paulsen et al, 2002). The whole genomic sequence of brucellae is comprised of a large chromosome, designated Chr I, and a smaller chromosome, designated Chr II. Chr I from *B. suis* and *B. melitensis* shares broad gene synteny or gene order with the large chromosome of *Mesorhizobium loti* but only limited synteny with the genomic sequences of *Agrobacterium tumefaciens* and *Sinorhizobium meliloti*. Chr II has limited synteny to genomic sequences of *M. loti*, *A. tumefaciens* or *S. meliloti*. Approximately 70% of the ORFs of *Brucella* are shared with at least one of the following species, *M. loti*, *A. tumefaciens* or *S. meliloti* and approximately 55% are shared by all

three. The closest bacterial species to *Brucella* are the *Ochrobactrum* spp. (Jumas-Bilak *et al.*, 1998). These are free-living soil bacteria but are also opportunistic human pathogens.

The high degree of relatedness of the genomes of the classical *Brucella* species was first demonstrated over thirty-five years ago by DNA-DNA hybridisation (Hoyer and Mc Cullough, 1968 a, b). This observation was extended by more recent pulsed field gel electrophoresis (PFGE) studies. Genomes of the Office International Des Epizooties (OIE) type species for the *Brucella* classical species and their biovars digested with *Xba*I produced similar but distinct profiles (Allardet-Servant, 1991). Further PFGE studies, revealed that the genomes of *Brucella* except for one biovar of *B. suis* have two chromosomes (Michaux, 1993).

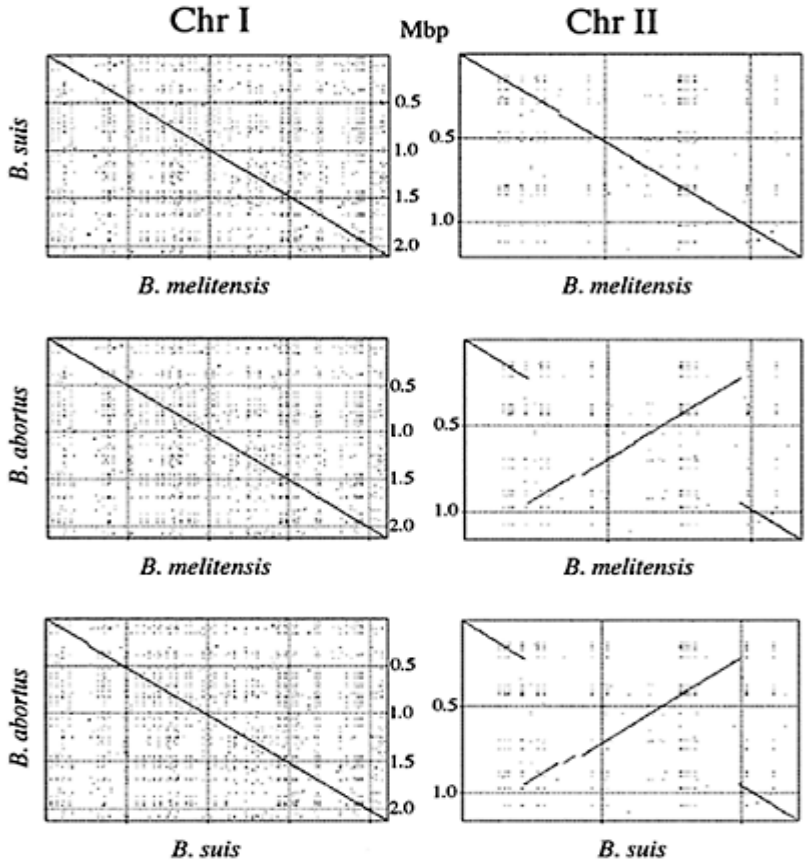

Figure 1. Pustell analyses (McVector 7.2, Accerlys Inc., San Diego, California, US) were carried out using the genomics sequences of *B.*

melitensis, B. suis, and *B. abortus*. The chromosomes were oriented in the same direction and start at a common locus. The window size was 50. The minimum score was 90% and the hash value was 6. The number of base pairs is given in Megabasepairs (Mbp).

The degree of similarity among genomes of the *Brucella* is illustrated also by the number of single nucleotide polymorphisms (SNPs) found when genomic sequences of *Brucella* were compared. The number of SNPs between the genomic sequences of *B. suis* and *B. melitensis* was 7,301 over 3.2 Mbp (Paulsen *et al.*, 2002). This is less than that for *Streptococcus pneumoniae* strains TIGR4 and R6 and *Escherichia coli* strains K-12 and O157:H7. The SNPs between strains of these two species were 8,303 over 1.9 Mbp and 36,676 over 3.9 Mbp, respectively.

The similarity of the genomic sequences and synteny among *B. melitensis*. *B. suis,* and *B. abortus* is evident by direct comparisons of their genomic sequences (Figure 1). There are more differences between the small chromosomes than the

Table 1. Unique, shared, and conserved genetic islands among *B. melitensis, B. suis,* and *B. abortus*

Size (bp)	**Genetic Island (Loci)**	[1]**Chr**	[2]**S**	[3]**M**	[4]**A**
18,290	BRA0362-BRA0379; tra genes and phage related genes	II	+	−	−
3,538	BR0588-BR0593. phage related	I	+	−	+
20,883	BMEI1674-BME1702; many small hypothetical ORFs, Phage integrase, Flj	I	−	+	+
25,245	BRA0419-BRA0439	II	+	+	−
3,952	BR1852-BR1854; Tn*2020*	I	+	−, +*	+

[1]Chromosome; [2]*B. suis;* [3]*B. melitensis;* [4]*B. abortus*

* Tn*2020* is not in the genomic sequence of *B. melitensis* 16 M but there is one copy of IS*2020* (Halling and Zuerner, 2002).

large. There are two large genetic islands (Table 1) among the genomic sequences of *B. suis* and *B. melitensis* that are not in Chr II of *B. abortus*. Given that Chr II has a plasmid-type replication origin; encodes homologs of two secretion systems (type IV secretion first described for DNA translocation and flagella); has a large putative composite transposon encoding amino acid, dipeptide, and sugar transport genes; contains at least two genetic islands encoding phage related proteins, it is reasonable to hypothesise that Chr II and many of its genes were horizontally acquired by *Brucella* (Paulsen *et al.*, 2002; Tsolis, 2002). Likely, some of the genes on Chr II were acquired at the same time

as the plasmid replication locus. This chromosome became essential after acquiring chromosomal genes.

Many features of the genome of the classical *Brucella* species were established before any of the whole genomic sequences was determined. The whole genomic sequences confirmed previous observations and provided details that furthered our understanding of other observations (DelVecchio *et al.* 2002; Paulsen *et al.*, 2002). The base composition was calculated to be 56 to 58 % G+C from DNA hybridisation studies (Hoyer and McCullough, 1968a). This is in agreement with that determined from the whole genomic sequences of *B. suis* and *B. melitensis*, 57%. The PFGE maps established that the genomes of *Brucella* contained about 3.3 Mbp distributed on two large circular DNA chromosomes (Michaux *et al.*, 1993) of approximately 2.1 Mbp and approximately 1.15 Mbp. The whole genomic sequences of both *B. suis* and *B. melitensis* are consistent with these sizes (DelVecchio *et al.*, 2002; Paulsen *et al.*, 2002).

The larger chromosome has a bacterial-like origin of replication while the smaller chromosome has a plasmid-like origin of replications. Small plasmids have not been isolated from *Brucella*. Attempts to isolate small plasmids from 600 strains of *Brucella* including all the classical species and their biovars using three different protocols at the University of California were not successful (Meyer, 1990). The

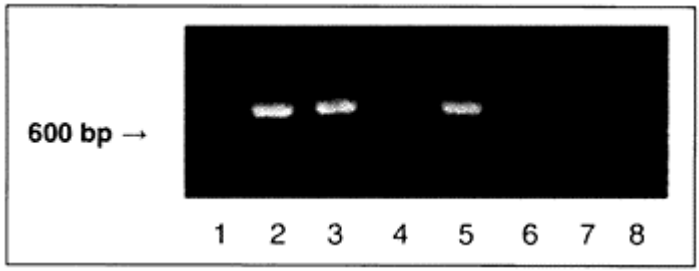

Figure 2. PCR products amplified using inversion specific primers. Lane 1: *B. suis* 1330; lane 2: *B. abortus* 544; lane 3: *B. abortus* biovar 2; lane 4: *B.* abortus biovar 3; lane 5: *B. abortus* biovar 4; lane 6: *B. abortus* biovar 5; lane 7: *B. abortus* biovar 6; lane 8: *B. abortus* biovar 9. The size marker is in base pairs (bp) on the left size of the figure. Forward primer was 5′-CCT-TTT-CCG-GAG-GCC-AAA-ATA-TGA-GCC-AT-3′ and reverse primer was 5′-CGC-CCG-ATA- TTT-CTC-TTC-ACT-TGA-CGC-CA-3′. Cells were heated to 95°C for 5 min.

Melting, annealing and elongation temperatures and times were 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for one and one-half minutes.

smaller, plasmid-based DNA molecule meets the criterion for a chromosome as it carries two tRNA synthetases and tRNA-Cys; these are essential genes. Most of the genes necessary for protein synthesis reside on the large chromosome while those encoding enzymes for sugar metabolism, protein regulators, and membrane transport proteins for sugars, dipeptides, and amino acids, reside on the small chromosome. Many of the transport protein coding sequences are within a 50 Kbp putative composite transposon, Tn*1953* (Bricker, Acc. No. AF454951).

There is a large inversion within the Chr II of *B. abortus* (Michaux-Charachon, 1997). The inversion was identified by PFGE maps prepared from genomic DNA digested with the restriction endonuclease *Pac*I. The inversion was identified in *B. abortus* 544, a biovar 1 strain and the type species for *B. abortus*. They also reported an inversion in Chr II of *B. abortus* biovars 2, 3 and 4. We designed primers based on the sequence flanking the inversion site of *B. abortus* 9–941 and confirmed the inversion in *B. abortus* biovars 1, 2, and 4 (Figure 2). We were unable to amplify genomic DNA from *B. abortus* biovar 3 Tuyla using these primers. This result was confirmed by use of primers that flank the genomic sequence of *B. suis* 1330 at the site where the inversion occurs relative to the genomic sequence of *B. abortus* 9–941. These primers amplified *B. suis* biovar 1 and *B. abortus* biovar 3 but not *B. abortus* biovar 1 strains 544 and 9–941, *B. abortus* biovar 2 and *B. abortus* biovar 4. The difference in results between the two laboratories may be due to different sources or strains of *B. abortus* biovar 3 used.

The relative small difference in the lengths of Chr I of *B. suis* and *B. melitensis* (1,207,381 bp and 1,177,787 bp, respectively) is mostly due to an unique genomic island in *B. suis* that appears to have been acquired by horizontal transfer. Note that here DNA fragments are referred to as genetic islands if they are at least 500 bp and encode phage-related ORFs, insertion sequences (IS), or fragments that either vary significantly in their G+C content from that of the genome or vary in codon usage from that of the genome statistics even though the G+C content may not vary. Only one unique island (Table 1) that was not transposon related was identified among the genomic sequences of *B. suis, B. melitensis* and *B. abortus* when the *B. abortus* draft sequence was compared with that of both *B. suis* and *B. melitensis* and that genetic is in the genome of *B. suis*. This island is carried on Chr II and encodes bacterial transfer functions and phage related ORFs and hypothetical proteins. No homologs of virulence factors were confirmed on any of the islands. The report of the genomic sequence of *B. melitensis* was accompanied by an excellent review by Moreno and Moriyón (2002). They listed the factors shown previously to affect the virulence and pathogenicity of *Brucella* and identified new putative factors from the genomic sequence of *B. melitensis*. *Brucella* have none of the obvious virulence factors identified for many bacterial pathogens such as capsules, pili or fimbriae, proteases, exotoxins, phages and cytolysins.

3. Genomic Diversity: Polymorphisms and Insertion Sequences

By the early 1990's, a number of genes had been cloned and sequenced from *Brucella*. The sequence data revealed that there was a high degree of similarity among the genomes, suggesting that it may be difficult to develop assays to distinguish the classical *Brucella* species and their biovars. Sequence diversity among the brucellae at the DNA level was observed among outer membrane proteins (Omps). Much of this work began with the observations that Omp profiles varied among brucellae isolates (Verstreate and Winter, 1984). Polymorphism observed among the Omps could be due to selection based on host, as the Omp are in contact with cells in the first phase of infection, adherence and attachment. The study of DNA based polymorphism at loci encoding Omps, *omp2a*/*omp2b* (Ficht *et al.*, 1989; 1990), *omp31,* and *omp25* (Cloeckaert *et al.,* 1995), lead to development of polymerase chain reaction-restriction length polymorphism (PCR-RFLP) assays that could identify *Brucella* and to varying extents distinguish the classical species and their biovars. This work is summarised in a recent review by Vizcaino and colleagues (Vizcaino, *et al.,* 2000; see also Chapters 1 and 2).

Insertion sequences (IS) are a source of genomic instability and diversity by moving within the genome causing gene reassortment by chromosomal deletions and inversions (Mahillon *et al.,* 1999). These events create polymorphism concomitantly. The insertion sequence, IS*711*/IS*6501* (Halling *et al.,* 1993, Ouahurani *et al.,* 1993) appears to be an ancestral sequence as not only do all the *Brucella* have the element but they also have common copies. It is a source of genetic diversity among the *Brucella* as it has transposed to at least one unique locus in the classical species of *Brucella*. So far, it has not been shown to be involved in genomic inversions and large deletions or rearrangements.

Insertion sequences can benefit bacteria by transposing and modifying gene expression, aiding in their adaptation to a new environment such as a different host. Given that *Brucella* has little exposure to other bacteria, IS may be an important internal source of diversity especially during selection. The copy number of IS711 ranges in *Brucella* from as few as seven to well over 30 in the marine isolates. Whether or not the other IS from *Brucella* are able to transpose is not known. Transposition of transposable elements is usually tightly regulated and, if the elements arose in *Brucella* from horizontal transfer, their promoters may not be recognised or functional in *Brucella*. Several of the elements in *B. suis* have pseudogenes and these would not be predicted to be mobile unless complemented by a second copy elsewhere in the genome.

The movement of IS alters gene linkages within the genome. This results in polymorphism that can be used as targets to identify and distinguish strains (see Chapter 1). Though the classical species have copies of IS*711* that are found at the same loci, they also have at least one IS*711* copy at a unique loci so that the classical species can be distinguished on Southern blots when they are probed with IS*711* (Ouahrani *et al.,* 1993; Bricker and Halling, 1994). Other assays based on IS*711* have been developed and continue to be developed (Bricker, 2002).

Though IS*711* elements transpose in *Brucella,* they do not appear to transpose at a high frequency (Halling and Zehr, 1990). This element in *B. ovis* isolates from different time periods and geographical locations did not appear to differ in copy numbers, even

though there are approximately 30 copies. The element has been observed to be mobile in *B. abortus*. The *B. abortus* biovar 1 strains 2308 and RB51 (Schurig *et al.,* 1991) have more copies of IS711 than the *B. abortus* type species, strain 544 (Bricker and Halling, 1995; Vemulapalli *et al.,* 1999). In *B. abortus* S2308, there are two tandem direct copies of IS*711*. During selection of the rough vaccine strain RB51 (Schurig, *et al.,* 1991), IS*711* mobilised again (Vemulapalli *et al.,* 1999). This copy interrupts the *wbo*A gene encoding a glycosyltransferase that is essential for the synthesis of O-side chain. These copies of IS*711* made it possible to develop PCR assays to identify and distinguish the challenge and vaccine strains, *B. abortus* S2308 and RB51. Though there are other insertion sequences in *Brucella,* there are not any reports of their transposition yet.

Genetic diversity by IS may be directed to a certain extent as most IS do not transpose randomly but, rather, transpose to target sequences. What other factors may be involved in internal transposition of an element such as structural changes caused by palindromes or other sequences is not known. The target sequence for IS*711* is pyrimidine-A-T-purine, often CATG, but IS*711* elements do not randomly transpose to those sequences either. Several of the elements from *B. ovis* were found to flank a repeated DNA sequence, Bru-RS1 (Halling and Bricker, 1994). There did not seem to be a pattern with regards to the relative orientation of IS*711* and Bru-RS1. The Bru-RS1 elements might have an effect on DNA secondary structure that affects the efficiency of either the transposition process or the exposure and recognition of target sites. Thus, Bru-RS1 may be a source of genetic instability and diversity for *Brucella* working hand and hand with IS*711*. As *B. ovis* has more than 20 copies of IS*711,* the genomic sequence of *B. ovis* will further our understanding of the association of IS*711* and Bru-RS 1. Also, it will be of interest to note if phenotypic traits of *B. ovis* can be traced to transposition of IS*711* in *B. ovis.* These data may be useful in generating hypotheses regarding both transposition targets of IS*711,* the most mobile transposable element in brucellae, and their effect on genomic structure.

Repeat sequences can generate polymorphism during replication due to mispair slippage. Slippage backwards or forwards can result in deletions or duplications of sequences, respectively. If the repeats reside in or near genes, gene expression can be affected and have been associated with genetic diseases in humans (Cummings and Zoghbi, 2000). Similar or identical tandem repeats of variable lengths are often found at several loci in a genome. These are designated as VNTRs for variable nucleotide tandem repeats. VNTRs have been used for strain typing for epidemiological trace back for bacterial pathogens, such as *Bacillus anthracis* (Keim and Smith, 2002) and *Mycobacterium tuberculosis* (Frothingham and Meeker-O'Connell, 1998). A VNTR was identified in *Brucella,* AGGGCAGT (Bricker *et al.,* 2003). Most of the loci that have VNTRs in *Brucella* have ORFs annotated upstream of them but the effect of the VNTR on expression of the ORFs is not known. Interestingly, most of these VNTRs in *Brucella* are physically linked to a conserved region that may influence the frequency of slippage.

4. Genomics and Taxonomy

Moreno and Moriyón hypothezised that *B. melitensis* and *B. abortus* share a common ancestor and became isolated at the same time that their hosts did, about 20 million years

ago (Moreno and Moriyón, 2001). The ability of *B. melitensis* to cause abortions in cattle, sheep and goats may be due to its having a common ancestor with *B. abortus*. *B. suis* can cause abortions in swine but not cattle. A number of studies support that *B. abortus* and *B. melitensis* are more closely related to each other than either one is to *B. suis*. This relative closeness of the genomes of *B. melitensis* and *B. abortus* is demonstrated by dendrograms constructed from data generated from the classical species of brucellae and their biovars by data obtained using: arbitrarily primed PCR (Fekete *et al.* 1992); random amplified polymorphic DNA (Tcherneva *et al.* 2000); PFGE (Michaux-Charachon *et al.*, 1997, Jumas-Bilak *et al.*, 1998); Western blot protein patterns including whole cell antigens and soluble antigens reacted with sera from rabbits hyperimmunised with either *Ochrobactrum anthropi* or *B. suis* 1330 (Velasco *et al.*, 1998), transcript analyses (see below) and metabolic capabilities (Meyer, 1990).

Comparison of genomic sequences entails not only the DNA sequence itself but their ORFs as well. Confidence in an annotated genomic sequence is higher for those ORFs for which conserved homologs are identified in protein databases searches. Even though sequences of *B. suis* and *B. melitensis* are highly similar they have been annotated differently in some instances. In some cases, ORFs are on opposite strands or overlap. Annotation of the *Brucella* genomes contains a number of hypothetical proteins and conserved hypothetical proteins. The function of some of the hypothetical proteins will likely be discovered with further experimental work on *Brucella* and other bacteria. In time, it is likely that it will be determined that some of these putative ORFs do not encode gene products. In any case in order to have an inclusive microarray, it is better to saturate the genome with ORFs and use data from other studies including microarray studies to identify which ORFs encode gene products. Proteomics can aid in the identification of start codons and expressed ORFs.

One of the most difficult tasks in annotation of any genome is to identify which short ORFs express polypeptides. There are many short ORFs annotated for the *B. suis* genome but very few for the *B. melitensis* genome. Mostly, selection of ORFs when there are no homologs in the databases is done by use of software such as Glimmer (Salzberg *et al.*, 1998; Delcher *et al.*, 1999). This software identifies ORFs based on using highly conserved homologous regions within the target genomic sequence. This approach works best to identify genes in regions of the chromosome where the G+C content matches the statistical G+C content for the whole genome. The ability to detect ORFs that are expressed, especially small ORFs, may be improved by studies to identify promoters from *Brucella*. Another difficulty in the annotation of genomic sequences of *Brucella* is that the α-Proteobacteria may use a start codon other than ATG. Annotation is an ongoing process and data from microarrays, RT-PCR, and transcript mapping will be needed to identify all the genes and their ORFs. These data will be needed to determine the genetic basis for differences in pathogenicity, virulence, and infectious cycle of the *Brucella*.

Various kinds of repeated sequences have been reported among bacteria. The accumulation, distribution and function of these elements are still largely speculative. They may affect gene expression indirectly by stabilising m-RNA (Hulton *et al.*, 1991). In enterobacteriae, two such sequences, designated Enterobacterial Repetitive Intergenic Consensus (ERIC) and Repetitive Extragenic Palindromic (REP) sequences, have been described (Stern *et al.*, 1984; Hulton *et al.*, 1991). Two sequences designated Bru-RS1 and Bru-RS2 that are similar to ERIC sequences in size and occurrence in non-coding

regions but not in DNA sequence were discovered in brucellae (Halling and Bricker, 1994). The Bru-RS sequences like ERIC sequences occur singly. A comparison of the genomic sequences supports that the elements have not been involved in genomic rearrangements. The two Bru-RS elements are 65% similar and are flanked by inverted repeats. Within the element, there is another copy of the repeat. This results in a 70 bp subsequence in Bru-RS1 that is bounded by 8 bp direct repeats and a 38 bp sequence bounded by 8 bp inverted repeats. Some of the Bru-RS1 elements are truncated, having only the 70 bp subsequence. While the Bru-RS2 elements have a similar structure, their inverted repeats are shorter, 6 bp. However, 17 bp of the left end of Bru-RS2 is directly repeated within the element to bound an 81 bp subsequence. The internal direct repeat pairs with the left end to form a 40 bp element. Both Bru-RS1 and Bru-RS2 have several repeats of the sequence 5′-GAAA-3′, a sequence shown to stabilise RNA hairpins (Heus and Pardi, 1991). As discussed above Bru-RS1 sequences are hot spots for IS*711* insertion. This may be due to the highly palindromic nature of the elements that could disrupt DNA structure. The sequences of the elements are not identical and appear to have drifted (Halling and Bricker, 1994).

5. Genomics and Diagnostics

Relatively small differences in the predicted gene content of *Brucella* are unlikely to completely explain differences in virulence and host preference. However, these minor gene content differences are sufficient to uniquely identify each of the species in an expression experiment.

In a genomic sequence comparison, we identified a total of 101 genes that are uniquely present, or uniquely absent, in one of the three *Brucella* genomes (Figure 3). Gene boundaries were defined based on existing annotation for *B. suis* and *B. melitensis,* and predicted on a draft sequence of *B. abortus* using Glimmer 2.0 (Delcher *et al.,* 1999). Complete nucleotide sequences were compared directly using an early prototype of the GenoMosaic system (Gibas *et al.,* 2003), and differentiating regions were identified and localised within gene boundaries. RT-PCR was used to test for expression of these differentiating genes *in vitro,* and 54 of the genes identified produced transcripts under the conditions of our experiment (Sturgill *et al.,* unpublished).

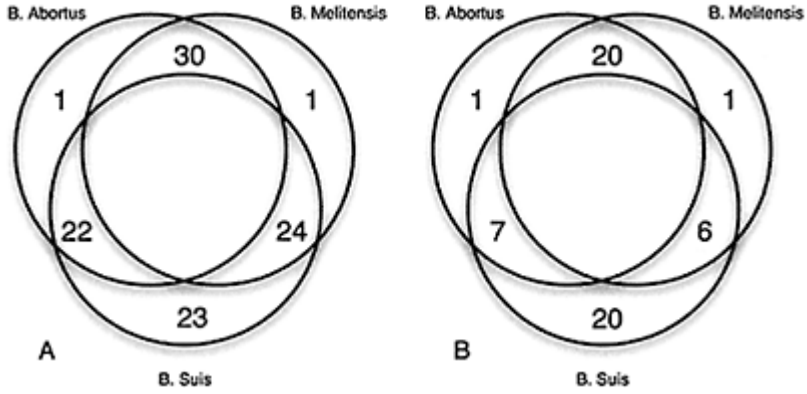

Figure 3. Distribution of unique and common genes in three *Brucella* genomes as A) predicted by sequence comparison and detected in the genomic DNA by PCR, B) detected by transcripts by RT-PCR.

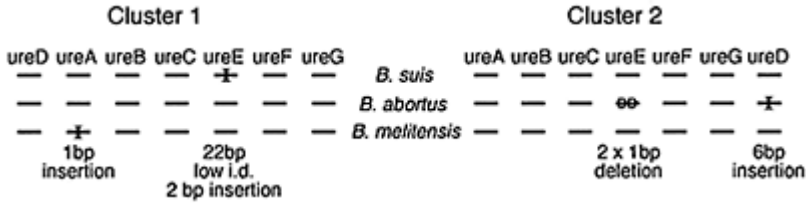

Figure 4. Schematic of Brucellaand 2 clusterswithin *Brucella* spp. genomes. I represent insertion(s) and D represents deletion(s) within indicated urease subunit gene.

xx`x

Genes with homologs in all three species were surveyed to identify additional differentiating features comprising less than a complete gene. Of 3,163 genes with homologs in all three genomes, nearly all were found to be more than 95% identical in sequence to their homologs at the nucleotide level, with 95% or greater coverage of the sequence. In every case where homologs were present in all three genomes, high scoring sequence match regions among them had sequence identity greater than 90%. Therefore, the only additional group of genes that could be considered as potential differentiating features in an expression context was a group of 214 genes where the sequence match

detected extended over less than 80% of the sequence, leaving a distinguishing region as a target for a unique primer or probe. These partial homologs may be useful as additional differentiating features but have not yet been tested in expression experiments (Sturgill, 2003).

6. Differential Gene Expression

The small number of truly unique coding sequences in the *Brucella* genomes suggests that differences in virulence and host preference are most likely a function of differential gene expression as well as unique gene content. Very few comparative studies of differential gene expression in *Brucella* exist, but recent results of a comparative study of urease gene expression (S.Boyle, unpublished) suggest that a differential approach to expression will yield interesting results.

There are two separate urease operons on Chr I of the three *Brucella* spp. genomes located at approximately 0,.273 and 1.316 Mb. Species-specific differences are present in the two gene clusters of urease subunits present in *B. suis, B. abortus,* and *B. melitensis* (*ure*A-G-1 BR0267-BR0273 and *ure*A-G-2 BR1356-BR1362 in *B. suis*). In the *ure*A-1 gene (BR0268) of *B. melitensis,* there is a 1 bp insertion representing a potential frameshift. In the *ure*D-2 (BR1362) gene of *B. abortus,* a 6 bp insertion was identified. In the *ure*E-2 gene (BR1359) of *B. abortus,* two separate single base deletions are present and representing possible frame shift in translation. Finally, the last 22 bp of *ure*E-1 (BR0271) were shown to be 100% identical in *B. abortus* and *B. melitensis* but significantly diverged in *B. suis,* including a 2 bp deletion. This variation within these urease gene clusters could prove to be significant to the expression of functional urease subunits (Figure 4).

Using primers specifically designed to amplify mRNA transcripts from the three species of *Brucella* grown in trypticase soy broth, we detected transcripts of a predicted size from all the subunit genes in both clusters except for *ure*D-1 and a smaller than expected transcript from ureC-2. All 3 *Brucella* spp. exhibited urease activity when tested in urea broth (Difco). However, when a kanamycin resistant marker was introduced and most of the *ure*A-1 and *ure*B-1 genes were deleted by recombination, *B. suis* did not produce urease activity. In contrast, when a kanamycin resistant marker was introduced and most of the *ure*B-2 and *ure*C-2 genes were deleted, *B. suis* retained urease activity. These results suggest that there is likely some type of post-translational complementation occurring among the subunits of the two urease gene clusters. A similar observation was reported (Sangari and Garcia-Lobo, 2003) when the *ure*C cluster was mutated by transposon insertion, only *ure*C-1 but not *ure*C-2 mutants exhibited loss of urease

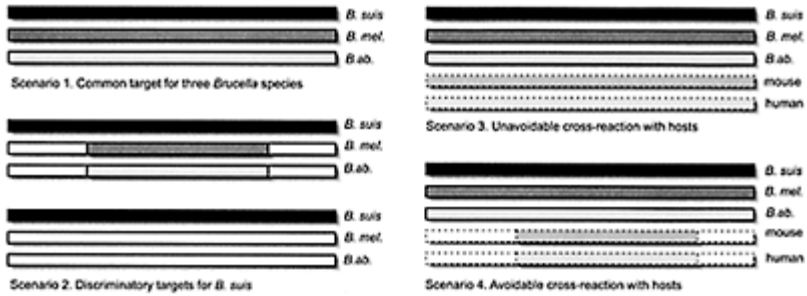

Figure 5. Some probe design scenarios for a multiple-genome array design problem. In scenario 1, a gene homolog exists in each of the three species with high identity along the full length of the gene. Scenario 2 describes a situation in which a differentiating probe can be made; the gene homolog in one or more of the genomes is missing, partially or completely. In scenario 3, this extensive identity includes genes in the host genomes and cross-reaction is unavoidable. In scenario 4, cross-reaction with the host can be avoided where identity to host genes is low.

activity in all three *Brucella* species. Certainly more experimental work needs to be performed in order to clarify the nature of the expression of the two urease operons of the *Brucella*.

7. Designing Arrays that Address Closely Related Sequences

DNA microarrays are widely used to study global transcription levels. A comparative expression study of the *Brucella* presents a unique challenge and therefore a unique opportunity in DNA array design. The extensive identity between gene homologs in the three *Brucella* species suggests a comparative genomics approach to construction of a DNA microarray. Rather than using separate arrays, or separate probes designed for each gene in each species, it would be possible to construct a generic *Brucella* array that would include probes designed for sequence regions common to all three genomes. Probes for differentiating genes or regions could also be added, to serve as a built-in diagnostic.

In fact, constructing such a design presents a significant challenge. Currently available oligonucleotide array design software packages have no capabilities for optimising probe picks to fall within highly similar regions of related targets. Complicating the issue further is the need to avoid regions of the target sequence that are highly similar to host sequences, in order to avoid cross-reaction with host transcripts in experiments where *Brucella* samples are isolated from host cells, as shown in Figure 5.

Even in sequences greater than 90% identical, it is difficult to avoid mismatches, and while the effects of single base pair mismatches on hybridisation is well-studied for oligonucleotides 25–30 nt in length (Lipshutz *et al.*, 1999; Ramakrishnan *et al.*, 2002; Peterson *et al.*, 2002; Riccelli *et al.*, 2003), there has been little corresponding work for longer oligonucleotides or for cDNA arrays. However, longer oligonucleotides have been shown to provide optimal sensitivity and specificity (Kane *et al.*, 2000; Hughes *et al.*, 2001). In the absence of quantitative study of the thermodynamic effects of slight mismatches in long oligonucleotides and development of a modelling approach to compensate for these effects in a comparative study, mismatches should be avoided when possible. A truly "generic" *Brucella* array for comparison among sequenced species would be limited to probes for regions that are 100% identical in all three species.

In practice, we were limited to designing an array using existing oligonucleotide selection methodologies pick70 (Bozdech *et al.*, 2003) and OligoArray (Rouillard *et al.*, 2002; 2003). These programs select probes based on commonly used design criteria, among them sequence uniqueness, low self-complementarity, and specific GC content or sequence complexity. OligoArray adds biophysical criteria which affect hybridisation—duplex T_m and T_m of secondary structures predicted to form within the probe—but at the present time these criteria are applied in a rather rudimentary fashion and information needed to predict hybridisation behaviour is not available.

Neither of these design methodologies addresses the problem of design for multiple transcriptomes, so it was not possible to eliminate mismatch entirely in the first round of oligonucleotide selection. *B. suis* was chosen as the base sequence for design of the array, and probes from specific differentiating targets to address unique regions in *B. melitensis* and *B. obortus* were subsequently added. The prototype array designed using these methods was analysed and found to contain approximately 10% of probes that contained unusually stable secondary structures, and approximately 10% of probes that did not match equally well with targets in each of the three genomes (Ratushna and Gibas, unpublished).

Because we can identify precisely where secondary structure and mismatch effects may interfere with hybridisation on this prototype array, the experiment becomes a valuable exploration not only of comparative *Brucella* functional genomics, but of our ability to design, optimise and interpret data gathered from a multi-transcriptome experiment. Multi-transcriptome approaches are likely to become more common as rapid genome sequencing allows microbiologists to take the approach of sequencing families of closely related microbial genomes to identify the basis of virulence and other phenotypic differences (Bhattacharyya *et al.*, 2002a; 2002b).

The differences in host specificity and virulence among the brucellae can be more easily investigated now that genomic sequences are available. The similarity among the genomic sequences suggests that those differences may be due in part to sequence variations such as SNPs and pseudogenes. The RT-PCR studies support that differential

gene expression is also likely to play a part as well. Differences in gene expression will also be applicable to rapid identification of agents of brucellosis. Clearly the genomic sequence is not the end but is another beginning.

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Chapter 6
Comparative Proteomics of *Brucella* Species

Vito G.DelVecchio* and Cesar V.Mujer

From: *Brucella:* Molecular and Cellular Biology. Edited by: Ignacio López-Goñi and Ignacio Moriyón

Abstract

The field of proteomics is the most prominent post-genomic discipline. Although this field is relatively young compared to other established sciences, proteomics has provided vast amounts of information crucial to a comprehensive understanding of system biology in various organisms. Proteomics provide the necessary tools to elucidate the interplay of metabolism, function and phenotypic expression. Ultimately proteomics will aid in the understanding of evolutionary relatedness and interrelationships among various species. Comparative proteomics has been used for identifying, characterising and comparing the proteomes of several nomen species of *Brucella*. A global overview of *B. melitensis* proteomes has been accomplished in a relatively short period of time primarily because a completely sequenced and annotated genome of this organism is now available. Consequently, proteins that are differentially expressed between the virulent 16M and vaccine Rev 1 strains of *B. melitensis* have been identified and have led to a better understanding of key metabolic pathways that may be crucial in attenuating virulence and the production of vaccine strains. Soluble and membrane-bound proteomes of all the recognised nomen species *(B. melitensis, B. abortus, B. suis, B. ovis, B. canis and B. neotomae)* are currently being examined to identify proteins involved in host preference and virulence. Additionally, the secretomes of *B. abortus* and attenuated *vir*B mutants have been investigated. Protein identification in these mutants will allow identification of protein candidates for use in vaccine development and as potential drug targets.

1. *Brucella melitensis* Proteome

The ultimate goal of post-genome sequencing is to understand protein function not in the context of individual proteins but on a holistic scale involving networks of other proteins that regulate various interacting metabolic pathways (Liebler, 2002). Proteomic tools are available for such a high throughput and rapid global analysis of proteins (Wilkins *et al.*, 1996; O'Connor *et al.*, 2000; Patton, 2000; Jenkins and Pennington, 2001). For instance, by using two-dimensional gel electrophoresis (2-DE), Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS) and Mascot search engine for protein identification, and the availability of a completely sequenced genome (DelVecchio *et al.*, 2002a), a rapid and

* For correspondence email vimbm@aol.com

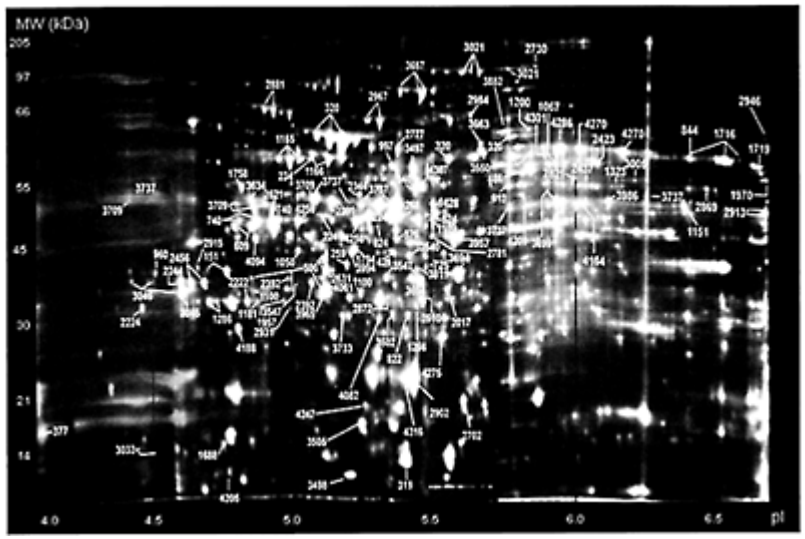

Figure 1. Partial global proteome map of *B. melitensis* 16M from pH 4.0 to 6.7. The map is a manually seamed, composite image of four narrow pH range 2-D gels, each containing 40 to 120 μg total protein extracted from chloroform-killed cultures of *B. melitensis* 16M. Gels were stained with SYPRO® Ruby for 12 h before imaging under 470 nm light.

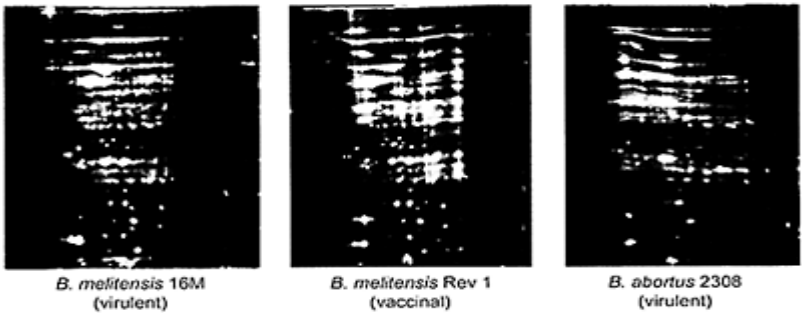

Figure 2. Membrane-associated proteomes of *B. melitensis* strains 16M and Rev 1 and *B. abortus* 2308

global proteome analysis of the *B. melitensis* virulent and vaccine strains was accomplished (Eschenbrenner *et al.,* 2002; Wagner *et al.,* 2002).

In a first paper on the global analysis of *B. melitensis* strain 16M proteome, Wagner *et al.* (2002) reported the identification of 883 protein spots between pH 3.5 to 11 (Figure 1). Of the 883 protein spots, 440 have been identified by peptide mass fingerprinting and represent 187 discrete open reading frames (ORFs). To date, a total of 937 protein spots have been identified that corresponded to 269 discrete ORFs. As indicated previously, the number of expressed ORFs is much less than the 3,197 predicted ORFs in the 16M genome which may be due to a number of factors (DelVecchio *et al.,* 2002b; Wagner *et al.,* 2002). One major limitation to the previous analysis was the type of proteins analysed. A majority of these proteins were hydrophilic and only a few were hydrophobic, with transmembrane domain-containing proteins being underrepresented. To obtain a sample preparation enriched for membrane-bound proteins, a ProteoPrep Membrane Extraction kit (Sigma, St. Louis, MO) was utilised for isolating hydrophobic proteins from the different species of *Brucella* (Figure 2). It is anticipated that when these results are combined with the number of soluble proteins visualised on 2D gels the total number of ORFs encoding for the expressed proteins will significantly increase.

The expressed ORFs identified to date by proteomics analysis reveal that they are evenly distributed over both chromosomes, suggesting that the two chromosomes are indispensable for the survival of this organism. Proteins involved in membrane transport as well as in carbohydrate and protein metabolism composed the majority of proteins identified in *B. melitensis*. Additionally, a significant number of proteins with unknown functions were included under the "hypothetical proteins" category. This category was assigned by Integrated Genomics, Inc. based on putative genes predicted from the annotated sequence of the *B. melitensis* genome (DelVecchio *et al.,* 2002a; 2002c).

One interesting finding from the analysis of the *B. melitensis* proteome is that several virulence genes including those that encode for VirB8 and VirB9 virulence factors, the 25 and 31 kDa immunogenic protein precursors, invasin protein B, the iron-regulated outer membrane protein FrpB, and the periplasmic immunogenic protein, were expressed even though the organism was cultured under growth conditions outside of the host (Wagner, Mujer and DelVecchio, unpublished). The detection of protein products for virB8 and

virB9 under laboratory conditions on Schaedler Blood agar raises important questions in regard to the conditions that activate the *vir*B operon in *B. melitensis*. Previous studies have demonstrated that the *vir*B operon, which includes *vir*B8 and *vir*B9, in *B. suis* grown in macrophage culture is transcriptionally activated in response to nutritional stress and acidic pH conditions (Boschiroli *et al.*, 2002). These conditions are critical during the early stages of infection and bacterial establishment inside the phagosome. The observation that *vir*B8 and *vir*B9 are expressed in Schaedler blood agar suggests that environmental factors aside from pH and nutritional stress may be involved in the coordinate induction of the *vir*B operon in *B. melitensis* (Mujer *et al.*, 2003).

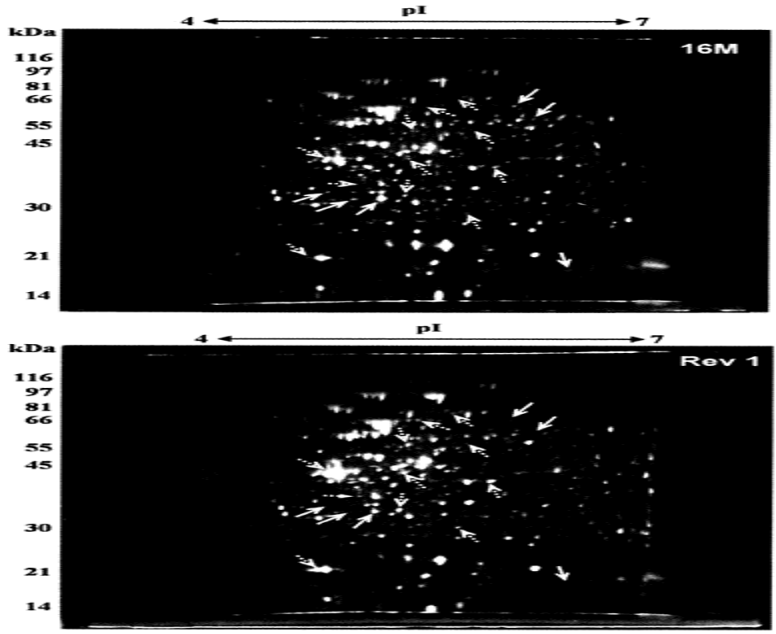

Figure 3. Proteomes of laboratory-grown *B. melitensis* strains 16M and Rev 1 in the pH range of 4.0 to 7.0. Forty μg proteins of each strain were separated in the first dimension by isoelectric focusing using IPG strips and run on SDS-10% Duracryl gels in the second dimension. The gels were stained with SYPRO Ruby and imaged at 470 nm. Overexpressed and underexpressed proteins are indicated

by dashed and solid arrows, respectively.

2. Vaccine Strain *B. melitensis* Rev 1 Proteome

Rev 1 is an attenuated, live vaccine strain that originated from a virulent *B. melitensis* isolate. This isolate initially became resistant to streptomycin but eventually lost this characteristic upon further culture (Elberg and Faunce, 1957). Rev 1 is one of the most successful vaccines used for the prevention of brucellosis in sheep, rams and goats (Elberg and Faunce, 1957; Blasco *et al.*, 1987; Blasco, 1997). The proteomes of Rev 1 and 16M are highly similar in terms of the number of spots and the general patterns on 2D gels (Figure 3). This is not surprising since strains Rev 1 and 16M belong to the same species and most likely share a high level of sequence homology. A total of 513 and 522 protein spots were detected for strains 16M and Rev 1, respectively, at pH 4 to 7. However, computer-assisted comparison of the two *B. melitensis* proteomes revealed differential expression of several proteins that regulate iron acquisition, sugar binding, protein biosynthesis, and lipid degradation. Protein expression levels may reflect differences in metabolic properties and may explain the ancestral lineage or attenuation of the vaccine strain. A listing of these differentially expressed proteins and their possible effects on the metabolism of the vaccine strain has been described previously (Eschenbrenner *et al.*, 2002).

The utilisation of iron is associated with the survival of pathogens inside a host cell. During infection, a macrophage reduces iron availability by producing chelating agents and actively exporting iron from the phagosome where the pathogen multiplies. In response, the pathogen synthesises other proteins that compete with the host for iron. Proteomics analysis of Rev 1 indicated that the bacteria produce bacterioferritin, a protein whose function is to store iron in the cytoplasm. Interestingly, bacterioferritin accumulation in Rev 1 is significantly higher than in 16M. Also overexpressed in Rev 1 are the iron-regulated outer membrane protein and an iron (III)-binding periplasmic protein which are both typically derepressed during low iron availability. The overexpression of proteins essential for both low and high iron availability suggests a misregulated system for iron metabolism and capture, leading to possible unnecessary expenditure of energy (Eschenbrenner *et al.*, 2002). To compensate for energy wastage, Rev 1 has upregulated other pathways such as those responsible for the β-oxidation of fatty acids and protein synthesis to generate more reducing equivalents, which ultimately leads to the production of ATP. This was supported by the observation that enoyl-CoA hydratase and acyl-CoA dehydrogenase proteins accumulated at a higher level in Rev 1. Both enzymes are involved in the synthesis of acetyl-CoA that consequently enters the TCA cycle.

4. *Brucella abortus* Proteome

B. abortus primarily infects cattle but is transmitted to buffaloes, camels, deer, dogs, horses, sheep and humans. A comparative analysis of *B. abortus* 2308 and *B. melitensis*

16M proteomes at pH 4.0 to 6.0 has been conducted using 2D-PAGE (Horn, 2002). A total of 575 and 549 protein spots were noted for *B. melitensis* and *B. abortus*, respectively. Since the *B. abortus* genome is not completely annotated, matched spots between the two species have been assigned putative identification based on the previously identified spots from *B. melitensis* (Wagner *et al.*, 2002).

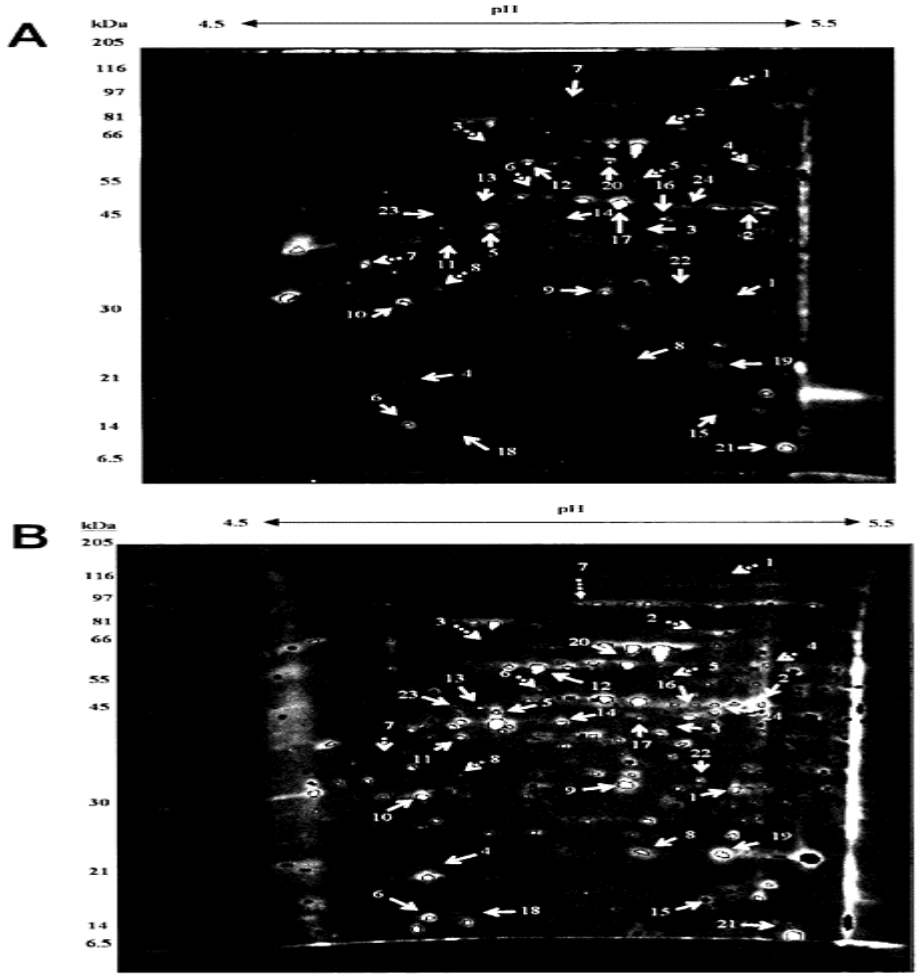

Figure 4. Differentially expressed proteins in (A) *B. abortus* 2308 and (B) *B. melitensis* 16M. Overexpressed and underexpressed proteins are indicated by dashed and solid arrows, respectively.

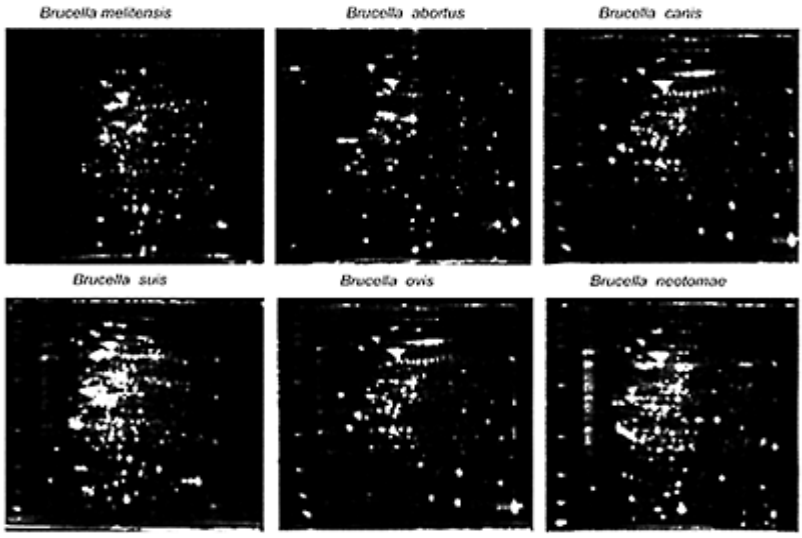

Figure 5. Proteomes of *B. melitensis, B. abortus, B. canis, B. suis, B. ovis and B. neotomae*.

When matching was done based on the similarities of pIs, MWs and general protein patterns on 2D gels between the two species, a total of 312 differentially expressed proteins were detected (Figure 4). These *B. abortus* proteins were categorised as overexpressed, underexpressed, variable, and unmatched. Of the 312 differentially expressed proteins, 15 were overexpressed, 70 were underexpressed, 25 were variable (levels of expression varies from gel to gel) and 202 were unmatched. Five overexpressed proteins were tentatively identified as succinyl-CoA synthetase beta chain (BMEI0138) (7.8-fold), two 25 kDa outer membrane immunogenic protein precursors (BMEI1829) (3.0- and 17.2-fold), a glycosyl transferase (BMEII0846) (3.5-fold), and a heat-inducible transcription repressor HRCA (BMEI1776) (7.7-fold).

Of the 70 underexpressed proteins in *B. abortus*, 44 were tentatively identified. Topping this list was a 32 kDa periplasmic immunogenic protein with a pI of 5.23. The level of this protein was 71.5-fold lower in 2308 compared to 16M. Other proteins that were highly repressed (>10-fold) included an isovaleryl-CoA dehydrogenase (47.4-fold), a protein translation elongation factor Tu (12.7-fold), an SSU ribosomal protein S1P (12.6-fold) and a leucine-, isoleucine-, valine-, threonine-, and alanine-binding protein precursor (12.1-fold). A highly repressed protein (46.3-fold) with a molecular mass of 26.4 kDa and a pI of 5.73 was not identified.

There were 89 unmatched spots identified in *B. melitensis* and 113 in *B. abortus*. Unmatched spots are those that are present in one species but absent in the other. These spots may represent the ORF products unique to a particular species. However, it is also likely that these proteins are the products of point mutations, duplicate ORFs, post-translational modifications or all of the above. The molecular masses of the unmatched proteins ranged from 13.2 to 91.9 kDa in *B. melitensis* and 9.9 to 113.6 kDa in *B.*

abortus. Overall, the differential proteomes found between *B. melitensis* and *B. abortus* indicate extensive metabolic differences between the two species.

4. Proteomes of Other *Brucella* Species

The proteomes of other *Brucella* species that have been investigated by 2D gel electrophoresis are presented in Figure 5 (Wagner, Mujer and DelVecchio, unpublished). A global and rapid proteome analysis and identification of proteins from these species was not conducted because of the unavailability of a complete genome sequence. However, it is clear from the 2D protein patterns that one species of *Brucella* can be distinguished from the other by comparing respective proteomes (Figure 5).

5. *Brucella* Secretomes

Knowledge of proteins that are induced during infection and those that contribute to pathogenicity would aid in the design of safe and efficient vaccines, new immunodetection assays, and could lead to the discovery of another generation of effective antimicrobial drugs. Possible candidates for such a study are the large quantities of proteins secreted into the growth medium, also termed the secretome

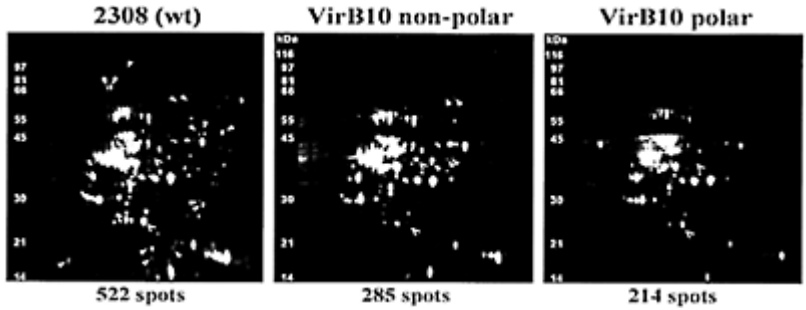

Figure 6. Differentially expressed proteins in *B. abortus* 2308 wild type and VirB10 polar and non-polar mutants. Protein spots missing in wild type or mutant strains are indicated by closed arrows. Differentially expressed proteins are indicated by open arrows.

(Tjalsma *et al.*, 2000). The secretomes from the wild type and *virB* polar and nonpolar mutants of *B. abortus* 2308 are presented in Figure 6 (Delpino, Ugalde and DelVecchio, unpublished results). For each sample, one litre of filter sterilised supernatant from an overnight culture of *B. abortus* strains in trypticase soy broth was used. The supernatant

was concentrated 1,000x by sequential lyophilization and dialysis and subjected to 2D SDS-PAGE and MALDI-TOF MS for protein analysis and identification. At least seven proteins were identified with confidence by MALDI-TOF MS. Some of these proteins absent in the *virB*10 polar or non-polar mutants include dihydrolipoamide dehydrogenase (DLDH), the chaperone protein DnaK, cytosol aminopeptidase, phosphoserine aminotransferase, choloylglycine hydrolase, and the ABC-transporter periplasmic sugar binding protein. Other proteins absent in both polar and non-polar mutants were tentatively identified as polyribonucleotide nucleotidyl transferase, aspartate aminotransferase, isocitrate dehydrogenase, 6-phosphogluconolactonase, alkylhydroperoxide reductase and maltose/maltodextrin-binding protein. Some highly expressed proteins detected in the wild type were the leucine, isoleucine, valine, threonine, and alanine binding proteins.

An interesting finding from the proteome analysis of *vir*B mutants is the absence of DLDH in the mutant strains. In *Streptococcus pneumoniae,* a lack of DLDH results in a deficiency in alpha-galactoside metabolism and galactose transport (Smith *et al.,* 2002). The DLDH-negative mutants produced only 50% of the normal capsular polysaccharide, a phenotype that may be associated with impaired carbohydrate metabolism, lack of virulence, and survival of the pneumococci within the host. These results suggest that knockout mutants for DLDH in *Brucella* would be useful for studying virulence attenuation and survival. The potential use of DLDH knockout mutants as a vaccine warrants further investigation.

One technical limitation of studying the secretome is the contamination of the culture media with proteins released from dead or dying *Brucella* cells. Since most of the proteins identified using MS are soluble proteins that lack a signal peptide, it is likely that some of these proteins come from burst dead cells. However, it is interesting to note that there are many differentially expressed proteins between the *vir*B mutants and the virulent strains of *Brucella*. Most of these proteins remained unidentified and may represent proteins secreted by the Type IV secretion system encoded by the *vir*B operon. The difference in their expression levels indicates basic metabolic differences that are essential in attenuating virulence and survival of *B. abortus* inside the host cell.

6. Conclusions

Since the first publication of a completely annotated *B. melitensis* genome in early 2002, the number of expressed proteins identified in this organism has increased significantly. Aside from the availability of a completed genome, this feat would not have been possible without advances in protein separation technology and mass spectrometry. Consequently, the global view of the proteome has lead to an integrative perspective of *Brucella* metabolism and a clearer understanding of phenotypic expression and evolution. The sequencing and annotation of the genomes of other *Brucella* species will be crucial in future proteomic investigations and will provide answers to basic questions relating to host preference, virulence, and survival inside the host. These future studies will also impact on the development of next generation antibiotics, antimicrobials, and vaccines. Proteomics will provide more definitive answers to these fundamental questions that describe the biology of the *Brucella*.

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Chapter 7
Brucella Virulence: A Matter of Control

Jean-Jaques Letesson* and Xavier De Bolle

From: *Brucella:* Molecular and Cellular Biology. Edited by: Ignacio López-Goñi and Ignacio Moriyon

Abstract

The publication of the complete genome sequences of several *Brucella* species allowed us to make a survey of the genomic information available on the regulation of gene expression and hence on the possible control of virulence. We identified 146 transcriptional regulators in the genome of *B. melitensis*. More than fifty percent of these are located on the small chromosome that, however, represents only one third of the total genome. With regard to two-component signal transduction systems, *B. melitensis* contains nineteen predicted histidine kinases and twenty one predicted response regulators allowing the formation of between ten and twelve pairs of proteins. The unmatched ones either belong to a phosphorelay system or are orphans. A phosphoenolpyruvate-dependent phosphotransferase transduction pathway also appears to be present and is predicted to function in a way resembling the phosphotransferase systems of Gram-positive bacteria. With regard to specialised global regulators, *B. melitensis* has five sigma factors in addition to the housekeeping sigma 70 factor, two quorum-sensing linked regulators and one regulator involved in the stringent response. Where possible, we compared the organisation of the mutated locus in the a *Proteobacteria* and tried to predict putative functions by using, as the starting point, the previously reported attenuation of *B. melitensis* mutants in regulatory factors.

"Name me someone that's not a parasite,
and I'll go out and say a prayer for him"

(Bob Dylan, 1966, "Vision of Johanna")

1. Introduction

The overall adaptability of an organism is a function of both the genetic information available and the mechanisms that control gene expression. The general adaptability rule for a bacterium in a given environment is to express the gene products allowing a growth or survival advantage and to repress unnecessary or deleterious ones.

Brucella has a partially understood infectious cycle (Letesson *et al.*, 2002; Moreno and Moriyón, 2002) whose key feature is the capacity to survive and multiply intracellularly in both phagocytic and non phagocytic cells. This ability

* For correspondence email jean-jacques.letesson@fundp.ac.be

relies mostly on the subversion of the vacuolar traffic of the infected cells (Gorvel and Moreno, 2002). During its odyssey in the host and its cells, *Brucella* has to adapt to an array of environmental conditions. From the *Brucella* viewpoint, the infection can be viewed as a developmental or differentiation process characterised by a succession of temporally and spatially defined events. Stepwise, and according to the conditions encountered at each specific stage of the infectious cycle, a tight and co-ordinated fine tuning of the expression of the virulence genes is needed while no necessary functions are shut down. The deciphering of these regulatory processes is of paramount importance for a better understanding of the pathogenesis.

On the basis of the phenotype of the mutants attenuated in various infectious models described in the last five years (Foulongne *et al.*, 2000; Lestrate *et al.*, 2000; Delrue *et al.*, 2001; Ficht, 2002; Kohler *et al.*, 2002; Kim *et al.*, 2003) and the genome sequences available (DelVecchio *et al.*, 2002; Paulsen *et al.*, 2002), we will review the regulation of virulence gene expression in *Brucella* and make predictions about signals or factors that could be part of the intricate regulatory network that governs the overall virulence.

2. The Regulatory Network

The expression of virulence genes is usually governed by signalling pathways and regulatory mechanisms similar to those that control genes that are not specific for pathogenesis. *Brucella* makes no exception to this rule. The following subsections deal with these classical signalling or regulatory mechanisms in *Brucella:* classical transcriptional regulators, signalling pathways based on reversible phosphorylation of proteins [two-component systems (TCS) or phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS)], and specialised global regulators acting either at the individual cell level or on a population basis. Each of these subsections contains a brief general introduction followed by the lessons we can learn about the particular mechanism by analysing the *Brucella* genome. Then, a short description of the relevant *Brucella* mutants and the ensuing predictions, if any, is included.

2.1. Transcriptional Regulators: GntR, LysR, AraC and Other Families

The main general signature in the DNA binding transcription factors of prokaryotes is the Helix-Turn-Helix (HTH) motif (Luscombe *et al.*, 2000). This motif also provides the main signature differences allowing the internal distinction among different families (Karmirantzou and Hamodrakas, 2001). Additional differences can be found in other domains also shared by members of the same family (e.g. sugar binding, oligomerisation domain, etc.). There are approximately twenty families defined in prokaryotes, among which the most frequent in the genomes sequenced so far are LysR, AraC/XylS, Cold, EBP, GalR/LaI, and GntR. It has also been suggested that the relative position of the HTH motif in the sequence correlates with its role as negative or positive regulator (Perez-Rueda and Collado-Vides, 2000). A protein with the HTH in the C-terminal part is often assumed an activator. On the contrary, an HTH motif in the N-terminal part is predicted to belong to a repressor. There are, of course, exceptions to this general scheme: the

Table 1. Characteristics of the main families of transcriptional regulators identified in the genome of *B. melitensis*

Family	Position of the HTH domain[1]	Regulator Role[2]	Number on CI[3]	Number on CII[3]	Total	%
GntR	N	repressor	6	14	20	13.7
LysR	N	dual	9	11	20	13.7
AraC	C	activator	5	8	13	8.9
MarR	V	?	5	7	12	8.2
AsnC	N	dual	5	5	10	6.8
TetR	N	repressor	6	2	8	5.5
IclR	N	repressor	2	5	7	4.8
LacI	N	repressor	3	4	7	4.8
FnR	C	dual	2	4	6	4.1
ArsR	M	?	3	3	6	4.1
Xre	N	repressor	5	1	6	4.1
MerR	N	repressor	4	2	6	4.1
DeoR	N	repressor	2	3	5	3.4
RpiR	N	repressor	0	3	3	2.1
Other		variable	11	6	17	11,6

TOTAL			**68**	**78**	**146**	**100**

[1] N: N-terminal; C: C-terminal; V: variable; M: middle.
[2] According to Perez-Rueda and Collado-Vides, 2000.
[3] CI: chromosome I; CII: chromosome II.

dual regulator families that are either activators of several genes and repressors of their own expression (e.g. the LysR family; Schell, 1993) or activators and repressors of different sets of genes (e.g. Crp/Fnr family). Within these families, the domain linked to the DNA-binding domain is more or less conserved. For example, the N-terminal nucleotide binding domain of the Crp/Fnr family, which shows homology with cAMP-dependent kinase (Kolb *et al.*, 1993), the C-terminal sugar binding domain of the RpiR and GntR families or the C-terminal ligand binding (sensory function) of the LysR family.

Using the signature consensus of these different families, we undertook the identification of the *B. melitensis* proteins with a known HTH motif excluding the members of the TCS, the sigma factors and the quorum sensing regulators which will be discussed separately. In addition, regulators potentially missed in the first approach were identified by keyword searches in two databases: the High quality Automated and Manual Annotation of Microbial Proteome (HAMAP proteomes) http://ca.expasy.org/sprot/hamap/hamap_stat.html, and the *B. melitensis* genome database (see Chapter 4) http://serine.urbm.fundp.ac.be/~seqbruce/GENOMES/Brucellamelitensis/.

2.1.1. What the Genome Tells Us

We identified one hundred and forty-six transcriptional regulators (TR) in the genome of *B. melitensis*. Of these, one hundred and twenty nine clustered in fourteen families represented by at least three members (Table 1). The remaining seventeen predicted Coding Sequences (pCDS) have been placed in one common heterogeneous category either because they are less than three members of one known family or because they were not assigned to a well defined family. There is an obvious disequilibrium between both chromosomes, since more than fifty percent of these TR are localised on the small chromosome that represents only one third of the total genome. Except for the TetR, MerR and Xre families, all the other TR families are equally or more represented on chromosome II. The LysR and the GntR families, both with twenty members (representing more than 25% of the total TR number) are the most represented ones. The LysR family is known to be more represented in the α and γ *Proteobacteria* (e.g. *Escherichia coli* has 45 LysR type TR) (Perez-Rueda and Collado-Vides, 2000). The GntR family appears to be overrepresented in *Brucella* as compared to other members of the α- *Proteobacteria* or even *E. coli* where the LysR family is almost twice as large as the GntR family. The latter is divided into four subfamilies (Rigali *et al.*, 2002) mostly according to the fold of the C-terminal effector binding oligomerisation domain: FadR (the most common) is exclusively α-helical; HutC has a C-terminal domain with several β-sheets and α-helixes; YtrA has the shorter domain with two α-helixes; and Moc R has the longest domain up to 350 amino acids which is highly homologous to the class I aminotransferase proteins. The GntR TR family of *Brucella* is composed of 14 FadR-like,

5 HutC-like and 1 MocR-like regulators. It is worth noting that among the six TR belonging to the Fnr/Crp family none is homologous to a *bona fide* CRP protein that could be involved in mediating a catabolic repression process (this point will be discussed below). It has to be emphasised that these are computational predictions that need experimental confirmation.

2.1.2. Mutants

Taking into account the number of genes of *B. melitensis* predicted to be involved in transcriptional regulation it is surprising that only five of them have been identified as attenuated in the various screenings published in the literature (Table 2). This relative paucity could be linked either to a redundancy in the regulatory mechanisms of the virulence genes or a lack of induction of certain virulence genes under the conditions used for those screenings. With this in mind, and based on the predictions described above, we undertook a systematic disruption of the predicted TR of *B. melitensis*. Due to the high number of mutants to be generated and tested, we focused our strategy on the TR families containing genes homologous to those known to be involved in either pathogenicity or symbiosis in other bacteria. All of the identified members of the following families were mutated by integrative

Table 2. List and characteristics of the mutant of *B. melitensis* TR identified so far as attenuated.

Family[1]	Name	N° pCDS	Reference	Models used
GntR	GntR1[2]	bmeII0475	V. Haine[4]	mice
	GntR4[2]	bmeI0169	V. Haine[4]	mice
	GntR5[2,3]	bmeI0881	V. Haine[4]	mice
	GntR10[2,3]	bmeII0116	V. Haine[4]	mice
	GntR 18	bmeII1066	Lestrate, 2003	mice, macrophages, HeLa
LysR	LysR12[2,3]	bmeII0390	V. Haine[4]	mice
	LysR13[2,3]	bmeI1913	V. Haine[4]	mice
	LysR18[2]	bmeI1573	V. Haine[4]	mice
	LysR21	bmeI0513	Foulongne *et al.*, 2000	macrophages, HeLa
AsnC	AsnC11	bmeI0357	Lestrate, 2003	mice, macrophages, HeLa
RpiR	RpiR[2]	bmeII0573	V. Haine[4]; Lestrate, 2003	mice, macrophages[5], HeLa[5]
DeoR	DeoR[3]	bmeII1093	Kohler *et al.*, 2002	macrophages
Other	NikR	bmeII0486	Lestrate, 2003	mice, macrophages, HeLa

[1]see Table 1; [2]mutants identified in the systematic disruption strategy; [3]no special function prediction can be made for these TR because we failed to identify unambiguously a conserved locus organisation in other Rhizobiaceae; [4]personal communication; [5]no attenuation was recorded

in these models.

disruption: LysR (n=20), GntR (n=20), AraC (n=13), DeoR (n=5), CrP (n=6), TetR (n=8), IclR (n=7), MerR (n=6), ArsR (n=6) and RpiR (n=3). All of these mutants were validated by Southern blot and some of them were analysed for their residual virulence in cellular models and mice (Table 2). The putative function of these regulators was predicted through observation of their genomic loci and, if feasible, the conservation of this locus organisation in other bacteria (mostly α-*Proteobacteria*) (Moreno-Hagelsieb *et al.,* 2001). This genomic co-localisation of a TR and some of its target genes allows suggesting testable hypothesis about their role in virulence. These data are summarised in Table 2 and discussed briefly hereunder.

The GntR1 TR is encoded by a gene located within a cluster of genes potentially involved in glucuronate metabolism (*uxuA,B* and *C*) and is homologous to *B. stearothermophilus uxuR* which encodes the repressor of the *uxuAB* operon (Shulami *et al.,* 1999). The *gntR4* gene is positioned downstream and in opposite direction of a membrane protein putatively involved in transport. This locus is conserved in *Mesorhizobium loti, Sinorhizobium meliloti* and *Bradyrhizobium*. This TR belongs to the MocR subfamily of GntR regulator and is homologous to MocR of *S. meliloti* which is described as a probable rhizopine catabolism regulator (Rossbach *et al.,* 1994). We are currently investigating its role in the control of the *moc* locus of *Brucella* and its relation with the RpiR regulator also involved in rhizopine metabolism (Lestrate 2003; see below).

The gene encoding GntR18 lies downstream and in opposite direction of an «operon» encoding subunits (GlcD, E and F) of a glycolate oxidase. An additional TR gene of the LysR family is located between the *glc* operon and the *gntR18* gene. This organisation is conserved in *S. meliloti* except that one additional gene is present between the *lysR* and *gntR18* homologs. Like in *E. coli,* in *M. loti* there is no *lysR* homologue in this region, and the *gntR18* homologue is just upstream and in opposite direction of the *glc* operon. This GntR regulator has been demonstrated to regulate the expression of the *glc* operon in *E. coli* (Pellicer *et al.,* 1996). This enzyme is involved in the metabolism of glycolate to glyoxylate. The carbon source is of crucial importance for an intracellular pathogen (Lorenz and Fink, 2002) and the glyoxylate cycle allows the utilisation of compounds with two carbons such as acetate to satisfy cellular carbon requirements. The two key enzymes in this pathway are isocitrate lyase *(icl)* and malate synthase *(glcB),* and it is known that *Mycobacterium tuberculosis* mutants in the *icl* gene are attenuated (McKinney *et al.,* 2000). *B. melitensis* has genes that are highly homologous to *icl* and *glcB* and it can be predicted that the corresponding mutants are attenuated in the chronic phase of infection. The adjacent organisation of the AsnC TR identified in the screening performed by Lestrate *et al.* (2000) is conserved in *M. loti*. The gene is located upstream of a gene encoding a dNTP pyrophosphatase and downstream of two genes in opposite orientation.

The NikR regulator identified in the STM screening published by these same authors (Lestrate *et al.,* 2000) is located upstream and in the opposite orientation to a gene (bmeII487) identified as a nickel periplasmic binding protein. In *B. suis,* the latter pCDS was shown to be specifically induced in human macrophages (Jubier-Maurin *et al.,* 2001)

but the corresponding mutant was not attenuated in this cellular model. This peculiar regulator is not conserved in either *S. meliloti* or *M. loti*.

The RpiR regulator is located in a region comprising the *iolC,D,E(mocC),B* genes, all of which are potentially involved in the catabolism of inositol-based compounds. The same organisation is also found in *M. loti* and *S. meliloti*. In these three species, this operon is in the immediate vicinity of the *mocA* gene, which encodes a myoinositol dehydrogenase. A mutant in the *mocC* gene has the same phenotype as the *rpiR* mutant: no attenuation in cellular models but attenuation in the mice model four weeks after infection. It appears that the catabolism of this peculiar carbon source is important for *Brucella* virulence. Other clues on the importance of this pathway are the identification of a *Brucella* STM mutant disrupted in a gene homologous to *mosA* (described as a methylase converting the scylloinosamine to 3 methyl scylloinosamine) (Saint *et al.*, 1993) and the demonstration by Eskra *et al.* (2001) in a DFI screen that the region upstream of the *mocC* gene of *B. abortus* is induced intracellularly in macrophages. This is reminiscent of the «rhizopine concept» which postulates that *Rhizobium* strains able to catabolise rhizopine have a competitive advantage in the nodulation process (Gordon, 1996).

LysR18 lies upstream and in the opposite orientation to two genes encoding respectively a hypothetical membrane spanning protein and a guanine deamylase, and upstream of three genes, *xdhA, B* and *C,* involved in xhantine dehydrogenase. This last enzyme is involved in purine metabolism. This organisation is conserved both in *M. loti* and in *S. meliloti* except that two additional genes are located between the *lysR18* homolog and the three *xdh* genes. The LysR21 regulator lies upstream a gene encoding a thioredoxine reductase. This locus is conserved in both *S. meliloti* and *M. loti*. This enzyme has a role in pyrimidine biosynthetic reaction (Kuriyan *et al.*, 1991). It is well known from previous work that these purine and pyrimidine metabolic reactions are crucial for *Brucella* intracellular survival (Kohler *et al.*, 2003).

2.1.3. Signals

Whereas few (if any) signals have been identified or can be predicted for the two-component regulatory systems (see below), the situation is somewhat better for the classical TR, opening once more the way for an experimental validation of the predictions. To the best of our knowledge, the only TR of *Brucella* for which a cognate signal has been experimentally tested is the RpIR regulator located in the operon for the catabolism of inositol based compounds. Using a transcriptional *gfp* fusion to the *rpiR* promoter, we demonstrated that synthetic 3-OMSI induces the expression of this promoter, which also happens in a *rpiR* mutant background without addition of exogenous 3-OMSI. This observation is coherent with the existence in the RpiR family of the TR of a sugar-binding domain (in addition to the HTH domain). It also indicates also that RpiR is a repressor of its own expression and probably of the whole catabolic operon. All these data reinforce the evidence for the biological relevance of the rhizopine story in *Brucella* pathogenesis.

Some other non-exhaustive predictions are summarised below where greater attention has been given to the Crp/Fnr family. As already mentioned, nickel or other divalent cations could be the signal for the so-called NI responsive regulator. Another obvious

example is pCDS II0427 *(eryD),* which encodes a putative erythritol repressor; this gene is in direct vicinity of other genes known to be involved in erythritol catabolism (*eryA, B* and *C*). EryD could be predicted to be responsive to erythritol (Sangari *et al.,* 2000). Recently, Kohler *et al.* (2002) have identified *eryC* and *B* mutants as being attenuated in a screen of *B. suis* mutants performed in human macrophages.

The Crp1 regulator (bmeI1294) is homologous to FixK1 of *B. japonicum* and AadR of *Rhodopseudomonas palustris,* both involved in the regulation of micro aerobic or anaerobic dependent processes (Anthamatten *et al.,* 1992; Egland and Harwood, 1999). In addition to the classical conservation of five glycine residues of the sensor domain and of the typical HTH motif in the C-terminal domain, this protein exhibits four cysteine residues which are reminiscent of those found in the Fnr protein of *E. coli* (Kiley and Beinert, 1998). This suggests that Crp1 in *Brucella* could also be a sensor for O_2 concentration via a 4Fe-4S centre. If this is correct, Crp1 could play a role in adapting the bacteria to the microaerophilic environment encountered intracellularly, perhaps by regulating the denitrification process or the use of an alternative cytochrome oxidase. Crp4 (bmeII0966), Crp5 (bmeII0986) and Crp6 (bmeII0947) are also worth investigating. On the basis of their HTH consensus motif, they all belong to the first class of the Crp/Fnr family

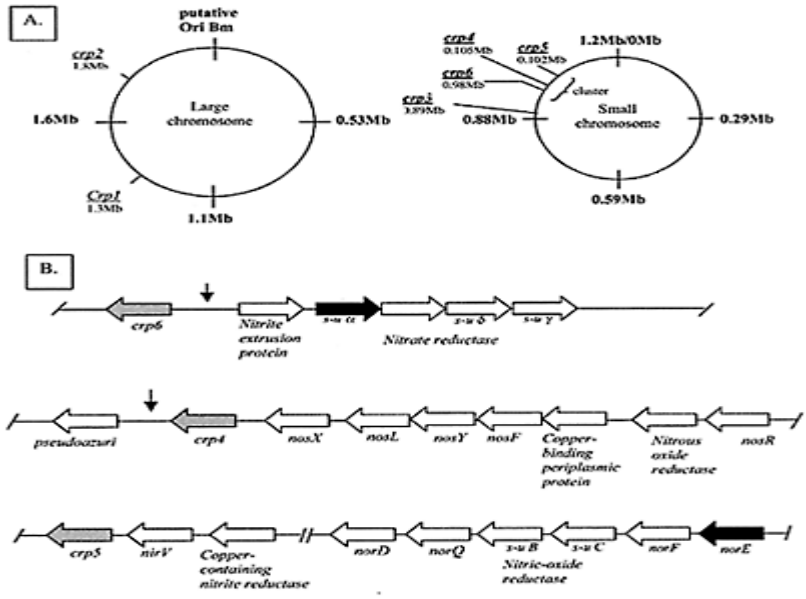


Figure 1. The Crp genes of *B. melitensis*. (A) Chromosomal maps of the *crp* genes. Cluster indicate the localization of the genes involved in the denitrification process. (B) Schematic representation of the cluster

of genes involved in the denitrification process. The filled grey arrows represent the TR of the Crp family. The filled black arrows represent genes for which attenuated *Brucella* mutants have been described. The small black arrows denote the predicted Crp1 binding motifs (FnR box).

(Fischer, 1994). The closest homologues are the NnrR of *R. sphaeroides* (for Crp4 and 5) and the NarR of *Paracoccus pantotrophus* (only for Crp6). These have been respectively described as involved in the regulation of the nitrite/nitric oxide and nitrate reductase during denitrification, (Sabaty *et al.*, 1999). In addition to these informative homologies, these pCDS cluster in chromosome II within a group of genes predicted to be involved in a complete denitrification process (Figure 1). In fact, *crp6* is upstream of the operon encoding both a transporter for oxidised nitrogen and the subunits of nitrate reductase. This overall organisation is conserved for the *nar*R of *P. pantotrophus*. The *crp4* gene is located upstream a cluster of genes involved in the nitrous oxide reduction process, and the *crp5* pCDS is located downstream of genes involved in nitrite reduction and nitric oxide reduction. Such a general organisation leads us to hypothesise that Crp6 could regulate nitrate reductase, Crp5 nitrite and possibly oxide nitric reductase, and Crp4 nitrous oxide reductase. Mutants in these regulators were constructed by integrative disruption and we demonstrated that, contrary to the wild type *B. melitensis* 16M, the *crp6* mutant was unable to anaerobically reduce nitrate to nitrite. The *crp5* mutant accumulated higher doses of nitrite when grown anaerobically on nitrate. These data confirm the functionality of the anaerobic respiratory chain predicted by the genomic analysis. They also open the way to deciphering the regulation of a denitrification process that has recently been shown to be important for the virulence of *Brucella*.

The *narG* (encoding a subunit of the nitrate reductase; Kohler *et al.*, 2002) and *norE* (encoding a subunit of the nitric oxide reductase; Lestrate *et al.*, 2003) mutants have been identified as attenuated in human macrophages and in mice, respectively. This means that these genes are needed either for an anaerobic respiration or eventually to detoxify reactive nitrogen intermediates produced during cellular activation, as hypothesised by Gary Splitter's team in a recent paper (Wang *et al.*, 2001).

Putatively, all these Fnr-like regulators could be responding to either low oxygen tension or to NO_3 or NO driven signals as previously shown in *Pseudomonas* and *Rhodobacter* (Zumft, 2002). Obviously, the major co-ordinator of this response should be the Crp1 regulator since it is able to sense the O_2 tension through its 4Fe-4S centre. In fact, there are well-conserved Fnr boxes (TTGATNNNNATCAA) both between the *crp6* and nitrate reductase genes and upstream of the nitrous oxide reductase genes. Amazingly, a *crp1* mutant has the same phenotype as the *crp6* mutant with respect to nitrate reduction.

pCDS I1776 is also of interest. This pCDS is highly homologous to the *hrcA* (heat shock regulation at CIRCE elements) which is located just upstream the essential chaperone gene *grpE* and downstream of a gene encoding a ribonuclease. This peculiar

organisation is conserved in *B. japonicum*, *S. meliloti* and *Caulobacter crescentus*. In this latter organism, both the promoter of the *hrcA*/ *grpE* transcriptional unit and an independent promoter of *grpE* are heat-inducible, and *hrcA* appears to regulate negatively (i.e. it is a repressor) the *groESL* operon (Roberts *et al.*, 1996). In this case, the signal is a rise in temperature.

2.2. Signal Transduction

Signal transduction systems allow cells to sense their environment, to transduce the stimuli through their membrane(s) and to build an appropriate response: e.g. to adapt their structure, their metabolism or their mobility. These systems are based on reversible protein phosphorylations (Reizer and Saier, 1997), and can be classified into three different categories: the two-component systems (TCS) in which sensor histidine kinases function in phosphoryl transfer to aspartyl residues in response regulators (see below); PTS, which functions in phosphoryl transfer to protein histidyl and cysteinyl residues; and the ATP-dependent protein kinases, which phosphorylate seryl, threonyl and tyrosyl residues in target proteins (STK). This last one is more commonly used in eukaryotes whereas prokaryotes generally rely on TCS or PTS (Bakal and Davies, 2000).

2.2.1. Two-component Systems

Few TCS have been described in eukaryotes and they are only found in yeast (Posas and Saito, 1998) and the plant *Arabidopsis thaliana* (Chang and Meyerowitz, 1994), but never animal cells. TCS represent around 1% of a bacterial genome, although this number is highly variable among genera. For example, whereas no TCS have been identified the genome of *Mycoplasma genitalium* (Fraser *et al.*, 1995), over 80 TCS are present in the cyanobacterium *Synechocystis* (Mizuno *et al.*, 1996). In the simplest version, TCS involve three sequential enzymatic phosphate transfer reactions and two protein partners called histidine kinase (HK) and response regulator (RR):

-reaction 1 (autophosphorylation): HK-His + ATP ⇔ HK-His~P + ADP

-reaction 2 (phosphotransfer) : HK-His~P + RR-Asp ⇔ HK-His + RR-Asp~P

-reaction 3 (dephosphorylation) : RR-Asp~P + H_2O ⇔ RR-Asp + Pi

This transference follows the rule «H1-D1», where H is histidine and D aspartate. Before analysing the data provided by the genome of *B. melitensis*, we will describe briefly the structure and function of both protein partners.

2.2.1.1. Histidine Kinase

HKs have been reviewed recently and only the essential aspects are summarised here (Hoch, 2000; West and Stock, 2001). Orthodox HKs are generally cytoplasmic membrane sensors which, upon stimulation, dimerise and undergo autophosphorylation of a conserved histidine residue before transferring the phosphate group to a RR, with which they are mated monogamously. In addition to their essential kinase activity, some

HKs could also stimulate the phosphatase activity of their cognate phosphorylated RR. On the other hand, some proteins not homologous to HKs are specialised in this dephosphorylation reaction (e.g. SpoOE). The architecture of HKs is based on several modular domains whose organisation allows a classification into families (Parkinson and Kofoid, 1992; Foussard *et al.*, 2001). The N-terminal sensor domains, also called input domains, of the various HKs are rather heterogeneous both in size and in sequence, reflecting the variability of the inducing stimuli. Usually, the sensor domains present several transmembrane regions, and these HK belong to the classical IT family (Input and Transmitter). However, some HKs are soluble and lack transmembrane regions: these belong to the IcT family (Input cytoplasmic and Transmitter) and are believed to sense directly intra-cytoplasmic stimuli or to interact with cytoplasmic domains of other proteins acting as sensors. The linker domain contains «coiled coil» motifs (Singh *et al.*, 1998) which could stabilise the homodimeric interaction between the N-terminal part of the autokinase domain. In contrast to the variable sensor domain, the C-terminal autokinase domain has a constant size (around 250 aa) and presents conserved motifs that allow a subdivision in two sub-domains: a N-terminal dimerisation sub-domain formed by two α-helices giving a «four helix bundle» structure when dimerised. The conserved histidine residue target of the auto-phosphorylation process is bore by this sub-domain together with the consensus motif SHELRTP (also called H-box) involved in the phosphotransfer to the RR. The C-terminal catalytic domain contains four conserved motifs (i.e. the N, G1, F and G2 boxes) all involved in Mg-ATP binding. This domain is homologous to the ATPase domain of type II topoisomerases and of Hsp90 chaperones.

In addition to orthodox HKs, more complex HKs, called hybrid-HKs, have also been described. In addition to the classical domains associated with HKs, they also contain a receiver domain characteristic of the RR (see below) in their C-terminal region: they form the ITR family (Input, Transmitter and Receiver; Parkinson and Kofoid, 1992). The modular architecture of such hybrid-HK allows a more convoluted signal transduction pathway as illustrated by the phosphorelay concept (see below). In the specialised cascades involving hybrid-HK, one also often finds a histidine phosphotransferase module (HPt), devoid of catalytic activity, covalently linked to the hybrid-HK. The function of this HPt is to relay the phosphate group issuing from the hybrid-HK to the destination RR (Perraud *et al.*, 1999).

The HKs can also be divided into subfamilies according to the presence of domains also present in other proteins (e.g. the PAS domain known to sense a signal through the binding of a co-factor).

2.2.1.2. Response Regulators

RRs have also been reviewed recently (Hoch, 2000; West and Stock, 2001). Usually, RRs are bifunctional proteins that contain a conserved N-terminal regulatory domain separated by a short linker from a variable C-terminal effector domain. According to the presence and type of the effector domain, RRs are also classified into several families (Parkinson and Kofoid, 1992; Foussard *et al.*, 2001). The function of this C-terminal domain is usually to bind to DNA and to activate the initiation of transcription (thus resulting in the RO_{II}, RO_{III} and RO_{IV} families, for Receiver and Output). However, enzymes or domains of other types (forming a heterogeneous family called RO_{I}) may be controlled in this

manner. For example, in *E. coli*, 25 out of 32 RRs have a DNA-binding domain. Alternatively some RRs can be devoid of the effector domain (the R family, for Receiver) or combined to other domains as described above for the hybrid-HK.

The conserved regulatory domain, also called receiver domain, is approximately 125 aa in size, and its three dimensional fold consists of a central five-stranded parallel β-sheet flanked by five amphipathic helices. Several amino acid residues are extremely well conserved within this structure. Four of these participate in the active site (also called the acidic pocket): one aspartate (D57, the phosphorylation site), two other acidic residues (D12 and D13, involved in Mg^{++} binding), and a lysine (K109, interacting with the phosphate group). In addition, other residues in the β-sheet also appear to be conserved, a serine/threonine around position 87 and an aromatic residue at position 106, either tyrosine or phenylalanine. The enzymatic activity of this domain is mostly to catalyse the transfer of the phosphoramidate group from the cognate HK to its own aspartate (D57). In addition, some of the RRs also have an intrinsic phosphatase activity. Moreover, when phosphorylated, this receiver domain can relieve the structural inhibition exerted on the effector domain and participate in the oligomerisation of the RR. The monodomanial RRs (the R family) always interact with other proteins (e.g. CheY).

Although all the DNA-binding effector domains of RRs have «helix-loop-helix» motifs, they can be divided into three families (RO_{II}, RO_{III} and RO_{IV}) according to the structural differences in this domain. The major representatives of these three families are, respectively, OmpR, NarL and NtrC. In *E. coli*, of the twenty-five DNA-binding RRs, fourteen belong to the OmpR family, seven to the NarL and four to the NtrC. Whereas the DNA-binding domain of the NarL and NtrC families (either as a monomer or as a dimer) contain the the «four-helix bundle» type topology, the OmpR family represents a novel sub-family of transcriptional factors of the «winged-helix» type (Kondo *et al.*, 1997). These structural differences between RRs correlate with divergences in the mechanisms of activation and with variability in the interactions with the transcriptional machinery of Cis-acting elements. For example, the effector domain of the OmpR family is known to interact not only with its target DNA motif but also with the housekeeping sigma factor (σ70) and sometimes with the C-terminal domain of the RNA polymerase (αCTD). In the same way, the RRs belonging to the NtrC family are able to interact co-operatively with σ54 dependent promoters.

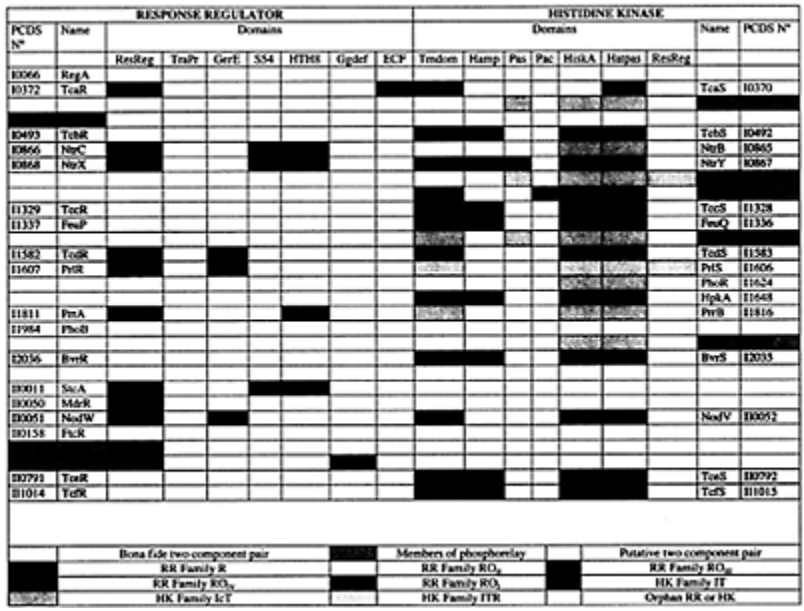
2.2.1.3. The Phosphorelays

In addition to the archetypal system of «one HK-one RR» described above, a huge variety of circuits has evolved over time and these are based on the modular architecture of the basic TCS. These more complex pathways also use phosphorylation for communication between modules but they obey to the rule H1-D1-H2-D2 (Appleby *et al.*, 1996). This rule implies three sequential phosphotransfers as compared to the single phosphate transfer H1-D1 described for the simple TCS. These phosphorelays involve an increase in the number of participating modules leading to the hybrid-HK (with a receiver domain in addition to the classical HK domains) so as to the HPt, a transmitting module devoid of catalytic activity. These multiple phosphotransfers can be inter or partially intra-molecular, the H1, D1, H2, D2 modules being either on the same or separate proteins (theoretically from one to four different proteins). The first obvious evolutionary

advantage given by such a phosphorelay is the capacity to integrate a huge variety of inputs.

Based on the structural domain characteristics described above, we undertook the task of identifying all of the pCDS in the *B. melitensis* genome that could be part of the TCS of *Brucella*. To this end, we took advantage of the availability of the following web facilities: the Conserved Domain Database (CDD) (Marchler-Bauer *et al.,* 2003) http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=cdd; the SMART (Simple Modular Architecture Research Tool) web tool (Letunic *et al.,* 2002) http://smart.embl-heidelberg.de/; the Pfam protein families database (Bateman *et al.,* 2002) http://www.sanger.ac.uk/Software/Pfam/; and the *B. melitensis* genome database (see Chapter 4) http://serine.urbm.fundp.ac.be/~seqbruce/GENOMES/Brucellamelitensis/.

Table 3. The RR and the HK are coded according to their respective family and presented according to their pCDS number and the chromosome on which they are located. Their domain content is represented from left (N-term) to right (C-term) except when otherwise stated



2.2.1.4. What the Genome Tells Us

We identified the RRs using as signature the response regulator receiver domain (Pfam 00072) and the HK using first the HATPase-c (Pfam 02518) and then the histidine kinase A (phosphoacceptor) N-terminal domain (Pfam 00512). The translated sequences of all *B. melitensis* pCDS were searched for homology with the respective consensus motif of

the domains. The pCDS identified as either RRs or HKs were submitted to a multiple sequence alignment using ClustalW in order to ascertain the conservation of the critical residues. Afterwards, features of the *B. melitensis* database allowed us to identify other conserved Pfam domains for each pCDS, the presence of transmembrane segments, and finally the genetic neighbours of the pCDS under analysis. The resulting data are summarised in Table 3. The RR and the HK are colour coded according to their respective family (see text and legend) and presented according to their pCDS number and the chromosome on which they are located. In addition, for each pCDS a graphical output allows the visualisation of the Pfam identified domains oriented, except otherwise stated, from the left (N-terminal) to the right (C-terminal). Finally, a name was given to each pCDS using either the previously published one or a four letters code (R. Hallez *et al.*, unpublished).

B. melitensis has 23 pCDS containing a RR domain, and of these 15 located on chromosome I and 8 on chromosome II. Two belong to hybrid-HK (CckA and PrlS) and these will be discussed with the other HKs. On each chromosome, two of the pCDS (namely CtrA and CckA on the large chromosome, and DivK and PleD on the small chromosome) were identified as belonging to a phosphorelay, which will be briefly described in the next section. Also on each chromosome, three pCDS containing the RR domain are orphans, in the sense that they are not located close to a HK or part of an identified phosphorelay. Among these the pCDS bmeII0158 (called FtcR for Flagellar Two-Components Regulator) is located in a flagellar locus and will be discussed in the next section. The small chromosome contains three RRs in close association with their putative cognate HK, none of which has been described or analysed previously. Seven RRs linked to their cognate HK are located on the chromosome I, among which BvrR, NtrC and FeuP have been studied previously. In addition, a special case has to be made for the PrlRR (pCDS I1607) that is in the reverse orientation compared with the adjacent HK (*prlS*, 11806). The latter appears to be a peculiar hybrid-HK (see below). The pCDS 11811 *(prrA)* on chromosome I is separated from its putative kinase (*prrB*, 11816) by four open reading frames (ORF) which could be a remnant of a transposable element. In addition the DNA binding domain of the *prrA* product is quite degenerate and could lack functionality. Four of the pCDS identified as RR lack a DNA-binding domain. Among these, two were identified as being part of a phosphorelay: PleD with two C-terminal domains of unknown function (Pfam 00990) called GGDEF domain, homologous to the adenylyl cyclase catalytic domain, and DivK which is strictly monodomanial (only the RR domain). Of the remaining pCDS that lack a DNA-binding domain, one (*mdrR*, for Mono Domain Response Regulator, II0050) is also monodomain (much like DivK) and could tentatively be part of a phosphorelay. The other (*tcaR*, I0372) has its RR domain at the C-terminal section of the protein, and its N-terminal domain is divergently related to the σ-24 factor (COG1595, RpoE). This peculiar domain organisation is only found in other α-*Proteobacteria* (*Agrobacterium tumefaciens*, *C. crescentus*, *S. meliloti* and *R. sphaeroides*). Seventeen out of the twenty-one pCDS containing a RR domain also have a DNA-binding domain and, according to the architecture of the latter domain, they can be subdivided into three families. The largest OmpR family (RO_{II}) is represented by ten members (among them CtrA, FeuP, BvrR and FtcR). The NarL family (RO_{III}) contains three members (VsrR, NodW, I1582) and the NtrC family (RO_{IV}) four members (NtrC, NtrX, I1811, II0011). Except for pCDS I1811, for which we discussed the DNA-binding

domain previously, all members of the NtrC family exhibit a σ-54 interaction domain (pf00158) with an ATPase activity (placed between the DNA-binding domain and the RR domain) that may promote the conformational changes necessary for the interaction.

The *B. melitensis* genome contains nineteen pCDS with a *bona fide* phosphoacceptor and dimerisation domain of HK (Pfam 00512) followed in the C-terminal part by the catalytic HATPase-c domain (Pfam 02518). Three of these pCDS are located on chromosome II and sixteen on chromosome I. Despite the fact that the Pfam protein family database fails to detect a phosphoacceptor-dimerisation domain and detects only the catalytic domain, an additional pCDS located on the large chromosome (I0370) was selected as HK. This pCDS exhibits a N-terminal extracellular sensory domain (Pfam 05227.1), similar to those found in the various classes of transmembrane receptors (HK among others) involved in the signal transduction pathways in bacteria. This putative HK is separated from a *bona fide* RR (I0372) by a pCDS encoding an extra-cytoplasmic sigma factor. This organisation is conserved with respect to that found in *S. meliloti*. The three HK on the small chromosome are predicted to be anchored in the membrane (IT family) and belong to classical TCS. On the large chromosome, five predicted HK belong to a phosphorelay family (PdhS, CckA, PleC, DivJ and DivL). Except for PleC and DivJ, which are internal membrane proteins, the others are cytoplasmic soluble proteins and should react to an internal stimulus or interact with another protein in the membrane (IcT family). Together with the pCDS 11606, also called PrlS (see below), CckA are the only two hybrid-HKs detected in *Brucella* spp (ITR family). Most of the HKs with a Pas domain are linked to a phosphorelay. Taking into account the previously discussed I1583 pCDS, eight HKs belong to classical TCS sensors, some of which have been previously described (e.g. NtrB, NtrY, FeuQ and BvrS). Except for NtrB, which is known to be cytoplasmic in *E. coli* (Weiss *et al.*, 2002), all these kinases are predicted to be internal membrane proteins. The three remaining predicted HKs are orphans (in the sense that they are not close to a cognate regulator) and one, I1624, is predicted to be cytoplasmic and could sense an unknown internal signal. Except for the pCDS I0370, for which we have already discussed the lack of kinase domain, and DivL (I2027) all the other HKs identified have the consensus histidine residue in the right environment. From the *C. crescentus* story, DivL is known to have a tyrosine instead of a histidine as the phosphorylated residue on the kinase domain (Wu *et al.*, 1999).

Table 4. Characteristics of the *Brucella* mutants in signal transduction pathways described as attenuated

Systems[1]	Function[2]	Name	N° pCDS	Reference	Models used
TCS	HK	BvrS	bmeI2035	Kohler *et al.*, 2002 Sola-Landa *et al.*, 1998	mice, macrophages, HeLa
	RR	BvrR	bmeI2036	Kohler *et al.*, 2002 Sola-Landa *al.*, 1998	mice, macrophages, HeLa
	HK	FeuQ	bmeI1336	Lestrate, 2003	mice, macrophages, HeLa
	RR	FeuP	bmeI1337	Dorrell *et al.*, 1998	mice, macrophages
	HK	NtrY	bmeI0867	Foulongne *et al.*, 2000	macrophages, HeLa
	RR	NtrC	bmeI0866	Dorrell *et al.*, 1999	mice, macrophages
	RR	PlrR	bmeI1607	Lestrate *et al.*, 2000	mice, macrophages, HeLa
	HK	NodV	bmeII0052	A. Dricot[3]	mice
	ORR	FtcR	bmeII0158	D. Fretin[3]	mice, macrophages[4], HeLa[4]
	PR RR	CtrA	bmeI0947	Bellefontaine, 2001	essential gene
PTS	EI^{ntr}	pstP	bmeI0190	Delrue, 2002	macrophage
	$EIIA^{ntr}$	glnL	bmeI1786	Kohler *et al.*, 2002	macrophage, Hela

[1] TCS: two-component system; PTS: phosphoenolpyruvate dependant phosphotransferase system.
[2] HK: histidine kinase; RR: response regulator; ORR: orphan response regulator; PR RR: phosphorelay response regulator.
[3] personal communication.
[4] no attenuation in these models.

2.2.1.5. Mutants

Owing to their importance in integrating internal or external environment with gene expression, it is not surprising that some of the HKs or RRs of *B. melitensis* have been described as involved in the virulence process. Except for PrlR, which could belong to a phosphorelay, FtcR pCDS, which appears to be orphan RR and CtrA, which is an essential gene, all of the other mutants belong to classical TCS (Table 4). Up to now, no signal has been unambiguously identified as involved in the activation of TCS of *Brucella*.

BvrS/R Until now, this is by far the best characterised *Brucella* TCS (Guzman-Verri *et al.*, 2002; see Chapter 10). Highly homologous to the ChvI/ExoS and ChvI/ChvG systems involved

in symbiosis and pathogenicity in *S. meliloti* and *A. tumefaciens,* respectively, it is involved in the regulation of outer membrane constituents (Omps and lipid A) (López-Goñi *et al.,* 2002). Mutants in both partners of the TCS are attenuated in all models of infection tested. Its co-localisation in all α-*Proteobacteria* with genes encoding partners of the PTS (see below) suggests that it could be controlled through one of the predicted modes of PTS-mediated regulation and, therefore, that it could be linked to the availability of carbon or nitrogen sources.

FeuP/Q This was also one of the first TCS described in *Brucella* (Dorrell *et al.,* 1998). While the homologous system is involved in the control of iron assimilation in *R. leguminosarum,* the growth of an isogenic mutant of the *feuP* gene of *B. suis* was unaffected on iron-depleted media. Survival and multiplication of this mutant in the mouse model and in macrophages were also unaffected. Recently, we isolated a *feuQ* mutant in a STM screen of *B. melitensis* which was attenuated both in mice and in a cellular model of infection (Lestrate P., 2003). The reason for this discrepancy could come from species differences.

NtrC Dorrel *et al.* (1999) also identified the *B. suis* homologue of the NtrC RR. Together with its cognate HK (NtrB), this RR participates in nitrogen metabolism in several bacterial species and could play a role in virulence in some cases, e.g. by regulating the urease gene in *Klebsiella pneumoniae*. The *ntrC* deletion mutant of *B. suis* had a growth curve comparable to the wild type strain but it showed a reduced metabolism on certain amino acid sources. It was not attenuated in macrophages but was less virulent in the mouse model. Both the structure of the NtrB protein and what is known in other bacteria, allow us to predict that this sensor responds to an intracellular signal.

NtrY The *ntrY* gene was identified in a STM screen of *B. suis* as affected in intracellular survival in a human macrophage infection model (Foulongne *et al.,* 2000). This gene encodes a sensor HK that, together with the adjacent *ntrX* gene, could be part of a ntr-related regulon that may be play

a role in glutamine synthesis, a process known to be important for *Brucella* intracellular survival. This NtrY/NtrX TCS is encoded downstream of the *nifR3ntrBC* operon, a genomic organisation conserved in *S. meliloti, M. loti* and *Bradyrhizobium*. Moreover, it has been shown in *Azospirillum brasilense* that the *ntrYX* operon can complement a *nifR3ntrBC* deletion mutant for nitrate-dependent growth, thus suggesting a possible cross-talk between the NtrY/X and NtrB/C sensor/regulator pairs (Ishida *et al.,* 2002).

PrlR A mutant in pCDS I1607 was identified in a STM screen of *B. melitensis* in mice (Lestrate *et al.,* 2000). This mutant (called PrlR for Proline Regulator) was also attenuated in macrophages and in HeLa cells. This gene encodes a RR of the NarL family. Adjacent to it, but in reverse orientation, there is a big pCDS (11606) encoding a probable hybrid-HK showing eleven transmembrane segments related to the Na^+/solute symporters (Jung, 2001) plus a poorly conserved RR domain at its C-terminal part. Both this kinase and a RR in the opposite orientation are highly conserved in *S. meliloti* and *M. loti*. The possibility that this *Brucella* PrlR HK is sensing some kind of solute transport (e.g. proline, pantothenate, etc.) is worth investigating.

NodV pCDS II0052 encodes one of the three HKs belonging to the TCS in chromosome II. It has been disrupted in a search for *Brucella* genes homologous to genes involved in the symbiosis of *Rhizobiaceae* (Loh *et al.,* 1997). The mutant was attenuated in a mouse model of infection.

FtcR pCDS II0158 encodes an orphan response regulator located in one of the flagellar loci of *B. melitensis* and hence it was called FtcR. This pCDS was disrupted in an attempt to search

for a regulator of the expression of the flagellar structure. Although not attenuated in cell cultures, the mutant is attenuated in a mouse model of infection, and its attenuation is of the same order as that of a mutant in the very basal body of the flagellar apparatus (MS ring encoded by the *fliF* gene). This regulator positively controls the expression of the *fliF* gene as demonstrated by a lacZ reporter gene fused to the *fliF* promoter. This pCDS should be part of the master regulatory genes of the flagellar cascade. The presence of a quorum-sensing regulator in another flagellar locus of *B. melitensis* (VjbR) (see below) and in the same genetic environment as the quorum-sensing gene *(visR)* of *S. meliloti* (Sourjik *et al.,* 2000) provides a means of evaluating the influence of population density on the expression of flagellar genes.

2.2.1.6. The CtrA Phosphorelay

In this section, we will concentrate on the RR CtrA, an essential actor of the *C. crescentus* cell cycle (Quon *et al.,* 1996) that is present in *Brucella*. A brief summary of the data accumulated in *C. crescentus* will be followed by a description of the facts and hypotheses of interest in *Brucella*.

In *C. crescentus,* the known role of CtrA may be summarised as the co-ordinated control of cell cycle and morphogenesis. Indeed, CtrA controls the initiation of replication by binding to the chromosomal origin of replication (Quon *et al.,* 1998), and the methylation status of DNA by controlling the *ccrM* methyltransferase gene (Quon *et al.,* 1996). It also controls the *ftsZ* gene (Kelly *et al.,* 1998) that is involved in the initiation of septation. These are all essential steps in the cell cycle, and this explains why the *ctrA* gene is essential. The CtrA protein also controls flagellum and pili biogenesis (Skerker and Shapiro, 2000), thereby linking the cell cycle to morphological adaptations. Indeed, from a stalked cell, *C. crescentus* initiates DNA replication, and a flagellar structure is produced at the stage of pre-divisional cell, i.e. just before septation occurs. The resulting two daughter cells are different: one remains stalked while the other becomes a swarmer able to swim in the environment to find a better niche if available (Jones *et al.,* 2001).

Given its central role, CtrA must be controlled carefully, and the pathways involved are currently being deciphered. A first level of control is the transcription initiation at the *ctrA* promoter. CtrA is able to regulate its own transcription, which starts at two different +1 sites named P1 and P2 (Domian *et al.,* 1999). The CcrM-dependent methylation of the *ctrA* promoter is also able to modulate the transcription initiation at the *ctrA* promoter (Reisenauer and Shapiro, 2002). A second level of control is the phosphorylation of CtrA (Domian *et al.,* 1997), at the aspartate residue conserved in the RRs of the TCS. The direct interaction between CtrA and a kinase or a phosphotransferase remains to be discovered, but Jacobs *et al.* (1999) elegantly demonstrated that CckA, a hybrid-HK, is required for CtrA phosphorylation *in vivo*. According to classical models (Perraud *et al.,* 1999), a histidine pho sphotransferase (HPt) should transfer the phosphate from CckA to CtrA. The signals sensed by the CckA protein are unknown, but the membrane anchoring of CckA is essential for its activity (Jacobs *et al.,* 1999). The *in vitro* phosphorylation of CtrA by a DivL kinase has been reported (Wu *et al.,* 1999). DivL is homologous to HKs, but a tyrosine residue that is autophosphorylated similarly to the classical HKs occupies the position of the conserved histidine. The third known level of control of CtrA is by proteolysis. In the pre-divisional cell, the future swarmer and stalked cells form distinct

compartments, and CtrA is hydrolysed by a ClpXP-dependent process in the future stalked cell (Domian *et al.,* 1997). This allows a derepression of the initiation of replication in the stalked cell that can thus directly enter in a new cell division process. The control of CtrA proteolysis is dependent upon DivK since CtrA is no more proteolysed in a *divK-cs* mutant (Hung and Shapiro, 2002). The DivK protein is a monodomain RR that, according to the current model, is phosphorylated by the DivJ histidine protein kinase at the stalked pole of the pre-divisional cell (Lam *et al.,* 2003). DivK is afterwards dephosphorylated by PleC, another HK homologue to DivJ (Lam *et al.,* 2003). Contrary to DivJ and PleC, the DivL tyrosine protein kinase is unable to phosphorylate DivK *in vitro* (Wu *et al.,* 1999), but it is able to interact with DivK in a yeast two hybrid test (Ohta and Newton, 2003).

A homologue of CtrA has been described in *B. abortus* (Bellefontaine *et al.,* 2002). The *ctrA* gene is conserved in both the *B. melitensis* and *B. suis* genomes. The CtrA protein is phosphorylated *in vitro* on Asp-51 and it is produced during culture in rich medium. Overexpression of the *ctrA* gene using a P_{lac} promoter on a medium-copy plasmid yields a cell cycle defect phenotype, with branched cells larger than wild type cells. Among other phenotypes, branching is also observed in *R. meliloti* when inhibitors of septation are added (Latch and Margolin, 1997). This is consistent with the observation of a specific binding of purified CtrA on promoters of genes putatively involved in cell division, such as the *minB* operon (upstream *minC* pCDS) and the *ccrM* gene. It is interesting to note that the *minB* operon is absent in *C. crescentus,* and that the *Brucella ftsZ* promoter does not present CtrA binding sites. One interpretation would be that CtrA controls cell division in both species, but through the control of a set of genes that are not "orthologly" equal. The essentiality of the *ctrA* gene has been suggested because it was impossible to inactivate it despite numerous attempts (Bellefontaine *et al.,* 2002). This suggestion does also fit with a major role of *ctrA* in cell division.

The control of CtrA is unknown in *Brucella,* but several hypotheses may be proposed from the analysis of complete genomes. Concerning the transcriptional control of *ctrA* promoter by CtrA itself, two binding sites of low affinity can be identified, and it has been shown that phosphorylation of CtrA on Asp-51 strongly enhances the apparent affinity of CtrA for these sites (Bellefontaine *et al.,* 2002). Except for its N-terminal region, that lacks the predicted transmembrane segments described in *Caulobacter,* CckA is well conserved in *Brucella*. Accordingly, the phosphorylation of CtrA may be performed in the latter through the same pathway as the one proposed in *C. crescentus* (Jacobs *et al.,* 1999). The analysis of the *B. melitensis* genome suggests that the pathway controlling proteolysis of CtrA through DivK is conserved, except that PdhS (an additional HK of the PleC/DivJ family also called "bm1035" by Bellefontaine *et al.,* 2002) is present. There is also a strong homologue of DivL in *Brucella,* but it is not predicted to be associated to the membrane, as suggested by the analysis of the *Caulobacter* DivL sequence.

In summary, homology studies suggest that the central part of the pathways controlling DivK and CtrA is conserved in *Brucella*. The CtrA regulon and the mechanisms involved in controlling CtrA need to be studied *in vitro, ex vivo* and *in vivo*. It would be particularly interesting to establish if CtrA is involved in the inhibition of cell division that is observed during the first hours of a cellular infection.

2.2.1.7. Comparisons Among α-2 Proteobacteria

With twenty RRs and eighteen HKs identified, *B. melitensis* appears to have a smaller number of TCS as compared to *S. meliloti, M. loti, C. crescentus,* or *A. tumefaciens*. However, this number remains in the range of the 1% of the genome and is so in accordance with its smaller genome size and probably with its «facultative extracellular»-intracellular parasitic life cycle. Except for the BvrR/ BvrS system discussed in Chapter 10, few large-scale comparisons of TCS have been undertaken within α-2 *Proteobacteria*. Bellefontaine (2001) performed the deepest published analysis on the CtrA phosphorelay and target genes. Using the available genomes of *B. melitensis, R. capsulatus, R. palustris, S. meliloti, M. loti* and *A. tumefaciens,* she was able to identify in all but *R. capsulatus* the homologues of the upstream activation cascade described in *C. crescentus*. Moreover, additional HKs related to PleC/DivJ were identified in most of them (e.g., in *B. melitensis* pCDS I0417 also called PdhS for PleC/DivJ homologue sensor). Based on the CtrA box (TTAAN7TTAAC), which has been validated experimentally in *C. crescentus* and confirmed in *B. melitensis,* putative CtrA targets were searched *in silico*. From this survey, it can be proposed that: (i), CtrA may regulate its own expression, as described in *S. meliloti, C. crescentus* and *B. melitensis;* (ii), the striking plasticity of distribution of the CtrA binding motif in the promoters of the genes in the upstream activation cascade should be related to the various stimuli received by these kinases according to the various life styles of these bacteria; and (iii), the control of septation and replication by CtrA is conserved and could be mediated either via *ftsZ* and *hemE (*in *C. crescentus*) or via *minC, ftsE* and *ftsK* (1 and 2), in all sequenced α-2 *Proteobacteria* with an animal or plant host. No prediction could be made for *Rhodobacter* spp. which appear to lack both *minC* and *ftsE* homologues and do not show any CtrA box upstream of *ftsZ* or *ftsK*. Moreover, the CtrA role in *Rhodobacter* is non-essential and this regulator appears to control the release of Gene Transfer Agents as demonstrated by Lang and Beatty (2002).

2.2.2. The PTS System

The phosphoenolpyruvate (PEP) dependant sugar phosphotransferase system (PTS) is a complex protein system that mediates uptake and subsequent phosphorylation of incoming sugars or hexitols (for a review see Kuroda *et al.,* 2001). These systems are ubiquitous in *Eubacteria* but never occur in *Archeobacteria* or *Eukaryotes*. The basic components of the PTS are the cytoplasmic Enzyme I (EI) and Hpr, both of which lack sugar specificity, and a variable number of enzyme II (EII) complexes, each specific for one or a few sugars. EII consists of three functional units called EIIA, EIIB and EIIC, which can be either distinct proteins or domains of the same protein. The phosphoryl relay proceeds sequentially from PEP to hystidyl residues in EI, Hpr, EIIA, EIIB and finally to the incoming sugar which is simultaneously transported across the membrane and phosphorylated by EIIC. In addition, a paralogous system described in *E. coli* and other *Proteobacteria* functions in nitrogen metabolism regulation (Ntr; Rabus *et al.,* 1999). This nitrogen-related PTS catalyses a sequence of phosphoryl transfers from PEP to EI^{Ntr}, Hpr^{Ntr} (also called Npr) and finally to $EIIA^{Ntr}$. This latter chain was suggested to link carbon and nitrogen metabolism. The transfers performed by both systems can be summarised as follows:

PEP→EI(His-P)→Hpr(His-P)→EIIA(His-P)→EIIB(His-P)→EIIC→Sugar-P
PEP→EI^{Ntr} (His-P)→Npr(His-P)→$EIIA^{Ntr}$ (His-P)

In addition to their role in sugar translocation, some proteins in the PTS system play a critical role in signal transduction and regulate their targets either allosterically or by phosphorylation. In *E. coli*, some of the targets regulated by the PTS system are CheA (inhibited by dephosphorylated EI, thereby linking chemotactism and sugar availability), lactose permease (inhibited by dephosphorylated EIIA, thus linked to inducer exclusion), glycerol kinase (inhibited by dephosphorylated EIIA), adenylate cyclase (activated by phosphorylated EIIA-P, which is thus linked to catabolic repression through the production of cAMP and activation of the Crp protein), and dephosphorylated EIIB (which sequesters a transcriptional repressor of genes involved in glucose transport and metabolism). Finally, other regulatory mechanisms involve the activation (or inactivation) of transcription factors (repressors and anti-terminators) through PTS protein-mediated phosphorylation on conserved histidine residues (PTS regulatory domain) of the targets.

The basic principles of the PTS dependent catabolic repression in low GC Gram-positive bacteria (e.g. *B. subtilis*) are somewhat different (Saier *et al.*, 1995). In fact, these bacteria lack both adenylate cyclase and Crp, and the Hrp protein of the PTS contains a conserved regulatory serine phosphorylation site in addition to the conserved histidine phosphorylated by EI. In response to cytoplasmic metabolites (e.g. fructose1, 6 bisphosphate), a Hrp (PtsK) kinase becomes activated and, at the expense of ATP, phosphorylates the conserved serine residue of the Hrp protein. Then, Hrp (Ser-P) forms a complex with a repressor protein, which in this form is able to prevent transcription of the target operons (Reizer *et al.*, 1998).

Some bacteria are completely devoid of the PTS system (e.g. *M. tuberculosis*) while others lack the sugar permeases of the PTS. Nevertheless, the genomes of the latter encode phosphoryl transfer proteins of the classical PTS and these probably function as regulatory elements (Hu and Saier, 2002).

2.2.2.1 What about Brucella and the PTS

Brucella belongs to those bacteria that appear to be devoid of PTS sugar permeases and EIIB and EIIC components. Except for *C. crescentus* and *M. loti*, that display a permease of the glucose and dulcitol families, respectively, all the α *Proteobacteria* available on the Kegg web site (*A. tumefaciens*, *B. suis*, *B. japonicum*, and *S. meliloti*) lack PTS permeases. Two mutants of *Brucella* in genes of the PTS transduction pathway have been identified (Table 4), despite the fact that PTS permeases are lacking. Accordingly, the existence of mutants in genes encoding both an EI homologue (10190) and an EIIA homologue (I1786) is a strong indication that the PTS signal transduction pathway is at work in *Brucella*. This prompted us to have a closer look at the genome to uncover the complete set of PTS related gene products, to analyse their structure and to make comparisons within the α *Proteobacteria*.

Brucella has only one EI homologue (10190), and it is different from the classical sugar EI by having a larger size because of the presence of an N-terminal Nif-A-like sensory domain. This last feature suggests that it is an EI^{Ntr}. The situation is similar in all

other α *Proteobacteria* (Hu and Saier, 2002). It is worth noting that once more *C. crescentus* and *M. loti* differ from the other α *Proteobacteria* sequenced by possessing two additional EI homologues. In *E. coli*, EI^{Ntr} seems to function exclusively in regulation possibly controlling the activities of Npr and $EIIA^{Ntr}$. These last proteins are encoded within the *rpoN* (σ54) operon of *E. coli* and have been implicated in the regulation of the σ54-dependent transcription of genes concerned with organic nitrogen utilisation (Powell *et al.*, 1995).

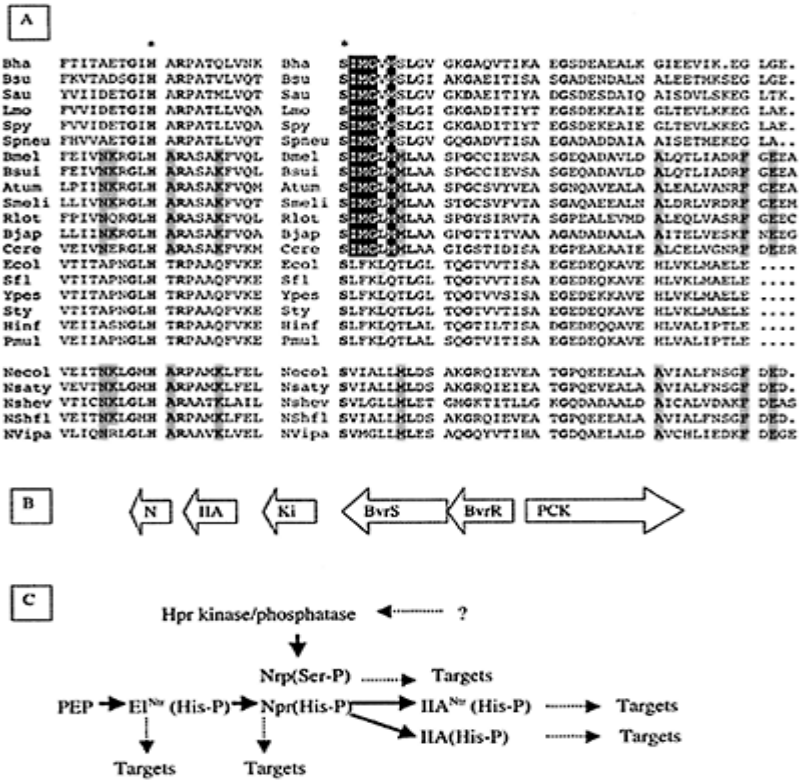


Figure 2. (A) Alignment of Hpr or Npr from Gram-positive and Gram-negative bacteria. Residues in bold are conserved. Star indicate the conserved histidine or serine residues. Residues in white with a blackground are next to the serine residue conserved between Gram-positive and α Proteobacteria. The residues boxed in light grey near the serine are conserved between

Gram-negative and α Proteobacteria. Residues boxed in dark grey all along the sequence are shared between the α Proteobacteria sequences and the *bona fide* Npr but not the Hpr. (B) Schematic representation of the Npr locus of *Brucella* (N: Npr; IIA: nitrogen regulatory protein IIA; Ki: putative Npr kinase; PCK: phosphoenolpyruvate carboxykinase). (C) Hypothetical PTS signaling pathway of *Brucella*.

There is no *hpr* homologue in the *rpoN* locus of *Brucella* or any other α *Proteobacteria* sequenced. Nevertheless, downstream *rpoN* there is a gene encoding a RpoN modulating protein, and further down there is a gene (I1786) homologous to the *pstN* of *E. coli*, which encodes $EIIA^{Ntr}$. A *B. suis* mutant in the *pstN* gene (also called "nitrogen regulatory protein gene") is attenuated in macrophage cell cultures. This gene is present in all α *Proteobacteria* sequenced and this locus organisation is conserved in *M. loti* and *S. meliloti* (Michiels *et al.,* 1998).

The *Brucella* genome encodes another EIIA protein (I2032) that seems to belong to the mannose family. The gene deserves further attention because of the conserved genomic organisation existing at both sides in all α *Proteobacteria* sequenced (Figure 2B; Hu and Saier, 2002; Boel *et al.,* 2003). In fact, there is a gene downstream pCDS I2032 that encodes a protein annotated as Hpr. In our opinion, this annotation is erroneous in *Brucella* and in all other α *Proteobacteria*. In fact, surrounding the conserved active histidine (GxHxR) site there is abundance of residues conserved in Npr (NKxGxHAR…K) but absent in Hpr (see Rabus *et al.,* 1999 for Npr consensus motifs). Moreover, a region found next to the regulatory serine residue (S(V/I)xL(L/M)ML) is also typical of Npr and clearly distinct from Hpr (SLFKLQTL). Finally, the C-terminus of pCDS I2032 also possesses the sequence FxE that is typical of Ntr and absent from Hpr. All these data probably make pCDS I2032 a good Npr candidate. Another astonishing observation is that the residues around the regulatory serine are much more like those found in homologous Gram-positive Hpr (which conserve the sequence SIMGxMxL) than those found in homologous Gram-negative Hpr (Figure 2A). This is particularly striking when the peculiar regulation of Hpr described for the Gram-positive bacteria is considered. Hpr is phosphorylated on the serine residue at the expense of ATP through an Hpr kinase (ptsK). In fact, there is a gene encoding a putative peculiar Hrp kinase with a *bona fide* serine kinase motif (pfam02603) upstream of the EIIA-encoding pCDS I2032. As already noted by Hu and Saier (2002), *C. crescentus, A. tumefaciens, M. loti* and *S. meliloti* encode at the same genomic locus a Hrp kinase homologous to the C-terminal domain of the Gram-positive Hrp kinase/phosphatase. They noted that the α *Proteobacteria* they studied lacked the allosterically regulated N-terminal domain but had the ATP and Hpr interaction domain and are thus likely to be functional

kinases/phosphatases. This is the only serine kinase we identified in the genome of *B. melitensis*.

In addition to these members of PTS, one can note the adjacent conservation of a TCS (BvrS/BvrR in *Brucella*) and the *pcK* gene in all of the α *Proteobacteria* sequenced. PcK is the key enzyme that carboxylates C3 glycolysis intermediates into C4 Krebs cycle intermediates (Hu and Saier, 2002). This suggests that there could be a link between PTS and the conserved BvrS/BvrR TCS regulating at least the expression of some outer membrane proteins in *Brucella* (see Chapter 10). A tentative overall picture of the PTS of *Brucella* is presented in Figure 2C.

Like other α *Proteobacteria*, *Brucella* could integrate in its own way elements of the Gram-negative and the Gram-positive signal transduction pathways for carbon and nitrogen metabolism regulation. The EI^{Ntr}, $EIIA^{Ntr}$ and EIIA (phosphorylated or not) could interact with the appropriate permeases, enzymes or transcriptional regulators and modify their activity either allosterically or by phosphorylating their targets. In addition, Npr could exist in different configurations depending upon the degree of phosphorylation (unphosphorylated, phosphorylated on serine, or histidine or both) and this could allow it to interact/phosphorylate various targets of the PTS system. Such an intricate signal network could assure the fine-tuning of *Brucella* carbon and nitrogen metabolism that has been repeatedly demonstrated to be crucial in the infection process. The impact of this PTS system on the regulation of the BvrS/BvrR TCS is also worth investigating.

2.3. Specialised Global Regulators

Most if not all the operons of bacteria belong to higher-level regulatory organisations. The term regulon was coined to encompass the sum of the operons regulated by the same regulator(s). The term stimulon is used to cover the co-ordinated expression of several operons in response to the same stimuli but not necessarily by the same regulators. A global regulator could be defined as the keystone of a regulon that co-ordinates the expression (i.e. activation or repression) of different operons. Hereunder, we will arbitrarily distinguish the global regulators that influence a regulon on an individual cell basis from those acting at the level of the population.

2.3.1. Intracellular Level

Indeed, several of the above-described regulators (belonging to the TCS, the PTS or to the classical TR) can be predicted to be key elements of a regulon (e.g. the CtrA phosphorelay regulates the co-ordination of cell cycle events such as DNA replication and septation). In addition, other specialised global regulators have to be considered: the alternative sigma factors that reprogram RNA polymerase to recognise the promoters of the member operons, and the stringent control network which appears to be devoid of protein modulator.

2.3.1.1. Alternative Sigma Factors

These factors have been recently reviewed by several authors (Burgess and Anthony, 2001; Borukhov and Nudler, 2003). The initiation of the transcription process requires

the reversible binding of a special subunit to the core RNA polymerase: the sigma factor that leads to the recognition of different types of promoters. The major sigma factor (also called RpoD or σ70 in *E. coli*) is responsible for the transcription of most of the housekeeping genes that are essential for bacterial survival and expressed during exponential growth. In addition to this principal σ70 factor, other sigma factors allow bacteria to adapt their gene expression to environmental changes. On the basis of sequence similarities, these alternative sigma factors cluster in two families: the σ70 family and the σ54 family. The members of the σ70 family (i.e. those structurally related to the major σ70) recognise a characteristic promoter sequence with two hexanucleotidic motifs centred on the −35 and −10 positions from the transcription initiation start site (+1 site). Some of these factors are also called ECF sigma factors (for extracytoplasmic function) because they couple a periplasmic or an extracytoplasmic signal with a change in gene expression. Each of these sigma factors directs the expression of a specialised set of genes: e.g., *E. coli* stationary phase genes (σ24 or RpoS), flagellum biosynthesis and chemotactism genes (σ28 or RpoF), heat-shock induced genes (σ32 or RpoH), extracytoplasmic stress induced genes (σ24), etc. On the other hand, the σ54 family contains only one member, which is structurally and functionally unrelated to the σ70 family. This factor, called RpoN in *E. coli*, is able to bind to positions −12 and −24 of the promoters even in the absence of RNA polymerase. Nevertheless, for the initiation to proceed, the σ54-RNApol complex needs the additional presence of a transcriptional activator. Among others, this σ54 factor drives the expression of genes involved in the metabolism of carbon and nitrogen sources, in the energy metabolism, some stress induced genes, etc.

In addition to the housekeeping RpoD sigma factor, *B. melitensis* has five alternative sigma factors: one σ54 factor and four belonging to the σ70 family with only two ECF sigma factors:

pCDS N°	**gene**	**sigma factor**
bmeI0280	*rpoH1*	σ32a
bmeI0371	*rpoE*	σ24
bmeI0378	*rpoH2*	σ32b
bmeI0532	*rpoD*	σ70
bmeI1789	*rpoN*	σ54
bmeII0072	*rpoV*	σ45

The presence of two RpoH sigma factors has also been noticed in the *Rhizobiaceae.* Like *A. tumefaciens* (Wood *et al*, 2001), *C. crescentus* (Nierman *et al.*, 2001), *M. loti* (Kaneko *et al.*, 2000) and *S. meliloti* (Galibert *et al.*, 2001), *B. melitensis* lacks a RpoS homologue. However, as compared to other a *Proteobacteria, B. melitensis,* has markedly fewer ECF sigma factors (thirteen in *C. crescentus,* and eleven in *A. tumefaciens* or *S. meliloti*). This is consistent with the lack of a stable extracellular life cycle for *B. melitensis,* which is defined best as a cultivable intracellular parasite. There are only three σ54-dependent transcriptional regulators (NtrC, NtrY and bmeII0011) in *B. melitensis* as compared to seven in *A. tumefaciens* and *S. meliloti*. As mentioned already in the section dealing with

the PTS, the overall organisation of *rpoN* locus is conserved in a *Proteobacteria*. Up to now, and no matter the screening process used, no mutant in a sigma factor has been identified in *B. melitensis*. This is noteworthy because the sigma factors have been described as regularly involved in the overall adaptation process to stressful conditions similar to these encountered by *Brucella* spp. during its infectious cycle. A certain functional redundancy may help to explain this observation. A systematic disruption strategy of all the genes encoding these factors is ongoing in our laboratory to solve this question.

2.3.1.2. Rsh and ppGpp

With the possible exception of the regulation of the cell cycle, no global regulator has such a profound impact on the global gene regulation as the stringent response network. This network is activated when bacteria encounter an amino acid or a carbon source starvation and culminates in a drastic modification of gene expression (inhibition of rRNA and tRNA synthesis, inhibition of DNA replication initiation, direct stimulation of biosynthetic or catabolic operons, etc.) that allows an optimised use of the resources remaining in the environment (Chatterji and Ojha, 2001). In *E. coli* (Gentry and Cashel, 1996), two proteins (RelA and SpoT) are the key players of the stringent response. They synthesise alarmones (derivatives of GTP bearing 3'pyrophosphate residues) collectively known as (p)ppGpp which mediate the global stringent response probably through an interaction with the beta subunit of the RNA polymerase, thereby affecting the sigma factor recruited by the RNA polymerase. Intracellular bacteria, either symbionts or pathogens, use starvation as a signal to regulate the genes needed for their infectious process (Chatterji and Ojha, 2001; Godfrey *et al.*, 2002). In fact (p)ppGpp alarmones and the stringent response are involved in the virulence of *Legionella pneumophila* (Hammer and Swanson, 1999) and the symbiosis of *S. meliloti* (Wells and Long, 2002). Except for the a *Proteobacteria,* all Gram-negative bacteria possess the two genes encoding the RelA and SpoT homologues. Like Gram-positive bacteria, the α *Proteobacteria,* have only one of these proteins which is called Rsh (for RelA SpoT homologue). In *B. melitensis,* this protein (encoded by ORF bmeI1296) has been shown to be necessary for the replication in macrophages and HeLa cell cultures (Kohler *et al.*, 2002; Kim *et al.*, 2003). This data is in accordance with the fact that the intracellular compartment in which *Brucella* replicates appears to be poor in nutriments. In fact, a large number of attenuated mutants are disrupted in genes involved in amino acid synthesis (Kohler *et al.*, 2003). Together with other signals, starvation could be used by *Brucella* to regulate its virulence genes. The team of David O'Callaghan (Boschiroli *et al.*, 2002) has shown that VirB is upregulated by starvation. We have recently demonstrated that a *Brucella rsh* deletion mutant is unable to drive the transcription of the *virB* operon as compared to the wild type *B. melitensis* strain.

2.3.2. Intercellular Level

In the last ten years, our view of the bacterial world has been profoundly modified by the finding that a bacterial culture is far more complex and organised than just a collection of individual unicellular organisms, as it was previously believed. The view of a population

of bacterial cells as a «multicellular organism» is now emerging and is clearly affecting the way we think about the regulation of cellular processes in bacteria. In addition to the intra-population communication systems known as quorum sensing (see below), it is also becoming clear that bacteria in the natural situation are usually not pure populations but extensively connected to other cells either prokaryotic (as exemplified in the biofilm situation) or eukaryotic. This latter point is particularly obvious when considering symbiotic or pathogenic microorganisms. These different aspects of intercellular communication (and hence regulation) are still in their infancy for *Brucella* and will be briefly described hereunder.

2.3.2.1. Quorum Sensing

Quorum sensing (QS) indicates the way by which bacteria communicate among them and co-ordinate an adaptive response when the cell density reaches a critical level. Among Gram-negative bacteria, the communication is mediated by N-acyl-homoserine lactones (AHL; Fuqua *et al.*, 2001). These are small diffusible molecules (also called pheromones) that, upon reaching at high concentration in the medium (this means a large population in an open space or a few cells in a closed environment), enter into cells and bind to a transcriptional regulator, thereby modifying gene expression. In an increasing number of symbiotic or pathogenic bacteria, adaptive responses are found to be regulated by AHL.

In a recent work by our group (Taminiau *et al.*, 2002), an AHL was identified in a cell free supernatant of *B. melitensis* 16M and characterised by mass spectrometry as a C12-AHL. This pheromone was also able to repress the transcription of one of the major virulence factor of *Brucella* spp, the type IV secretion system. This finding makes the first clear link between QS and virulence in *Brucella* spp. A second clear link was provided by a mutant in a QS regulator, which was identified in a systematic screen of transpositional mutants of *B. melitensis* unable to replicate in HeLa cells (Delrue, 2002). In addition to a set of mutants in the type IV secretion system, this screen identified a mutant in a QS regulator that has almost the same intracellular behaviour as the *virB* mutants (e.g., it is unable to reach its replicative vacuole). For this reason, this regulator was called VjbR for Vacuolar Jacking *B*rucella Regulator (pCDS II1116). This mutant is also clearly attenuated in other cellular models of infection and in the mice model. Experiments in our laboratory established that VjbR is able to regulate the transcription of the *virB* promoter but up to now we have no formal proof that this regulation is direct or that it involves binding of the C12-AHL. The genomic location of this regulator is also interesting because it lies downstream a TR gene of the TetR family and in the immediate vicinity of flagellar genes (namely *flhB* and *fliG*). This peculiar organisation is conserved in both *S. meliloti* and *M. loti* except for the fact that the TetR regulator is replaced by a second QS regulator (VisN) in these species (Sourjik *et al.*, 2000). The fact that QS is known to regulate flagellar genes in several *Rhizobiaceae* prompted us to have a closer look at the role of VjbR on the expression of flagellar genes in *Brucella*. This point is now under investigation.

A second regulator, called BabR (pCDS I1758) was identified in a genetic screening aimed at finding the pheromone synthase gene (Taminiau, 2003). This screening used a biosensor system based on *E. coli* bearing a plasmid with a reporter luciferase gene downstream of a promoter *(pluxI)* recognised by a QS regulator (LuxR) also encoded on

the plasmid. This system allows the detection of AHL either added to the medium or synthesised by a gene encoded on a compatible plasmid. Using a genomic library of *B. melitensis*, only the gene encoding BabR was identified and demonstrated as being a false positive able to bind and activate the *luxI* promoter even in the absence of exogenously added AHL (Taminiau, 2003). A mutant in the *babR* gene was not attenuated in any of the model tested.

Other regulators able to bind AHL were searched for in the genome of *B. melitensis* based on a typical HTH motif in their C-terminal part. This HTH domain was also found in some RR regulators of the TCS family (i.e. pCDS I1583, I1607, II0051, see also Table 1), but *bona fide* QS regulators have also a canonical AHL binding domain (called auto-inducer binding domain) in their N-terminus (Zhang *et al.*, 2002). Except for VjbR and BabR, no other regulator with such characteristics was found in the genome.

At this point, it is worth mentioning that, although the genes encoding these regulators are often linked with the genes encoding the AHL synthetases (Fuqua *et al.*, 2001), this is not the case in *Brucella*. In addition, up to now we have not been able to identify, either *in silico* or experimentally, the gene(s) responsible for the production of the C12-AHL synthesised by *Brucella*. The existence of two QS regulators in *Brucella* points also to the question of the integration of these regulators in a complex regulatory circuitry, and makes crucial the deciphering of their respective target genes. Although regulation of virulence genes is a crucial role of the *Brucella* QS system, it could also play a more central role in the physiology of the bacteria. Recent evidence suggests that the QS and starvation sensing pathways converge to regulate the entry of cell into the stationary phase in other bacteria (Lazazzera, 2000).

2.3.2.2. Brucella and the Host

Being a facultatively extracellular intracellular parasite (Moreno and Moriyón, 2002), *Brucella* has to integrate a tremendous amount of signals from the host environment in order to accomplish its infectious cycle. Some of these signals have been identified (oxidative stress, pH, iron concentration, and others). This section addresses another kind of communication between *Brucella* and its host dedicated to integrate the pair host-bacteria in a higher level, the «meta-organism». To the best of our knowledge, this higher level of interaction between *Brucella* and its host, although suspected to exist, has never been demonstrated experimentally. Likewise, the considerations that follow are purely speculative but they could be experimentally tested. To illustrate this line of thought, and taking into account the amazing proximity between the *Brucella* and *Rhizobium* genera (Ugalde, 1999; Moreno *et al.*, 2002), let us first focus on the symbiotic relationship between leguminous plants and the latter genus. When nitrogen availability in soil decreases, a complex set of interactions between the plant and its symbionts will ultimately lead to the formation of root nodules. There, the differentiated bacteroids will start to fix molecular nitrogen in a form used by its host. This «symbiotic harmony» (Broughton *et al.*, 2000) is directed by several signals exchanged between the plant and the rhizobiae. In a first step, flavonoid compounds emanate from the plant and interact with a rhizobial TR called NodD. This activates the transcription of bacterial genes involved in the synthesis of lipo-chito-oligosaccharidic factors (called Nod factors) that are mitogenic and induce the curling of root hairs which precedes the initiation of the

infectious thread. Afterwards, other rhizobial factors (extracellular polysaccharides and proteins secreted by a type III secretion system) seem necessary for a continued and efficient infectious thread development (van Rhijn and Vanderleyden, 1995). The *Rhizobium* NodD TR belongs to the LysR family and exhibits in its C-terminal part (known to interact with flavonoids) some resemblance to animal steroid receptors that are also known to interact with some flavonoids (Gyorgypal and Kondorosi, 1991).

Calves infected by *Brucella* in uterus or as neonate can become latent carriers and remain seronegative until the middle of their first gestation. Nevertheless, in the pregnant animal, *Brucella* preferentially replicates in placental trophoblasts during the middle and late stages of gestation only after these cells actively secrete steroids (Samartino *et al.*, 1994). The reasons for the "waking up" of latent bacteria, for the affinity for the trophoblast and for the process leading to abortion after midgestation are not known. Some authors have suggested that steroid hormones may influence *Brucella* (Meyer, 1976), or that *Brucella* uses them as growth factors (Misra *et al.*, 1976). It is also possible that some gestational steroids act as a signal for the bacteria, indicating that the time has come to close the infectious cycle and to disseminate by means of the abortion process.

We found, in *Brucella*, different homologues of genes involved in rhizopine and inositol degradation in different species of rhizobiae. We have shown that some of these genes are involved in virulence and that rhizopine has a biological effect on *Brucella* probably mediated by the RpIR regulator (Lestrate P., 2003). It is believed that the ability to catabolise rhizopine enhances survival of the rhizobiae in the rhizosphere. If this is true, because of natural selection, most of the rhizobiae in the rhizosphere should be able to use rhizopine as a carbon source. However, it has been observed that this ability is not widespread among the rhizobiae (Rossbach *et al.*, 1995). Thus, it is believed that rhizopine might be involved somewhere in the nodulation process, possibly by acting as a signal between the bacterium and the plant. Our results suggest that the rhizopine might be involved in *Brucella* pathogenesis (Lestrate P., 2003). The original rhizopine concept is difficult to transpose to *Brucella* pathogenesis. Instead, rhizopine might be a signal molecule, the role of which remains to be determined. This suggests that brucellae and rhizobiae would have conserved, from a common ancestor, a chemical language allowing them to interact with eukaryotic cells.

2.4. Post-transcriptional Regulation

Besides the transcriptional regulation of gene expression, there are other mechanisms to avoid the production of a gene product. Because of the discovery of global post transcriptional regulators, and because several global transcriptional regulators are themselves controlled at a post transcriptional level, there is an increasing number of examples of control of gene expression at the level of translation and mRNA stability (Nogueira and Springer, 2000). In addition, several mechanisms are at play to limit the presence of a gene product either temporally or spatially (compartmentalisation, proteolysis). It is out of the scope of this review to make an exhaustive survey of all the systems at play in *Brucella*. We intend only to address this point by means of some exemplary systems: Hfq as a post-transcriptional global regulator, Flbt a translational repressor and, finally, compartmentalisation and proteolysis.

Hfq, also called HF-1 (Host Factor I) was discovered in *E. coli*. Mutants in the corresponding gene have pleiotropic phenotypes such as decreased growth rate, increased cell length and osmosensitivity. Hfq is a small RNA binding protein that has many functions including to positively regulate the expression of *rpoS* (the stationary phase sigma factor) at a post-transcriptional level, probably by altering the structure of the rpoS mRNA and allowing enhanced translation (Muffler *et al.,* 1997). Hfq has also been shown to regulate negatively the expression of some genes (including the *hfq* gene) by causing a decrease in mRNA stability. The team of Marty Roop II (Roop *et al.,* 2002) has described a *Brucella hfq* mutant (I0872) that not only showed a behaviour reminiscent of a stationary phase defect but was also attenuated in murine macrophages and unable to sustained long-term persistence in mice. As already mentioned, because there is no *rpoS* homologue in *Brucella*, the action of Hfq is probably mediated through another global regulator (sigma factor) still to be identified.

In *C. crescentus*, FlbT is a translational repressor that controls the expression of flagellin (Anderson and Gober, 2000). If the pCDS of *Brucella* homologous to *flbT* (II0163) is functional, it can be predicted that the corresponding mutant should express higher levels of flagellin. In fact, preliminary data from our laboratory indicate that the *fliC* gene encoding flagellin is transcribed constitutively whereas the protein is only detected in a very narrow stage of the growth phase.

A recent paper demonstrates that in *S. meliloti* the protein DivK and its spatial regulation along the cell may play a critical role in the regulation of the cell cycle (Lam *et al.,* 2003). Because this locus is conserved in *Brucella* as well as in other α *Proteobacteria*, the asymmetric polar distribution of the DivK homologue could also regulate the complex signalling network that ultimately leads to an asymmetric division. If this is true, the real questions could be: what are the respective properties of these two daughter cells?; and, in addition, are they equivalent in their potential to infect cells? Recent research has demonstrated that, in bacteria also, conditionally unstable proteins tend to be essential regulators of major cellular processes such as cell cycle, differentiation and the stress response. To the best of our knowledge, such aspect has never been investigated in *Brucella*, and we will just point out some of the possible proteolysis targets that have been identified in other α *Proteobacteria*. In *A. tumefaciens*, the quorum sensing transcriptional regulator TraR folds into a protease resistant conformation upon binding of its cognate pheromonal effector (oxoC8 AHL). In the absence of this effector molecule, TraR is rapidly degraded by the Clp and Lon proteases (Zhu and Winans, 2001). The activity of CtrA (an essential cell cycle response regulator) must be eliminated before cells enter the DNA replication phase. In *C. crescentus*, a selective temporal and spatial CtrA degradation allows DNA replication during the differentiation of a swarmer cell into a stalked cell and in the stalked compartment of the predivisional cell. The degradation signal is bipartite. One part resides in the last fifteen amino acids that more likely represent the binding site for the ClpX ATPase. The second part is located in the first fifty-six residues which might constitute the binding site for a proteolysis activator itself under cell cycle control (Ryan *et al.,* 2002). In *Brucella*, CtrA is lacking the C-terminal tag that is required for ClpXP-dependent proteolysis in *C. crescentus*. Proteolysis of the *Brucella* CtrA protein in *C. crescentus* is observed when the C-terminal sequence of *Caulobacter* CtrA is genetically fused (Ryan *et al.,* 2002).

Since the C-terminal tag is not sufficient for proteolysis, we propose that another signal is present in the N-terminal domain of *Brucella* CtrA.

3. Conclusion

This review is merely a non-exhaustive catalogue of the actors which are believed or known to be involved in the regulation of *Brucella* virulence. We omitted (intentionally or not) certain aspects or certain partners of the complex regulatory network that could be used by *Brucella* to succeed in its infectious cycle. For example, we do not address the nucleoid proteins, which are also involved in modulating transcription in response to environmental clues. While their ability to alter the DNA structure is central to their activity, there are also clear interactions described between nucleoid proteins, with gene specific regulatory factors or even RNA polymerase (McLeod and Johnson, 2001). In addition to the regulatory partners and pathways we have described in this review, there might be others. Nevertheless, we are confident that the predictions made, always keeping in mind their tentative nature, could help in the near future to dissect experimentally the regulatory circuitry that controls in time and space the virulence genes of *Brucella*. In fact, having identified the partners involved in a regulatory process, we are still far from understanding the properties emerging from this regulatory network. The major point to address is to put the models we use to evaluate *Brucella* virulence under scrutiny. As quoted by Bull and Lewin in a paper in Science, “mice are not furry Petri dishes” (Bull and Levin, 2000). This is not just a provocative sentence but illustrates the difficulty we face when we try to extrapolate results obtained in culture media or even in animal models to the infectious process in the natural host. Consider for example that none of the mutants we made for various parts of the flagellar structure was attenuated in cellular models and, furthermore, that no attenuation was observable in mice one week after infection. Nevertheless, the same mutants were almost cleared from mice eight weeks after infection. The same holds true for the siderophore story because mutants unable to produce 2, 3, dihydroxybenzoic acid are attenuated in a pregnant cattle model but not in mice (Bellaire *et al.,* 2003). This challenge posed by the models will be of paramount importance when trying to understand the virulence gene regulation as a whole. Caution must be exercised when attempting to extrapolate relevant *in vivo* signals from environmental clues that regulate virulence gene *in vitro*. Because *in vitro* signals may operate through artificial processes bypassing the relevant *in vivo* signalling pathway, and also because the predictable complexity of a virulence gene regulation that will only be achieved *in vivo*, it seems clear that a detailed understanding of the relevant *in vivo* conditions necessitates a complete appreciation of the entire infectious cycle, and that pathophysiological studies are needed. A lot of work is still to be done but the complete genome data were obtained only one year and a half ago. Considering this and the rapid development of new experimental and theoretical approaches (for reviews see Pilpel *et al.*, 2001; Banerjee and Zhang, 2002; Stormo and Tan, 2002; Wolf and Arkin, 2003), we can hope that a more complete picture of *Brucella* virulence regulation will emerge in a near future.

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Chapter 8
Brucella Lipopolysaccharide: Structure, Biosynthesis and Genetics

Maite Iriarte, David González, Rose M.Delrue, Daniel Monreal, Raquel Conde, Ignacio López-Goñi, Jean-Jacques Letesson, and Ignacio Moriyón*

From: *Brucella:* Molecular and Cellular Biology. Edited by: Ignacio López-Goñi and Ignacio Moriyón

Abstract

Brucella lipopolysaccharide (LPS) is one of the key molecules involved in *Brucella* virulence. Despite this, its structure has not been fully elucidated. In this chapter the LPS structure will be reviewed in the light of structural and genomic comparisons, with emphasis on the core and lipid A sections. The *Brucella* genome contains homologues of the ORFs necessary to synthesise lipid IV_A and to acylate it with long-chain fatty acids. Despite the overall similarity with *R. leguminosarum*, the only homologue to those that modify the disaccharide backbone of the latter is a putative phosphatase which may act at position 1 to generate monophosphoryl lipid A. The comparisons support the absence of heptose, galacturonic acid and phosphate in the core, or of arabinosamine in lipid A. An ORF putatively involved in phosphoethanolamine transfer was found in *B. melitensis* and *B. suis* but carried a missense mutation in *B. abortus*, suggesting species differences. The evidence supports the hypothesis that *Brucella* is unique among α-*Proteobacteria* in that the lipopolysaccharide core lacks all negatively charged groups but 3-deoxy-D-manno-2-octulosonic acid while keeping amino-compounds. It is postulated that this reduced negative charge plus the presence of long chain fatty acids are the basis for the altered pathogen-associated molecular pattern of *Brucella* lipopolysaccharide.

1. Introduction

It is a textbook concept that the cell envelopes of *Bacteria* belong to two main types: those based on a single inner membrane, and those based on an inner and an outer membrane (OM) enclosing a periplasmic space. Usually, thick peptidoglycan layers surround the single-membrane envelopes, whereas thinner peptidoglycan layers are enclosed within the periplasmic space in the two-membrane design. With few exceptions, the second type of envelopes is identified as a negative Gram-staining and, accordingly, bacteria showing it are usually designated as Gram-negatives. Most of these belong to the *Proteobacteria,* and this class is divided into five main divisions. *Brucella* belongs to the a division where it groups with members of the *Rhizobiaceae* to form the α-2 subdivision. This subdivision

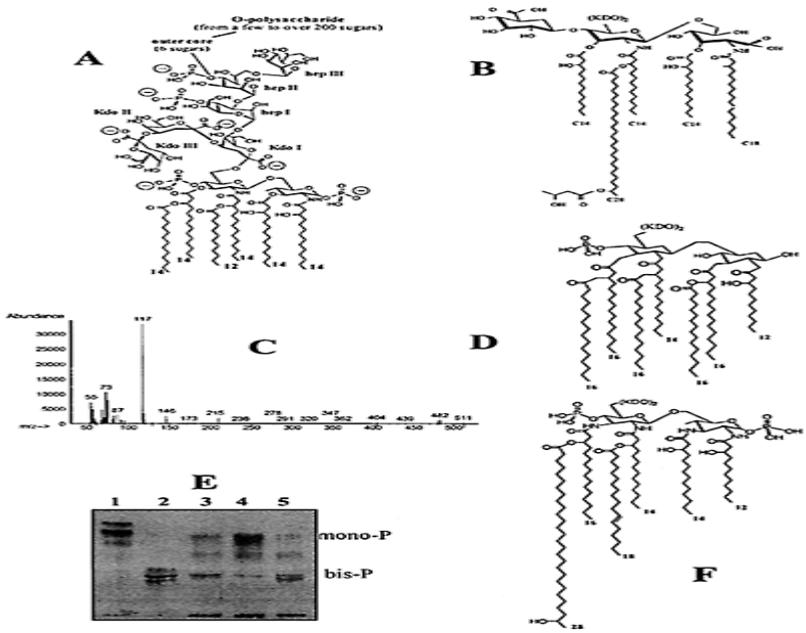

Figure 1. Bacterial LPS structures. (A), Core and lipid A of *E. coli* K12; (B), lipid A of *R. leguminosarum;* (C), Electronic impact mass spectrum of $C28_{27OH}$ of *B. abortus* 2308 showing the characteristic fragmentation pattern of the hydroxyl BSTF derivative (mass 73, 117, 215, 404, and 482) and the

mass of its unprotonated form (511); (D), monophosphorylated heptaacyl lipid A form of *B. abortus* 2308 LPS; (E), analysis by thin layer chromatography of the degree of phosphorylation of *Ochrobactrum* and *B. abortus* lipids A (lane 1, monophosphorylated lipid A of *Salmonella minnesota;* lane, bisphosphorylated lipid A of *Salmonella minnesota;* lipid A of *O. anthropi;* lane 4, lipid A of *O. intermedium;* and lane 5, lipid A of *B. abortus* 2308); (F), hexaacyl lipid A of *O. intermedium*. See text for references.

includes bacteria able to establish parasitic or endosymbiontic relationships with a variety of eukaryotic cells (Moreno, 1992; Moreno and Moriyón, 2002b), and evidence is growing that they are able to do so in part because they attenuate detection by the innate immune system (Rasool *et al.,* 1992; Gorvel and Moreno, 2002; Moreno and Moriyón, 2002a; Moriyón, 2003). In this regard, it has been stressed that many of the peculiarities of *Brucella* related to this ability are concentrated in the architecture of its cell envelope and OM (Moreno and Moriyón, 2002a). As expected, many distinctive characteristics of this structure are ancestral and shared in part by members of the *Rhizobiaceae* but not by taxonomically distant Gram-negative bacteria. Thus, comparisons with *Escherichia coli* and *Salmonella*, two γ-*Proteobacteria* whose OMs are often taken as the archetype, and with the closer phylogenetic neighbours have provided insight into the relevance of the *Brucella* OM in pathogenicity (Leong *et al.,* 1970; Moreno *et al.,* 1981; Moriyón and Berman, 1982; Rasool *et al.,* 1992; Martínez de Tejada and Moriyón, 1993; Martínez de Tejada *et al.,* 1995; Freer *et al.,* 1996; Velasco *et al.,* 2000; Moreno *et al.,* 2002; Moreno and Moriyon, 2002b).

The OM is an asymmetrical structure that owes many of its properties to a unique glycolipid, the lipopolysaccharide (LPS) or endotoxin, located in its outer leaflet. Figure 1A shows the structure of *E. coli* K-12 LPS and the sections that can be distinguished in this and other LPSs. The lipid A is a phosphorylated 2-amino, 2-deoxyglucose (glucosamine, GlcN) disaccharide carrying four ester- and amide-linked 3-hydroxy-myristoil groups, two of which (those corresponding to the non-reducing sugar) bear lauric and myristic acyl chains in acyloxyacyl linkages. This very hydrophobic structure anchors the LPS hydrophilic moiety into the OM, but in many bacteria additional

stabilisation by divalent cations and polyamines (see below) is necessary for OM stability. The core oligosaccharide is divided into an inner and an outer core. The former is made up of distinctive sugars (3-deoxy-D-*manno*-oct-2-ulosomc acid [Kdo] and L-D-*glycero*-D-*manno*-heptose) that, because they are either acidic or substituted in part with orthophosphate, create an area with a high density of negative charges. The outer core, which varies little between serotypes, is composed of a small number of, mostly neutral, sugars (Holst, 1999). On the other hand, the larger O-polysaccharide (O-PS), which is composed of monosaccharide or oligosaccharide repeating units, is highly variable among serotypes. Some mutants (termed rough [R] mutants) display a truncated sugar moiety—that is missing either the O-PS or the O-PS plus some sugars of the core oligosaccharide. In the OM, the densely acylated moiety of the lipid A is highly ordered (Labischinski *et al.,* 1985). This has a rigidifying effect which is complemented by the bridging of the phosphoryl groups and acidic sugars in the lipid A-inner core by divalent cations and polyamines (Nikaido and Vaara, 1985; Vaara, 1999). These two properties, and the strong hydrophobic interactions with the outer membrane proteins, create a barrier at the outer leaflet of the OM which retards the diffusion of hydrophobic substances into the cell, and makes the channels of porins the normal pathway of entry for hydrophilic solutes (Nikaido and Vaara, 1985; Vaara, 1992). This barrier action and the associated selective permeability are key functions of the OM, and they impose the structure of the LPS inner sections.

Whereas adaptive immunity can detect subtle variations in just one or few components of macromolecules, innate immunity relies on the recognition of the so-called pathogen-associated molecular patterns (PAMPs) (Janeway, Jr. and Medzhitov, 2002). These are motifs absent from self and repeatedly used by microbes, often because of functional and/or genetic constrains. The LPS bears one of such PAMPs, as manifested by its recognition by elements of the innate immune system such as the bactericidal peptides and proteins of phagocytes and mucosas, complement (in the absence of antibodies), and several cell receptors including the CD14-TLR4-MD2 complex (Moriyón, 2003; Takeda *et al.,* 2003). Through this last system, LPSs induce a powerful stimulation of the cytokine network (endotoxicity) thus linking the innate and the acquired immune defences against Gram-negative pathogens (Takeda *et al.,* 2003). It is in this context where the LPS of *Brucella* stands out as a structure gathering a related and remarkable set of properties: resistance to binding by antimicrobial peptides and proteins, low complement activation, low stimulation of cells triggering the cytokine network and low toxicity for the cells where the bacterium grows (Moreno *et al.,* 1981; Rasool *et al.,* 1992; Martínez de Tejada *et al.,* 1995; Gorvel and Moreno, 2002; Moreno and Moriyón, 2002a; Moriyón, 2003). Indeed, this set of properties reflects both an altered LPS PAMP and OM properties different from those of many Gram-negative bacteria.

Owing to its conspicuous place in the innate immunity-pathogenicity cross-roads, there is a renewed interest on the structure-activity relationships of LPS. With regard to *Brucella* LPS and immunity, some recent developments are presented elsewhere in this book. Thanks to the work of DelVecchio *et al.* (2002) Paulsen *et al.* (2002) and Halling *et al.,* (see Chapter 5), it is possible now to reexamine the *Brucella* LPS structural data in the light of the genomic sequences. In this approach, comparisons with the genomes of phylogenetically related bacteria and their LPS structure is essential.

2. *Brucella* LPS Structure

2.1. O-Polysaccharide

Nuclear magnetic resonance (NMR) analysis has been used to establish that the *Brucella* O-PSs are homopolymers of N-formyl-perosamine (N-formylated 4-amino, 4,6-dideoxyglucose) either exclusively in α (1–2) linkages (for example, in *B. abortus* and *B. suis* of biovar 1) or in α (1–2) plus α (1–3) in a ≥4:1 proportion (4:1 in *B. melitensis* biovar 1) (Perry and Bundle, 1990). These structural variations result in three basic types of overlapping epitopes: C (common to all chemical types of *Brucella* O-PS), M (present in those O-PS with α (1–3) linkages) and A (present in those O-PS with no α (1–3) linkages or with a proportion of α (1–2) to α (1–3) linkages higher than 4:1). NMR studies have also demonstrated that the smooth (S) LPS (S-LPS) of *Yersinia enterocolitica* O:9 carries a homopolymer of N-formyl-perosamine in α (1–2) linkages and, accordingly, it should be identical to the O-PS of *B. abortus* biotype 1 (Perry and Bundle, 1990). However, whereas some monoclonal antibodies (MoAbs) of O-PS specificity react equally with S *Brucella* and *Y. enterocolitica* O:9 (Cyb epitopes), others recognise epitopes common to all S *Brucella* but not to *Y. enterocolitica* O:9 (Cb epitopes) (Douglas and Palmer, 1988; Cloeckaert *et al.*, 1998). This evidence strongly suggests the presence of subtle structural differences that may be undetectable by NMR analyses.

2.2. Core Oligosaccharide

Although there are qualitative data on the composition of the *B. abortus* LPS, its structure is not known. Analysis of the *B. abortus* S-LPS reveals Kdo, GlcN (2-amino, 2-deoxyglucose), quinovosamine (2-amino, 2,6-dideoxy-D-glucose), glucose and mannose, and the absence of heptose and orthophosphate, two features in common with other α-2 *Proteobacteria*. However, in contrast to the core structures of the phylogenetic neighbours elucidated so far (which contain galacturonic acid (Holst, 1999)), the LPS core of *B. abortus* seems to lack acidic sugars other than Kdo. The absence of galacturonate in the *Brucella* LPS core is confirmed by the phenotype of galactose-epimerase mutants (see Section 3). This likely absence of negatively charged groups sets it apart from other Gram-negatives and helps to explain the marked resistance of *Brucella* to bactericidal peptides (Martínez de Tejada *et al.*, 1995). In addition, there is evidence that the structure of the core itself creates a steric impediment preventing polycationic peptides from reaching the Kdo and negatively charged groups of lipid A (see below) (Velasco *et al.*, 2000). It is not know whether there are differences in core sugar composition among the classical *Brucella* species and biovars.

2.3. Lipid A

It has been established that the lipid A backbone of *Brucella* is a β (1–6) linked 2,3-diamino, 2–3-dideoxyglucose (diaminoglucose; NGlcN) disaccharide (Qureshi *et al.*, 1994), a structure which is also present in some (*Ochrobactrum* and *Mesorhizobium*) but not all its phylogenetic neighbours. This disaccharide exists in both a mono and a

bisphosphorylated form in position 1 or 1 plus 4', respectively (Qureshi *et al.,* 1994; and Figure 1E). The basic sugar 4-amino-arabinose (arabinosamine) has not been detected in *Brucella* lipid A (Moreno *et al.,* 1990; Freer *et al.,* 1995). In several Gram-negative bacteria, this sugar is linked to the lipid A backbone, thereby neutralising its overall negative charge and increasing the resistance to polycationic peptides. Owing to the markedly lower binding of polycationic peptides to the *Brucella* LPS, its absence in this molecule is noteworthy (see below).

The NGlcN disaccharide bears four amide-linked 3-hydroxy fatty acids and, in addition, there can be up to three additional fatty acids in acyloxyacyl linkages. As in other bacteria, lipid A heterogeneity has been observed (Qureshi *et al.,* 1994; Freer *et al.,* 1995; López-Goñi *et al.,* 2002; Monreal *et al.,* 2003; Campos *et al.,* 2004) which arise from differences in the number of acyloxyacyl groups. Accordingly, forms ranging from tetraacyl (with no acyloxyacyl groups) to heptaacyl (with three acyloxyacyl groups, possibly on the 3-hydroxy fatty acids in positions 3, 2' and 4') lipid A have been reported (Campos *et al.,* 2004). As expected, each of these forms does not represent a single chemical species. Considerable micro-heterogeneity has been observed (Campos *et al.,* 2004) which is possibly accounted for by variations in the fatty acids (Qureshi *et al.,* 1994; Velasco *et al.,* 2000) and presence of one or two phosphoryl groups in 1 and 4' (Qureshi *et al.,* 1994; Velasco, 1997). The issue is complicated further by the fact that different authors have found important differences in the fatty acid composition. Moreno *et al.* (1990) reported ester (acyloxyacyl) linked $28{:}0_{(27\text{-}OH)}$ in the lipid A of *B. abortus* 1119–3, a long chain fatty acid (LCFA) also present in the lipid A of *Rhizobium* (Figure 1B). This and other ester-linked iso-hydroxy and keto LCFAs were also demonstrated in *B. abortus* 2308 lipid A by Velasco *et al.* (Velasco *et al.,* 2000) (Figure 1C). Quantification of these LCFAs showed almost equimolecular amounts with respect to beta-hydroxymyristic acid ($14{:}0_{3\text{-}OH}$) suggesting its importance in the lipid A structure (Velasco *et al.,* 2000). On the other hand, LCFAs were not observed by Qureshi *et al.* (1994) in a monophosphorylated heptaacyl fraction derived from *B. abortus* 45/20. Likewise, LCFAs have not been observed in the tetra, penta, hexa or heptaacyl lipid A forms recently identified in *B. abortus* 2308 and RB51 (Campos *et al.,* 2004) (Figure 1D). As suggested by Qureshi *et al.* (1994), the discrepancies probably represent the analysis of only a fraction of the total lipid A in these latter works. Moreover, lactobacillic acid ($19^{c}{:}0$) and its C18:1 precursor was reported in the study performed with *B. abortus* 2308 by Velasco *et al.* (2000) but not observed by others. These two fatty acids are present in the phospholipids (Thiele *et al.,* 1969) and ornithine-lipids of *Brucella* and their relatives (Thiele and Schwinn, 1973), and these lipids are known to co-extract with LPS (Holst *et al.,* 1983; Velasco, 1997). However, the lipid A preparations studied were analysed for ornithine with negative results, and enrichment of $19^{c}{:}0$ and C18:1 was observed during the purification steps aimed to remove free lipids (Velasco, 1997). An analysis carried out in parallel on the dephosphorylated lipid A of *Ochrobactrum intermedium*, the closest know phylogenetic neighbour of *Brucella*, led to the identification of an hexaacyl lipid A of MW 1935 and 1954. This evidence, and the demonstration of bisphosphorylated lipid A (Figure 1E), led to the tentative structure presented in Figure 1F. This type of molecules is in all likelihood present in *B. abortus*. In addition to the parallelism in overall composition existing between the lipids A of both bacteria and the genetic evidence (see below), the analysis of the acyl-chain fluidity

shows a remarkable overlap between the LPSs of *B. abortus* and *O. anthropi* (Velasco *et al.*, 2000). This parameter is directly related to the type of acyl-chain.

3. Genetics and Biosynthesis of *Brucella* LPS

The synthesis of LPS in *Brucella* has not been the topic of specific studies to date. However, the emerging genetic evidence (Godfroid *et al.*, 2000; DelVecchio *et al.*, 2002; Paulsen *et al.*, 2002; Monreal *et al.*, 2003) allows comparisons with the pathways that have been partially elucidated in some Gram-negative bacteria (Reeves *et al.*, 1996; Heinrichs *et al.*, 1999; Keenleyside and Whitfield, 1999).

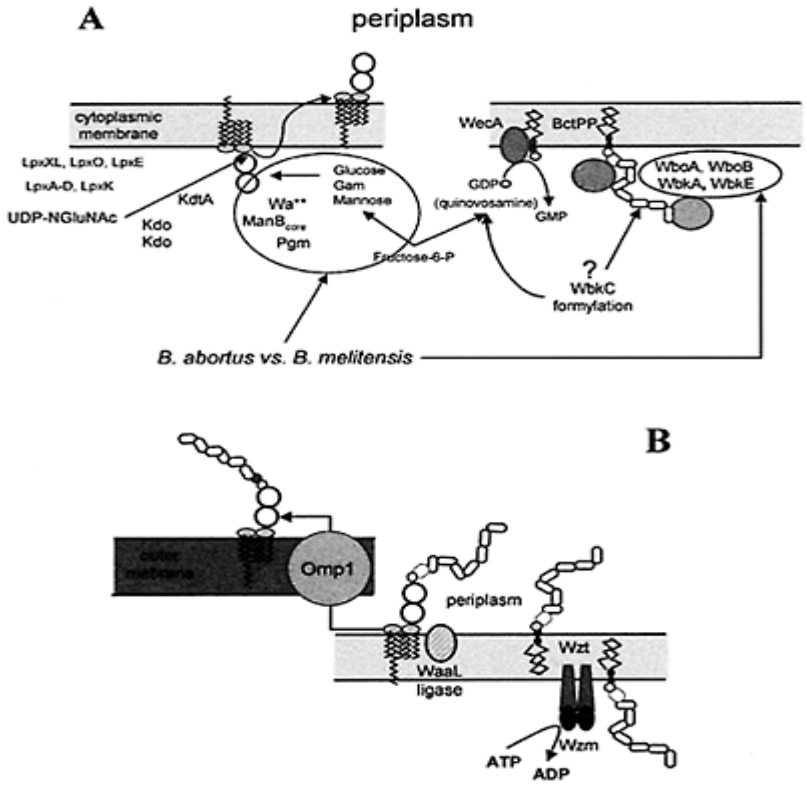

Figure 2. General pathways involved in the biosynthesis and export of the S-LPS of *B. abortus* and *B. melitensis*. Panel A summarises the two independent pathways that take place on the cytoplasmic membrane and that

lead to the synthesis and translocation of the lipid A-core (left side) or to the synthesis of the O-PS linked to the bactoprenol carrier. Panel B summarises the transport of the O-PS to the periplasmic space and its assembly on the lipid A-core and the final translocation to the outer membrane.

Lipid A is synthesised on the cytoplasmic face of the inner membrane and sugars are added both during and after lipid A synthesis through the sequential action of glycosyl-transferases to build the lipid A-core which is then transferred to the periplasmic side of the membrane. The O-PS is synthesised in an independent pathway, linked to the acceptor sugar of the complete lipid A-core in the periplasmic space, and the whole molecule translocated to the outer face of the OM (Figure 2). Moreover, there are subsidiary pathways (either exclusive to LPS biosynthesis or general pathways) that provide nucleotide-sugar and acyl-CoA precursors.

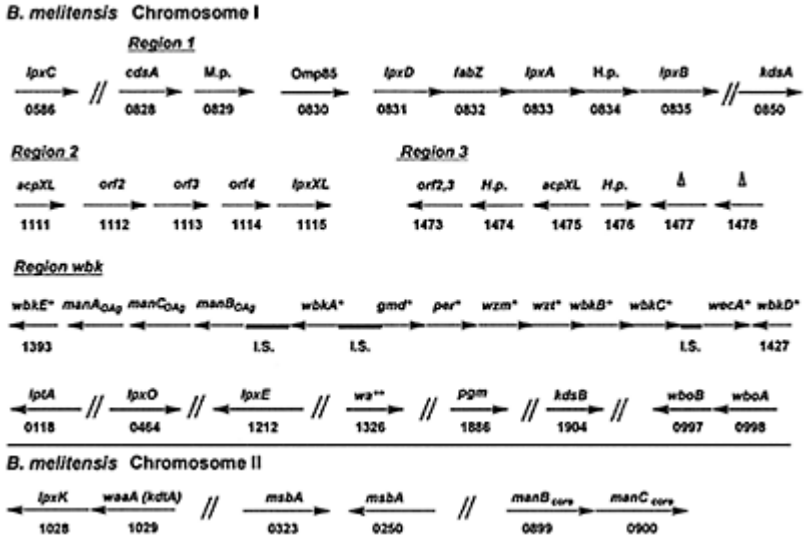

Figure 3. Chromosomal distribution of the genes identified in the *B. melitensis* 16M genome which have been shown (marked with an asterisk) or could be involved in the synthesis of LPS. Region 1 encompasses genes involved

in translocation to the outer membrane plus lipid A biosynthesis. Region 2 and 3 are highly homologous to those that are involved in the transfer of LCFAs to lipid IV_A in *Rhizobium* H.P., hypothetical proteins; shadowed triangles mark genes putatively implicated in fatty acid synthesis). Region *wbk* is implicated in the synthesis of the O-PS and its translocation to the periplasm. Other genes in either chromosome I or II are not clustered into large regions (see text for details).

3.1. O-Polysaccharide

Genetic evidence demonstrates that the O-PS is synthesised by the so-called ABC transporter-dependent (or *wzy*-independent) pathway, which is characteristic of homopolymeric polysaccharides (Keenleyside and Whitfield, 1999; Godfroid *et al.*, 2000). The first step in this pathway is the priming of the undecaprenol-pyrophosphate carrier with an amino sugar by the WecA protein. Subsequent steps involve the sequential addition of sugars to the non-reducing end of a growing polysaccharide chain through the activity of glycosyl-transferases acting on the nucleotide-linked sugars. When the O-PS reaches a certain size, the ABC proteins translocate the amino sugar-O-PS to the periplasmic side of the cytoplasmic membrane where it is ligated to the lipid A-core by WaaL (see Section 4). In *Brucella*, the (putative) genes implicated in the synthesis of the nucleotide-perosamine, the formyl-transferase, the undecaprenol pyrophosphate primase *(wecA)*, two glycosyl-transferases, and the ABC transporters are clustered in the *wbk* region of chromosome I (ORFs BMI1393 to BMI1427; Figure 3) (Godfroid *et al.*, 2000; González, 2003). The region is identical in the *B. suis* and *B. melitensis* genomes. But for the exceptions discussed below, disruption of these genes generates R phenotypes. The region shows also some intriguing features.

The *wbk* genomic region contains three homologues of genes that act sequentially in GDP-mannose synthesis from fructose-6-P: *manA* (mannose-6-P isomerase), *manB* (phosphomannomutase) and *manC* (mannose-1-P-guanylyltransferase). Since GDP-mannose is the precursor of perosamine, the location of these three *man* genes within the *wbk* region strongly suggests that they act co-ordinately with *gmd* (GDP-mannose dehydratase) and *per* (perosamine synthetase) (Figure 3). In many cases, both the mannose-6-P isomerase and mannose-1-P-guanylyltransferase functions are attributed to ManC, and *manA* is often absent. For example, *B. melitensis* and *B. suis* carry a *manB-manC* tandem in chromosome II which is involved in the synthesis of the inner core mannose (see below). In fact, the *manA, manB* and *manC* arrangement seems rare. It is not found in the genomes of *Brucella's* phylogenetic relatives (Kaneko *et al.*, 2000;

Galibert *et al.*, 2001; Goodner *et al.*, 2001; Wood *et al.*, 2001; Nierman *et al.*, 2001), and *manA* is not present in other *wb* regions sequenced so far that contain perosamine genes (Stroher and Manning, 1999; Heidelberg *et al.*, 2000; Awram and Smit, 2001; Perna *et al.*, 2001) including that of *Y. enterocolitica* O:9 (Lübeck *et al.*, 2002) which synthesises an OP-S possibly identical to that of *B. abortus* (see above). This is intriguing because, based on the GC content (about 48% for *wbk*, including *manA*, *manB* and *manC*, and 58% for the *Brucella* genome), it is postulated that *wbk* has been acquired horizontally by *Brucella* (Cloeckaert *et al.*, 2000). Moreover, mutagenesis of the *wbk manB* does not generate an R (O-PS deficiency) phenotype (J.J.Letesson, unpublished), which seems to preclude the involvement of *wbk manA*, *manB* and *manC* in perosamine synthesis and raises the question of their actual role. However it is possible that the $manB_{core}$ gene in chromosome II complements the *wbk manB* mutation. Therefore further work is necessary to clarify this. As in the case of *manA*, disruption of *wbkB* does not generate an R phenotype (Godfroid *et al.*, 2000), and its role remains also to be established.

The product of *wbkD* has a high homology with putative epimerase-dehydratase enzymes involved in LPS synthesis in several bacteria, including a dehydratase (LpsB) of *R. leguminosarum*, and its disruption generates a R phenotype (González, 2003). This suggests that it may take part in the generation of a keto, deoxysugar precursor of an LPS amino, dideoxysugar. So far, the only *Brucella* LPS compound other than perosamine which shows these characteristics is quinovosamine (2-amino, 2,6-dideoxyglucose) (Bowser *et al.*, 1974; Caroff *et al.*, 1984; Velasco *et al.*, 2000) whose presence in the outermost section of the core is deduced by comparisons of the sugar composition of R and S *Ochrobactrum* spp. and *B. abortus* (Velasco *et al.*, 2000). On this basis, it is tempting to speculate that quinovosamine is the substrate used by the WecA protein of *Brucella* to prime undecaprenol for OP-S synthesis, and the adjacent location of *wbkD* and *wecA* in the *wbk* region of both *B. suis* and *B. melitensis* is consistent with this possibility.

Additional genes coding for putative glycosyl-transferases involved in LPS synthesis have been identified outside of the *wbk* region. A *wboA*-*wboB* tandem is present in *B. abortus* and *B. melitensis* and their disruption generates R phenotypes (McQuiston *et al.*, 1999; González, 2003). Like the glycosyl-transferases WbkA and WbkE, and consistent with their postulated role in O-PS polymerisation, WboA and WboB show homology with mannosyl-transferases (perosamine is a mannose derivative). No other glycosyl-transferases have been associated with *Brucella* O-PS synthesis, and this raises the question as to which enzymes are associated with the AC and MC epitopic structures. *B. melitensis* 16M and *B. suis* 1330 belong to two different serotypes (MC vs. AC, respectively). However, identical genes coding for these four glycosyl-transferases are present in both genomes indicating that these glycosyl-transferases are unlikely to account for the MC and AC differences. Surprisingly, *B. abortus* RB51, which is a R *wboA* mutant derived from a AC serotype strain (Vemulapalli *et al.*, 1999), produces small amounts of a perosamine polysaccharide reacting with MoAbs against the M epitope (Cloeckaert *et al.*, 2002). This, and the fact that at least four glycosyl-transferases seem to be necessary for the synthesis of a relatively simple homopolymer (a single sugar with a single type of linkage in *B. abortus* 2308 or *B. suis* 1330), raises intriguing questions about the precise biosynthetic pathway of the *Brucella* O-PS.

3.2. Core Oligosaccharide

In *E. coli* and *Salmonella*, the first step in core biosynthesis (the addition of Kdo to the lipid A disaccharide backbone) occurs prior to completion of lipid A. Then, the lipid A is fully acylated and assembly of the core proceeds through sequential addition of sugar residues by glycosyl-transferases.

Brucella contains two ORFs whose deduced proteins sequences show clear homology with those of two of the enzymes required for Kdo formation. The product of BMEI0850 is homologous (65% similarity) to KdsA, the Kdo-8P-synthase that condenses arabinose-5P and phosphoenolpyruvate to yield Kdo-8P; and that of BMEI1904 resembles (54% similarity) KdsB, the Kdo-cytidyl-transferase (Goldman and Kohlbrenner, 1985; Woisetschlager and Hogenauer, 1986). Like in *E. coli* and *Salmonella*, the *kdsA* and *kdsB* genes of *Brucella* are situated in different regions of the bacterial genome. The gene encoding the Kdo-

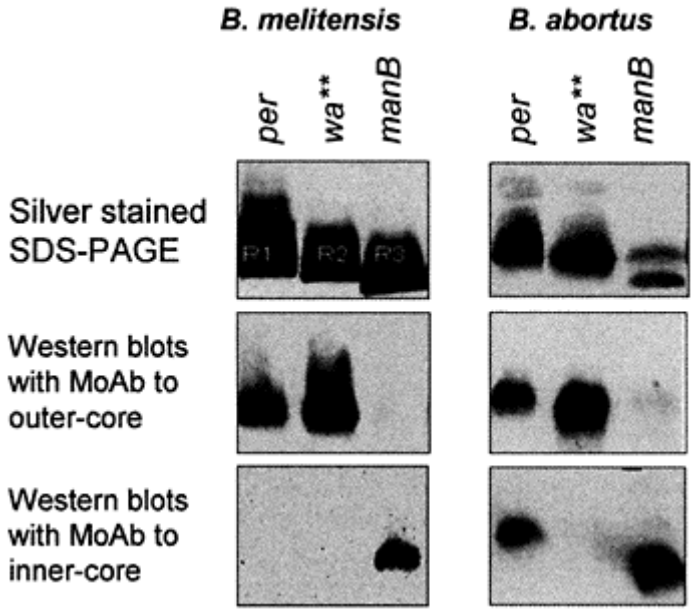

Figure 4. SDS-PAGE and Western blot analysis of LPS extracts of *B. melitensis* and *B. abortus* mutants in the O-PS *per* gene and in the *wa*** and $manB_{core}$ genes.

8-phosphatase acting between KdsA and KdsB has not been identified so far in *E. coli*. Transfer of two Kdo residues from CMP-Kdo to the lipid A disaccharide backbone could be mediated in *Brucella* by the product of ORF BMEII1029 that has a 51% similarity with the Kdo transferase (WaaA, formerly KdtA) of *E. coli* (Belunis and Raetz, 1992).

Brucella does not contain any clear homologue of *waaZ (rfaZ),* the gene encoding the alfa-2, 4 Kdo transferase activity that adds Kdo III in the inner core of *E. coli* and *S. enterica.* It also lacks homologues of *waaC, waaF* and *waaQ,* the genes encoding the L-glycero-D-manno-heptose transferases necessary to build the inner core of enteric bacteria (see Figure 1A). Indeed, these absences parallel those of Kdo III and heptose in the LPS core of *Brucella* and its phylogenetic relatives.

At least three genes have been identified in *B. abortus* and *B. melitensis* whose disruption generates truncated core oligosaccharides (Allen *et al.,* 1998; Monreal *et al.,* 2003; González, 2003). Mutants in gene $manB_{core}$ (also *pmm;* formerly *rfbk;* BMEII 0899 and BRA0348) (Allen *et al.,* 1998; Monreal *et al.,* 2003) show an R LPS with a deeply truncated core which fails to react with MoAbs specific for the outer core (Monreal *et al.,* 2003) (Figure 4). The gene is adjacent to $manC_{core}$ (BMEII 0900; BRA0347), which putatively codes for both mannose-6-P-isomerase and mannose-1-P-guanylyltransferase activities, so that this *manB-manC* arrangement suffices for GDP-mannose synthesis (see above). Moreover, the product of *Brucella* ORF BMEI 0509 shows significant homology to LpcC, a transferase recently identified in *Rhizobium* which links a mannose unit to Kdo I of the lipid A precursor $(Kdo)_2$-lipid IV_A (see below) at the same place where WaaC adds heptose I in *E. coli*. (Kadrmas *et al.,* 1996; 1998; Kanipes *et al.,* 2003). An appealing possibility is that the BMEI 0509 protein performs the same function as LpcC in *Rhizobium* and transfers mannose to Kdo I. In support of this, their similarity is remarkably high (76%) and mannose is directly linked to Kdo I in other phylogenetic relatives of *Brucella* (Holst, 1999) including *O. intermedium* (Velasco *et al.,* 1998).

A *pgm* (phosphoglucomutase) gene was first shown to be involved in the synthesis of *B. abortus* LPS by Ugalde *et al.* (2000). These authors reported an electrophoretic profile for the mutated LPS indistinguishable from that of the wild-type core, and suggested that glucose, galactose, or a derivative of these sugars may be part of the linkage between the core and the O-PS. However, the *pgm* (BMEI 1886) mutant of *B. melitensis* has a clearly truncated core with an electrophoretic mobility intermediate between that of the R-LPS of *per* (which can be taken as a reference of a complete core) and $manB_{core}$ mutants (González, 2003). Indeed, depending upon the electrophoretic conditions, single sugar deficiencies in the core may escape detection and, in addition, there are differences in the core of *B. melitensis* and *B. abortus* which may account for the discrepancies observed by these authors (see below). On the other hand, galactose has never been reported in the analysis of *B. abortus* LPSs, and galactose epimerase mutants of *B. melitensis* synthesise a complete O-PS, which demonstrates that this sugar is not part of the outer core of *Brucella* (Petrovska *et al.,* 1999). Presently, the phenotype of *pgm* mutants seems to relate directly to the absence of glucose and the electrophoresis analyses are consistent with its presence in the outer core. Moreover, galacturonic acid is derived from galactose by the action of a galactose-6-phosphate dehydrogenase, and the corresponding gene seems to be absent in *Brucella*. This evidence, and the smooth LPS phenotype of the galactose epimerase mutants, is consistent with the reported absence of galacturonic acid in *B. abortus* core (Velasco *et al.,* 2000).

Monreal *et al.* (2003) identified another *B. abortus* gene implicated in the synthesis of the core. This has been provisionally denominated *wa*** and it encodes a putative glycosyltransferase. A similar gene is present in *B. melitensis* (BMEI 1326) and *B. suis* (BR0615). At least in *B. abortus* and *B. melitensis, wa*** mutants produce an R-LPS of

electrophoretic mobility not clearly different from that of the *per* mutant. However, analysis with MoAbs of the appropriate specificity (Rojas *et al.*, 1994) shows that the *B. abortus wa*** mutant lacks the inner epitope and, unexpectedly, it retains the outer core epitope (Figure 4). Since the inner epitope comprises the two Kdo residues linked to some unknown sugars (Rojas *et al.*, 1994), it has been proposed that the core of *B. abortus* is branched and that Wa** links an unknown sugar to a branch corresponding to the inner core epitope.

The availability of *B. abortus* and *B. melitensis* R mutants with mutation in homologous LPS genes and the existence of MoAbs specific for the inner and outer core epitopes of *B. abortus* (Rojas *et al.*, 1994) has made possible a limited comparison of the core structure of these two species. The MoAb specific for the outer core of *B. abortus* reacts with all *B. abortus* or *B. melitensis* R mutants but those in the $manB_{core}$ gene (see above) (Figure 4). This shows, firstly, that mannose is located such that, when it is missing, the outer cores is not synthesised and, secondly, that the outer core is probably similar in both species. On the other hand, the MoAb to the inner core of *B. abortus* fails to react with *B. melitensis per* mutant (and also with mutants in *gmd, wzm, wecA, wbkA, wbkD, wbkE, wboA* and *wboB*) but not with its homologs in *B. abortus* (Figure 4). Since these mutants should have a complete core, this result shows that the inner cores of the *B. melitensis* and *B. abortus* strains tested are different. Surprisingly, the $manB_{core}$ mutant of *B. melitensis* reacts with this inner core specific MoAb (Figure 4). This suggests that the epitopic difference in the inner core of *B. melitensis* and *B. abortus* relates to a sugar(s) linked to the mannose so that when missing the reactivity of the MoAb is restored in *B. melitensis*. The possibility that this unexpected variation in the *Brucella* LPS core may be due to strain rather than species differences cannot be ruled out at present.

Finally, it is worth considering the presence of orthophosphate in *Brucella* core. In *E. coli* and *Salmonella*, such phosphoryl modifications (Figure 1A) depend on genes *waaP (rfaP)* and *waaY (rfaY)* both with homology to kinase proteins (Helander *et al.*, 1989; 1997; Yethon *et al.*, 1998). *Brucella* lacks clear homologues to *waaP* and *waaY* and this is in agreement with the reported absence of orthophosphate in the LPS core (Moreno *et al.*, 1990; Freer *et al.*, 1995). The presence of phosphoethanolamine is discussed below.

3.3. Lipid A

3.3.1. The $(Kdo)_2$-lipid IV_A Pathway

This pathway has been studied in detail in *E. coli* and *Salmonella* and is apparently conserved in *Rhizobium*. These bacteria synthesise the lipid A via a constitutive pathway mediated by seven conserved enzymes that converge in the production of $(Kdo)_2$-lipid IV_A (Figure 5). The first reaction is the incorporation of a β-hydroxymyristoyl residue from a hydroxymyristoyl-acyl-carrier-protein (ACP) to UDP-N-acetyl-GlcN. This reaction is mediated by LpxA (UDP-N-acetyl-GlcN transferase) which is specific for β-hydroxymyristate (Anderson and Raetz, 1987). The product (UDP-3-*O*-monoacyl-N-acetyl-GlcN) is very unstable, and quickly deacetylated by the enzyme LpxC (also called EnvA) to yield UDP-3-*O*-monoacyl-GlcN (Young *et al.*, 1995) which is in turn acylated by LpxD by incorporation of a second β- hydroxymyristoyl residue (Kelly *et al.*, 1993). The pyrophosphate bond in the resulting UDP-2, 3-diacyl-GlcN is then cleaved by a

specific pyrophosphatase (LpxH) to form 2, 3-diacyl-GlcN-1-phosphate (lipid X) (Babinski *et al.,* 2002). This enzyme is probably less well conserved among Gram-negative bacteria suggesting that another enzyme could also catalyse this reaction.

Once lipid X is synthesised, a disaccharide synthase encoded by *lpxB* is responsible for the β 1'-6 dimerisation that generates the tetracyldisaccharide-1-phosphate (Crowell *et al.,* 1986). The next step is the phosphorylation of the 4' position of the disaccharide by the specific kinase LpxK to yield lipid IV_A (Garrett *et al.,* 1997). As described above, once lipid IV_A is formed, the Kdo-transferase WaaA, (KdtA) transfers two Kdo residues from CDP-Kdo to form $(Kdo)_2$-lipid IV_A. (Clementz and Raetz, 1991; Belunis and Raetz, 1992).

3.3.2. O-Acylation of 3-Hydroxyacyl Residues

In *E. coli*, the incorporation of Kdo to lipid IV_A occurs prior to the incorporation of the two acyloxyacyl-linked fatty acids (laurate and myristate). The donors are specific lauryl and myristyl-ACPs and attachment to the $(Kdo)_2$-lipid IV_A requires

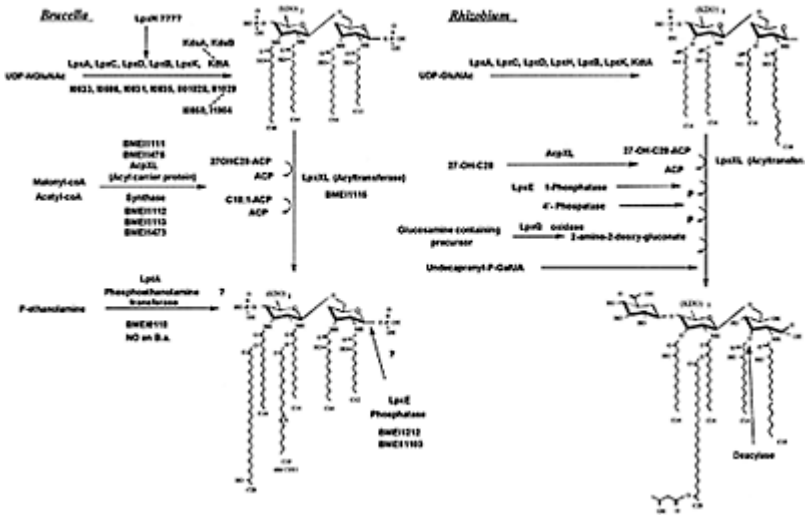

Figure 5. Comparison o the pathways and proteins putatively involved in the synthesis of *B. melitensis* (on the left) and *R. leguminosarum* (on the right) lipids A. The steps indicated in the *Brucella* pathway are hypothetical and based on the homology of the products of the indicated ORFs to those described in *E. coli* (Lpx and Kds and Kdt), *Rhizobium* (AcpX, LpxXL and

LpxE) and *Neisseria* (LptA) (see also Table 1).

the action of the lauryl transferase LpxL (also called WaaM or HtrB) (Karow *et al.*, 1991) and the myristoyl transferase LpxM (also known as WaaN) (Karow and Georgopoulos, 1992) (Figure 5).

3.3.3. Modifications of the Acylation Pattern

When *E. coli* cells are subjected to cold shock, the lipid A is modified by addition of palmitoleate and reduction in the levels of laurate, and it is thought that this probably helps to maintain an optimal outer membrane fluidity. The step requires a specific palmitoleoyl transferase, LpxP, which shows a 54% identity and 73% similarity to LpxL and uses the corresponding palmitoleoyl-ACP (Carty *et al.*, 1999).

Addition of palmitoleate can also be mediated by the OM protein CrcA in *E. coli*, and its *Salmonella* homologue PagP both of which O-acylate the N-linked hydroxymyristate of lipid A (Guo *et al.*, 1998). However, in contrast to LpxL, LpxM or LpxP, PagP is dependent on acyl groups derived from phospholipids (Bishop *et al.*, 2000). PagP is a PhoP-PhoQ regulated protein (see below) of considerable interest. At least in *Salmonella*, the modification it introduces in lipid A seems to alter the LPS PAMP to the point that it brings about an increase in the resistance to bactericidal peptides and a decrease in some endotoxicity-related activities (Guo *et al.*, 1997; 1998). Homologues of PagP are present in *Bordetella*, *Yersinia pestis* and *Legionella* but are apparently absent in other bacteria.

S. typhimurium lipid A can carry two additional modifications not found in *E. coli*. The first is the presence of S-2-hydroxymyristate as a result of the action of LpxO which hydroxylates the 3' secondary acyl chain (Gibbons *et al.*, 2000), and the second is the removal of the β-hydroxymyristoyl residue at position 3 mediated by PagL (Trent *et al.*, 2001a). The *pagL* gene is apparently exclusive to *Salmonella* since no homologous proteins have been described in other species. However other Gram-negative bacteria including *Pseudomonas*, *Rhizobium* and *Helicobacter pylori* also contain partially 3-*0*-deacylated lipid A, and a membrane-bound deacylase activity has been described in *Pseudomonas* and *Rhizobium.* In *R. leguminosarum* this deacylase selectively removes the 3-*O*-linked β-hydroxymyristoyl residue of lipid A. The gene encoding this deacylase remains to be identified (Basu *et al.*, 1999).

3.3.4. Modifications in the Lipid A Backbone and Adjacent Sugars

Several modifications that reduce the negative charge of LPS and consequently decrease the binding of antimicrobial peptides have been described in *E. coli*, *Salmonella* and a few other Gram-negatives. At lipid A level, they are the addition of 4-amino-4-deoxy-L-arabinose (arabinosamine) to the 4'-phosphate (and sometimes to the 1-phosphate) and phosphoethanolamine to the 1-phosphate (and sometimes to the 4'-phosphate) of the lipid A backbone. Moreover, phosphoethanolamine is sometimes added to Kdo and to the first heptose residue of the core (Helander *et al.*, 1994; Zhou *et al.*, 2001; Kanipes *et al.*, 2001; Stinavage *et al.*, 1989).

The proteins involved in arabinosamine addition to lipid A have been identified. They are encoded in the transcriptionally independent *unit pmrE* and the *pmrHFIJKLM* operon, the expression of which is regulated by the two component systems PhoP-PhoQ and PmrA-PmrB through the mediator *pmrD* (Gunn *et al.*, 1998; 2000; Trent *et al.*, 2001c; 2001d; Tamayo *et al.*, 2002; Breazeale *et al.*, 2002; 2003). Functions identified are those necessary to synthesise arabinosamine from glucose (*pmrE, pmrH* and *pmrI* [also *pagA, arnB,* and *arnA,* respectively]), its transfer *(pmrF [arnC])* to undecaprenyl-phosphate and the final incorporation to lipid A (*pmrK* [also *arnT,* orf5, or *yfbI*]) (Trent *et al.*, 2001b; 2001d). The role of genes *pmrJ, pmrL,* and *pmrM* as well as that of the adjacent *pmrG* is not known.

The genes involved in phosphoethanolamine addition to the core and lipid A in *E. coli* and *Salmonella* have yet to be identified. However, a transferase involved in the specific transfer of phosphoethanolamine to lipid A has been recently identified in *Neisseria* as the product of gene *lptA* (NME1638) (Cox *et al.*, 2003). LptA is homologous to a previously described protein (Lpt-3) responsible for the specific transfer of a phosphoethanolamine residue to the 3- position of the heptose II residue in the *Neisseria* core oligosaccharide (Mackinnon *et al.*, 2002).

3.3.5. Brucella Lipid A

Like other bacteria with classical peptidoglycans, *Brucella* contains homologues of the genes encoding the enzymes necessary to generate UDP-N-acetyl-GlcN, which is a common precursor to both classical lipids A and murein (however it is not presently known how NGlcN is generated). The presence of a homologue of LpxC (the deacetylase that converts UDP-3-O-(3 hydroxymyristoyl)-N-acetyl-GlcN into UDP-3-O-(3 hydroxymyristoyl)-GlcN) in *Brucella* suggests the existence of an UDP-3-N-(3 hydroxyacyl)-2-N-acetyl-NGlcN intermediate in the initial steps of the pathway and, accordingly, that 2, 3, NGlcN is synthesised before LpxA acts. Homologues of *lpxA* and of all the remaining genes coding for the enzymes involved in the $(Kdo)_2$-lipid IV_A pathway, with the not uncommon exception of LpxH (see above) are also present.

Once the $(Kdo)_2$-lipid IV_A is formed, specific enzymes should be responsible for the acylation of the 3-hydroxy primary acyl chains, and comparisons with other α-2 *Proteobacteria* are interesting. It has been recently shown that the $C28_{27OH}$ LCFA present in *Rhizobium* lipid A is incorporated into $(Kdo)_2$-lipid IV_A by LpxXL, a specific acyltransferase only distantly related to the LpxL protein of *E. coli* (Basu *et al.*, 2002). Moreover, its associated ACP has been identified as the product of *acpXL,* a new member of a specialised family of ACPs devoted to the transfer of hydroxylated LCFA and different from the constitutive ACPs involved in the synthesis of C12-C18 fatty acids (Brozek *et al.*, 1996a; Vedam *et al.*, 2003). Interestingly, *Brucella* contains an ORF (BMEI1115) with significant similarity (68%) to the LpxXL acyltransferase required for incorporation of LCFA $C28_{27OH}$ to *Rhizobium* lipid A. Moreover, it is very likely that the specialised ACPs for these LCFAs are encoded by *Brucella* ORFs BMEI1111 and BMEI1475 since both display very high or high similarity (95% and 66% respectively) to the *Rhizobium* AcpXL. It is noteworthy that BMEI1115 seems to be the only putative acyltransferase in *Brucella* with homology to acyltransferases involved in lipid A biosynthesis. Also, its homology with *Rhizobium* LpxXL (68%) is higher than with other

acyltransferases like the LpxL (38%) or LpxM (38%) required for the transfer of the chains acyloxyacyl linked to 3-hydroxymyristoyl in *E. coli* lipid A. It seems therefore, that the genomic data support the prediction of the presence of LCFAs in *Brucella* lipid A and of structures similar to those found in *O. intermedium* (Figure 1C).

In addition to this evidence, a global sequence comparison of the DNA region around BMEI1115 and BMEI1475 (Figure 3) with the GenBank database reveals remarkable similarities between the gene arrangements of the putative *acpXL* and *lpxXL* in *Brucella* and their homologous loci in *Rhizobium* (Table 1). The *Brucella* region that more closely resembles the one described in *Rhizobium* is the BMEI1115 region. Like in its *Rhizobium* counterpart, *acpXL* and *lpxXL* are separated by several ORFs with significant homology with proteins involved in fatty acid biosynthesis: BMEI1112 and BMEI1113 are homologous to fatty acid synthases and to orf2 and orf3 of *Rhizobium*, and BMEI1114 shares with orf4 of *Rhizobium* the homology with alcohol dehydrogenases. On the other hand, the BMEI1475 region contains a putative 3-oxoacyl-ACP synthase (BMEI1473), a short chain dehydrogenase (BMEI1477), and a malonyl co-acyl carrier protein transacylase (BMEI1478) but no homologs of the LpxXL acyltransferase.

Although the lipid A disaccharides are different (Table 1), comparisons with *Rhizobium* add also some interesting details. Although the genes of the $(Kdo)_2$-lipid IV_A pathway are common to all *Rhizobium* species (Price *et al.*, 1994), the final structure of the *R. leguminosarum* and *R. etli* lipid A is different. In these two species the lipid A contains 2-amino-2-deoxy gluconate in place of the GlcN 1-phosphate unit and a galacturonic acid residue instead of the 4'-phosphate group (Figure 1B). Incorporation of the latter residue occurs by unknown mechanisms and, even though a 4' phosphatase activity acting on $(Kdo)_2$-lipid IV_A has been detected in *R. leguminosarum* extracts, the corresponding gene has yet to be identified (Price *et al.*, 1995; Brozek *et al.*, 1996b). With regard to the modifications at the reducing end, the specific phosphatase cleaving the 1-phosphate linkage has been identified as the product of gene *lpxE* (Karbarz *et al.*, 2003). Once this phosphate is removed, 2-aminogluconate is generated by an OM oxidase (LpxQ) (Que-Gewirth *et al.*, 2003a; 2003b) (see Figures 1B and 5). As expected, no homologue of LpxQ is found in *Brucella* but there might be some reminiscence of the *Rhizobium* backbone modification pathway. As mentioned above, the *B. abortus* lipid A contains both mono and bisphosphorylated forms (Figure 1D and 1E) and the bisphosphorylated forms are the predicted result of the $(Kdo)_2$-lipid IV_A pathway. Thus, it seems likely that the lipid A phosphorylated only in 4' is produced by partial dephosphorylation of $(Kdo)_2$-lipid IV_A. Interestingly, two *Brucella* ORFs (BMEI1212 and BMEII1103) have a conserved phosphatase domain and show 49% and 43% similarity to the specific lipid A 1-phosphatase LpxE of *Rhizobium* (Figure 5).

With regard to possible fatty acid modifications occurring after completion of lipid A synthesis, it is worth noting that the product of ORF BMEI0464 shows 44% similarity to the LpxO of *Salmonella* (Gibbons *et al.*, 2000). As mentioned

Table I. Lipid A and core biosynthetic ORFs identified in the *B. melitensis* 16M genome, their homologies, putative functions and equivalent ORFs in *B. suis* and *B. abortus*

ORF	Homologous gene (alternative name) and bacteria	Homology at protein level	Function (when known)		*B. suis* ORF	Presence in *B. abortus* (chromosome)[1]
BMEI 0833	***lpxA*, *E.coli***	36 % identity; 54% similarity. 255 aa[2] overlap out of 282	UDP-GlcNAc acyltransferase	UDP-GlcNAc→UDP-3-*O*-monoacyl-GlcNAc	BR1151 (I)	+(I)
BMEI 0586	***lpxC*** *(envA),* ***E.coli***	43% identity; 66% similarity. 280 aa overlap out of 286	Deacetylase	UDP-3- *O*-monoacyl-GlcNAc→UDP-3-*O*-monoacyl-GlcN	BR1424 (I)	+(I)
BMEI 0831	***lpxD*** *(firA; ssc),* ***E.coli***	36% identity; 53% similarity. 312 aa overlap out of 351	Acyltransferase	UDP-3-*O*-monoacyl-GlcN→UDP-2,3-diacyl-GlcN	BR1153 (I)	+(I)
n.i.[3]	***lpxH*, *E.coli***	Missing in some Gram negatives	Pyrophosphatase	UDP-2,3-diacyl-GlcN→2,3-diacyl-GlcN-1-P (lipid X)	n.i.	n.i.
BMEI 0835	***lpxB*, *E.coli***	31% identity; 47% similarity. 362 aa	Disaccharide synthase	Lipid X→ Tetraacyldisaccharide 1-P	BR1149 (I)	+(I)

		overlap out of 395				
BMEII1028	***lpxK, Salmonella***	27% identity; 38% similarity. 244 aa overlap out of 341	Kinase	Tetraacyldisaccharide 1-P→Lipid IV_A (Tetracyldisaccharide 1,4-bis-P)	BRA0216 (II)	+(II)
BMEII1029	***waaA*** *(kdtA),* ***E.coli***	35% identity; 51% similarity. 363 aa overlap out of 446	KDO transferase	Lipid IV_A→$(KDO)_2$-Lipid IV_A	BRA0215 (II)	+(II)
BMEI 1115	***lpxL*** *(htrB),* ***E.coli***	23% identity; 38% similarity. 286 aa overlap out of 307	Lauroyl acyltransferase	$(KDO)_2$-Lipid IV_A→$(KDO)_2$—pentaacylated lipid A	BR0851 (I)	+(I)
BMEI 1115	***lpxM*** *(msbB),* ***E.coli***	25% identity; 38% similarity. 294 aa overlap out of 307	Myristoyl acyltransferase	$(KDO)_2$—pentaacylated lipid A→$(KDO)_2$—hexaacylated lipid A	BR0851 (I)	+(I)
BMEI 0850	***kdsA, E. coli***	45% identity; 65% similarity. 268 aa overlap out of 277	2-dehydro-3-deoxyphosphooctonate aldolase	D-Arabinosa 5P→3-Deoxy-D-mannooctulosonate-8P	BR1133 (I)	+(I)
BMEI 1904	***kdsB, E.coli***	42% identity; 54% similarity. 228 aa overlap out	3-deoxy-mannooctulosonate cytidyltransferase	3-Deoxy-D-mannooctulosonate→CMP-3-Deoxy-D-mannooctulosonate	BR0038 (I)	+(I)

		of 295				
BMEI 0832	***fabZ, E.coli***	46% identity; 64 % similarity in 138 aa out of 173	(3R)-hydroxymyristoyl(acyl-carrier-protein) dehydratase		BR1152 (I)	+(I)
BMEI 1115	***lpxP*** *(ddg),* ***E.coli***	24% identity; 38% similarity. 280 aa overlap out of 307	Palmitoleoyl transferase	Transfers palmitoleate from palmitoleoyl-ACP to $(KDO)_2$-lipid IV_A cold shock lipid A biosynthesis	BR0851 (I)	+(I)
BMEI 0464	***lpxO, Salmonella***	30% identity; 44% similarity. 130 aa overlap out of 173	α- ketoglutarate-dependent dioxygenase	Hydroxylation of lipid A (or a key precursor)	Present but not annotated	+(I)
BMEI 0118	***lptA, Neisseria meningitidis***	37 % identity; 54% similarity. 533 aa overlap out of 544	Phosphoethanolamine transferase for lipid A		BR1948	truncated protein
BMEI 1212	***lpxE, Rhizobium***	33% identity; 49% similarity. 119 aa overlap out of 235	Lipid A 1-phosphatase	Selective removal of 1-phosphate group from lipid IV_A	BR0740 (I)	+(I)
BMEII 1103		25%identity; 43% similarity. 147 aa overlap out of 292 Phosphatase domains are conserved in both			BRA0131 (II)	+(II)

ORF	*Homologous*	*Homology*	*Function*		*B. suis*	*Presence in B*

	gene (alternative name) and bacteria	*at protein level*	*(when known)*		*ORF*	*abortus (chromosome)*[1]
n.i.	***n.i. Rhizobium***	n.i.	Lipid A 4'-phosphatase		n.i.	n.i.
n.i.	***lpxQ Rhizobium***	n.i.	Outer membrane oxidase	GlcN containing precursor→2-amino-2-deoxy-gluconate	n.i	n.i
BMEI 1111	***acpXL Rhizobium***	91% identity; 95%similarity 90 aa overlap out of 93	Acyl carrier protein	Transfers (w-1) hydroxyl long (C28-C30) acyl chain to lipid A	BR0855 (I)	+(I)
BMEI 1475		41%identity, 66% similarity 53 aa overlap out of 78			BR0459 (I)	+(I)
BMEI 1115	***lpxXL Rhizobium***	56% identity; 68% similarity 302 aa overlap out of 307	Lipid A acyltransferase	Transfer of long (w-1) hydroxyl fatty acid onto lipid A	BR0851 (I)	+(I)
n.i.	***n.i. Rhizobium***	n.i.	Deacylase	Cleaves the 3-*O*-Linked β-Hydroxymyristoyl moiety of lipid A precursors	n.i.	n.i.

1. The presence of a the ORF is indicated with a +
2. Amino acid
3. Not identified

above, LpxO has been related to the oxygen-dependent hydroxylation of the 3' acyloxyacyl-linked myristic acid to generate an S-2-hydroxymyristate. The *Brucella* sequence contains the motif of the putative active site but, as far as it is known, there are no S-2-hydroxylated fatty acids of *Brucella* and the role of this putative acyl hydroxylase is intriguing. It might be that such fatty acids have escaped detection, or are not expressed under standard conditions. The alternative possibility that it participates in hydroxylations at other positions, such as that in the LCFA $C28_{27OH}$ seems presently unlikely as there are no matches in the genome of *R. leguminosarum*.

Finally, given the comparatively low binding of polycationic peptides by *Brucella* LPS, it is worth commenting on the genetic evidence for arabinosamine and ethanolamine modifications (see above). Concerning possible arabinosamine

substitutions, several *Brucella* genes have homology (up to 55% similarities in the protein) to genes of the *pmrHFIJKLM* operon and to *pmrE* (see above) required for arabinosamine synthesis (*pmrE, pmrH* and *pmrI*) and incorporation to lipid A *(pmrK, pmrL)*. However, *Brucella* lacks others both of unknown function *(pmrM, pmrD)* or required *(pmrJ)* for the addition of arabinosamine to lipid A in *Salmonella* and, moreover, the homologous *Brucella* genes are not organised in a operon but rather scattered in both chromosomes. This is in agreement with the reported absence of arabinosamine in the LPS of *Brucella*. With regard to ethanolamine, this compound has not been reported either in *Brucella* LPS. However, a close analysis of *B. melitensis*, *B. suis* and *B. abortus* genomes reveals an ORF of 545 amino acids (BMEI0118 in *B. melitensis* and BR1948 in *B. suis*) with significant similarity (about 54%) to LptA, the phosphoethanolamine transferase acting on *Neisseria* lipid A (Cox *et al.*, 2003). In *B. abortus*, the possible homologue of BMEI0118 and BR1948 contains a frame shift in codon 261. This would lead to the expression of only a fragment of the complete protein unlikely to be functional. If so, the lipid A from *B. melitensis* and *B. suis* but not *B. abortus* may have ethanolamine substitutions in the phosphoryl groups of the lipid A disaccharide. Since the reported absence of ethanolamine comes from the analysis of *B. abortus* LPSs (see above), it seems that this aspect needs to be reinvestigated.

4. Assembly and Transfer to the Bacterial Surface

Once lipid A and the core are assembled on the inner side of the cytoplasmic membrane, they are translocated to the periplasmic space. Transfer requires the activity of an ABC transporter, such as MsbA which was first described in *E. coli*. Msb A acts as a flippase that helps the lipid A-core to traverse the membrane (Polissi and Georgopoulos, 1996; Zhou *et al.*, 1998; Chang and Roth, 2001; Doerrler *et al.*, 2001; Doerrler and Raetz, 2002). It is thus worth noting that *Brucella* contains two ORFs (BMEII0250 and BMEII0323) that have significant overall homology at the protein level (55% and 49% respectively) to MsbA. Thus BMEII0250 and BMEII0323 are predicted to perform the same function as MsbA. In *E. coli*, *msbA* is situated in an operon containing *lpxK*. However *msbA* and *lpxK* are situated in different regions of the *Brucella*, chromosome, with *lpxK* forming an operon with *kdtA*, which encodes the Kdo transferase.

Completion of the smooth LPS requires translocation of the O-PS to the periplasmic side, where it is ligated to the nascent lipid A-core. This is mediated by the products of *wzm* and *wzt*, two *wbk* genes (see above). In addition to their homology with other ABC transporter genes, it has been shown that their disruption generates R mutants in which a cytoplasmic formyl-perosamine PS accumulate (Godfroid *et al.*, 2000; González, 2003). It is known that WaaL in *Salmonella* and *E. coli* mediates the next step, i.e. the ligation reaction, even though the exact mechanism remains unknown. It is noteworthy that, at least in these two closely related bacteria, there is little similarity between the primary structure of the WaaL proteins. Apparently, this reflects the specificity of the ligase for the core oligosaccharide section that serves as the acceptor and not the O-PS structure. No homologues of the *E. coli* and *Salmonella waaL* can be found in *Brucella* genomes, and it seems likely that the reason for this is the marked difference between core structure of these bacteria and that of *Brucella*.

Recently Genevrois *et al.* (2003) have identified an outer membrane protein of 85.000 Da (Omp85) required for lipid and LPS export to the outer membrane in *Neisseria meningitidis*. These authors also pointed out that *Brucella* contains a protein (corresponding to BMEI0830, BR1154) with a clear homology to Omp85 (46% similarity). This protein had been previously identified by S.W.Bearden and T.A.Ficht (Gene Bank accession No. U51683.1) as the precursor of *B. abortus* Omp1. The marked presence of Omp1 in extracts enriched in OM proteins and its association with LPS (Moriyon and Berman, 1982; Verstreate *et al.*, 1982) supports the prediction that BMEI0830 is the gene coding for the protein involved in the transfer of LPS to the OM. Furthermore, the *Brucella omp1* gene is immediately upstream of the putative *lpxD* and in the same chromosomal region as *lpxA, lpxB,* and the genes *(cdsA, fabZ)* encoding phospholipid synthesis enzymes. This is a location similar to the gene coding for the 85KDa protein of *Neisseria*.

5. Concluding Remarks

One of the intriguing questions concerning pathogens is the way through which they emerge in nature. In this regard, the lipid A and core sections of the LPS of Gram-negative bacteria have the dual interest in that firstly, they contain valuable phylogenetic information and secondly, they can be readily recognised by the innate immune system as potential pathogen markers. Both roles are particularly clear in the case of *Brucella* LPS: its lipid A and (possibly) core structure bears witness of its origin in a group of soil bacteria and its altered PAMP makes it an unusually effective and multi-faceted virulence factor.

Given the essential role of the OM as a permeability barrier, one may ask why and how the *Brucellae*, like several mucosal Gram-negative pathogens, gave up part of this function and became permeable to hydrophobic compounds. The core and lipid A negatively charged groups are critical for the functioning of this barrier and, at the same time, constitute targets for the bactericidal polycationic peptides of the innate immune system (Vaara, 1999). Accordingly, the *Brucellae* may have evolved from an α-*Proteobacteria* environmental microbe that, by colonising those host environments where no noxious hydrophobic permeants exist, would have been able to reduce to a minimum the acidic core sugars and lipid A phosphates as a response to selective pressure of antimicrobial peptides (Moreno *et al.*, 2002; Moreno and Moriyón, 2002b; Moriyón, 2003). The strategy of giving up those core and lipid A groups can be viewed as opposite to that followed by enteric bacteria i.e. these bacteria acquire positively charged groups (i.e. ethanolamine and arabinosamine) to become resistant to such peptides. The phylogenetic background of *Brucella* would have facilitated this type of evolution as only a few steps would have been necessary to make such a change. Genomic analyses suggests that the pathway for galacturonate synthesis has been lost in *Brucella* and that ancestral phosphatases capable of pruning off lipid A are present. With regard to the latter, there is only indirect but convincing evidence that the LCFA of *Brucella* are also important in pathogenicity. The most compelling one stems from the observations showing that, whereas the biological activities of *Brucella* and enterobacterial LPS are markedly different, the activities of the LPSs of *Brucella* and *Legionella* are similar

(Rasool *et al.*, 1992; Moreno and Moriyón, 2002b; Moriyón, 2003). Indeed, the LPS of the latter shows LCFAs similar to those of *Brucella*. Electron microscopy studies on the action of polymyxin B on the OM of *Brucella* and *Ochrobactrum* are also consistent with a role of LCFAs in the resistance to polycationic peptides (Velasco *et al.*, 2000). The analysis of the genome not only supports the presence of LCFAs in *Brucella* LPS but also demonstrates the ancestral character of this trait. Accordingly, the lipid A-core of the *Brucella* ancestor would represent a platform on which the acquisition of additional virulence factors by the bacterium would lead to pathogenicity. With regard to LPS itself, the evidence on the horizontal transfer of *wbk* genes (Cloeckaert *et al.*, 2000; Godfroid *et al.*, 2000) and the role of the O-PS as a negative modulator of *Brucella* adsorption and entry into cells and apoptosis (Sola-Landa *et al.*, 1998; Fernández-Prada *et al.*, 2003) suggest such an horizontal transfer as one of the next steps. Some of the hypothesis on the role of core and lipid A elements in pathogenicity and LPS evolution can now be examined by developing the appropriate mutants. These studies will also lead to a better understanding of the pathways for the synthesis of this remarkable molecule and of the multiple and contingent ways through which pathogens emerge in nature.

6. Acknowledgements

Research at the laboratories of the authors is supported by the European Commission (Research Contract QLK2-CT-2002–00918), Ministerio de Ciencia y Tecnología of Spain (Proyecto AGL2000–0305-C02) and Redes Temáticas de Investigación Cooperativa del FIS—Promoción de las Regiones (objetivo 2, 2000–2006) (Red Temática de Investigación en Brucellosis, G03/204).

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Chapter 9
Brucella Cyclic β-1,2-Glucans: Structure, Biosynthesis, Biological Activities and Role in Virulence

Nora Iñón de Iannino and Rodolfo A.Ugalde*

From: *Brucella:* Molecular and Cellular Biology. Edited by: Ignacio López-Goñi and Ignacio Moriyón

Abstract

Cyclic β-1,2-glucans are unique periplasmatic polysaccharides produced by *Brucella, Rhizobium,* and *Agrobacterium,* bacteria that belong to the a Proteobacteria group and display intimate interactions with plant or animal eukaryotic cells. Cyclic β-1,2-glucans are homopolymers with a degree of polymerisation ranging from 17 to 24 glucose residues substituted with a variety of non-glycosidic residues. Under low osmolarity conditions cyclic glucans are highly accumulated in the periplasmatic space reaching values ranging from 5 to 20 % of the total cellular dry weight, thus suggesting that they may play a role in adaptation and/or protection of the bacterial cells under certain environmental conditions. Moreover, *Rhizobium* and *Agrobacterium* cyclic β-1,2-glucans are required for effective plant interaction (symbiosis and virulence, respectively). In addition, *Brucella* cyclic β-1,2-glucan mutants displayed reduced intracellular multiplication and virulence, thus suggesting that cyclic β-1,2-glucan may be a common feature required for effective interaction of the bacteria with animal and plant cells. Cyclic β-1,2-glucans are synthesised by a glucosyl transferase, cyclic glucan synthase (Cgs), that uses UDP-glucose as sugar donor. The synthesis proceeds through a novel mechanism in which Cgs, an integral membrane protein of 300 kDa, acts itself as protein intermediate and contains all the enzymatic activities required for the synthesis: i.e. initiation, elongation and cyclization. Cyclic glucans are synthesised on the cytoplasmatic side of the inner membrane and secreted into the periplasmic space by inner membrane-bound ABC-transporters (*S. meliloti ndvA, A. tumefaciens chvA* or *B. abortus cgt*). *A. tumefaciens, S. meliloti* and *B. abortus* cyclic

glucan ABC-transporters are highly similar and functionally interchangeable. Moreover *ndvA, chvA* and *cgt* null mutants, unable to transport cyclic glucan into the periplasm, display defective host interaction, thus suggesting that secretion into the periplasmic space is required to exert its action. Little is known on the mechanism of action of cyclic glucan. It has been proposed that the glucan itself may be required for effective cell attachment. Other authors have proposed that the observed phenotype might be associated to the protection of the cells under stressing conditions, as for example low osmolarity. However, several evidences suggest that the mechanism may be more complicated than that originally thought.

* For correspondence email rugalde@iib.unsam.edu.ar

For example several pleiotropic effects on the structure and function of cellular envelope were observed in *cgs* mutants: i) defective assembly of the flagella in motile species like *Rhizobium* and *Agrobacterium,* ii) increased sensitivity to antibiotics and detergents, and iii) reduced stability of several components of the type IV secretion system. These results suggest that cyclic β-1,2-glucan may be involved in the correct assembly of membrane macromolecular structures required for effective host interaction.

Introduction

According to 16S rRNA sequence analysis, *Brucella, Rhizobium* and *Agrobacterium* belong to the α-2 subgroup of Proteobacteria (De Lay, 1987; Moreno *et al.,* 1990). All of these organisms have a complex genomic organization with multiple circular or linear chromosomes, a characteristic restricted to certain species within Proteobacteria. Moreover the recent complete genome sequence of *B. melitensis* (Del Vecchio *et al.,* 2002), *B. suis* (Paulsen *et al.,* 2002) and *B. abortus* (R.A.Ugalde, unpublished) revealed a high degree of gene similarity and synteny between *Brucellae*, *Rhizobium* and *Agrobacterium*. The α-2 subdivision includes, in addition to free-living species, Gram-negative bacteria that live in close association with eukaryotic cells such as *Brucella* which is considered a facultative extracellular, intracellular pathogen.

Brucellosis is a disease that affects a wide range of mammals, including humans, causing recurrent fever in humans and abortion in ungulates. The persistence of the pathogen inside the host cells (Smith and Ficht, 1990) causes no apparent cell damage, which makes the treatment of disease difficult during the chronic phase. Moreover, virulence usually correlates with the ability to persist in the host. It has been recently demonstrated that once inside the cells, *Brucella* avoids the fusion of the phagosome with the lysosome and reaches a compartment with endoplasmic reticulum (ER) markers where the proliferation takes place (Pizarro-Cerda *et al.,* 1998a). *B. abortus* escapes from the endocytic pathway, exploits the autophagic machinery of the host cell and establishes its replication niche in a endoplasmic reticulum-like organelle. On the other hand, the soil

bacteria *Rhizobium* and *Agrobacterium*, phylogenetically related to *Brucellae*, live in close association with plant eukaryotic cells. *Rhizobium* invades leguminous roots leading to the formation of root nodules where nitrogen fixation takes place. *Agrobacterium* causes the formation of crown gall tumours, a process that requires a close interaction of the bacteria with the plant cell to transfer into the plant genome the Ti-DNA. The role of polysaccharides in these bacteria-cell interactions is well known (Whatley *et al.*, 1976; Leigh *et al.*, 1985; Puvanesarajah *et al.*, 1985). Great importance was attributed in brucellosis to lipopolysaccharide (LPS) as a virulence factor and protective molecule (Cherwonogrodzky *et al.*, 1990). *Brucella* LPS is highly antigenic and induces specific antibody response (Moreno *et al.*, 1981). Rough mutants of *B. abortus*, defective in the synthesis of the O-antigen or the core region, lost virulence (Reeves, 1995). However, *B. ovis* and *B. canis* are natural rough fully virulent strains in their natural hosts, suggesting that in *Brucella* smooth LPS is not the only factor necessary for virulence.

Although smooth LPS of *Brucella* has been reported to be important as a virulence factor its precise role in pathogenesis is not yet clear. In a recent study *B. suis* LPS O-side chain was shown to be involved in the inhibition of the early fusion between *B. suis*-containing phagosomes and lysosomes, a role assigned to VirB (Porte *et al.*, 2003). The phagosomes containing rough mutants, which fail to express the O-antigen, rapidly fuse with lysosomes. In addition, the authors showed that rough mutants, contrary to smooth strains, do not enter host cells by using lipid rafts, a role also assigned to VirB. (Watarai *et al.*, 2002 a). Thus, it was proposed that the LPS O-chain might be a major factor that governs the early behaviour of bacteria inside macrophages.

Recently a *B. abortus* genetically defined phosphoglucomutase *(pgm)* rough mutant was characterised. Pgm is required for the synthesis of glucose-1-phosphate, an obligate intermediate during the synthesis of UDP-glucose, ADP-glucose, UDP-galactose and any other sugar-nucleotide that uses these sugar-nucleotides as intermediates. Pgm mutants displayed a pleiotropic phenotype derived from the fact that they are unable to form the sugar donors for the biosynthesis of molecules containing these sugars. A *B. abortus* 2308 *pgm* mutant does not form cyclic β-1,2-glucan and accumulates on the surface an LPS devoid of the O-antigen (rough phenotype). Pgm mutant lost virulence in mice but partially retained the ability to multiply inside HeLa cells (Ugalde *et al.*, 2000).

B. abortus S19 is the most frequently used vaccine against bovine brucellosis. Although it induces good protection levels it cannot be administered to pregnant cattle, and re-vaccination is not advised due to interference in the discrimination between infected and vaccinated animals during immune-screening procedures. Furthermore, S19 strain is virulent for humans. Due to these reasons, there is a continuous search for new bovine vaccine candidates that might confer protection levels comparable to those of S19 but without its disadvantages. Recently we analysed the protection, proliferative response and cytokine production induced by a deleted Δ*pgm* strain in BALB/c mice (Ugalde *et al.*, 2003). We showed that this strain synthesises O-antigen with a size of approximately 45 kDa, but is rough. This is due to the fact that Δ*pgm* is unable to assemble the O-side chain in the complete LPS. Vaccination with Δ*pgm* induced protection levels comparable to those induced by strain S19 and generated a spleenocyte proliferative response and cytokines profile typical of a Th-1 response. On the other hand, we were unable to detect specific anti-O-antigen antibody response by the Fluorescence Polarisation Assay (FPA).

In view of these results, it is possible that Δ*pgm* might be used as a vaccination strain in bovines.

The underlying molecular mechanism of *Brucella* pathogenesis and the bacteria's interaction with host cells remains unclear. No classical toxins, pili, flagella, plasmids, or pathogenicity islands were so far identified. Moreover the complete sequence of the genome did not reveal the presence of classical virulence factors. However, some interesting features suggesting that *Brucella* spp. have evolved form a common soil ancestor closely related to *Rhizobium* and *Agrobacterium* emerged from these studies. Moreover, the ability to invade and multiply inside the host cells causing no damage resembles an endosymbiont rather than a pathogen and revealed some common features between plant and animal pathogens with endosymbionts.

1.1. Intracellular Lifestyle of *Brucellae*

The mechanisms by which *Brucella* binds and penetrates host cells are not fully understood, but several observations suggest that the bacteria are actively internalised by local membrane extensions that resemble the zipper-phagocytosis mechanism observed during *Listeria* infection. It was proposed that macrophage plasma membrane cholesterol is required for the VirB-dependent internalisation of *B. abortus* and for the establishment of bacterial infection in mice (Watarai *et al.*, 2002 b; see also Chapter 14). It was also observed that *Brucella* internalise into macrophages by generalised membrane ruffling, after which the bacteria are enclosed by macropinosomes containing lipid raft-associated molecules such as (GPI)-anchored proteins, GM1 gangliosides and cholesterol. These results suggest that the entry route of *Brucella* into the macrophage may be modulated by lipid raft microdomains (Watarai *et al.*, 2002a). The same authors have also reported that *B. abortus* Hsp60 and VirB may be involved in bacterial internalisation and intracellular signal transduction (Watarai *et al.*, 2003). All these observations suggest that a complex interrelated set of events are involved in the process of cell invasion, trafficking and intracellular multiplication of *Brucellae*. Moreover there are evidences that there may be more than one mechanism of entry into the cells and that the intracellular fate of *Brucella* may be quite different depending on which mechanism is used.

It was described that during the initial steps of invasion of professional and non-professional phagocytes, *B. abortus* is transiently found in a vacuole characterised by the presence of early endosomal antigens like EEA1, Rab5 and the transferrin receptor (Pizzarro-Cerda *et al.*, 1998a; 1998b). At later post-infection time, virulent bacteria are found in an ER-like compartment in which replication occurs. Taken together, these data indicate that pathogenic *B. abortus* escapes from the endocytic pathway and establishes its replication niche in an ER-like organelle (Anderson and Cheville, 1986; Meador and Deyoe, 1989; Detilleux *et al.*, 1990).

1.2. Signal Delivering System in Animal and Plant Pathogenic Bacteria

Several intracellular pathogens have evolved different strategies for controlling the maturation of their membrane-bound compartments, circumventing host defences and further bacteria degradation and transforming the host cell into nutrient-rich

environments where bacteria can replicate. This complex interaction is not necessarily aimed at causing disease but rather to secure microbe multiplication and transmission to other hosts. In *Brucella*, virulence is strictly related to the ability of the strain to survive inside the host cells. Moreover evolution has secured the survival of the pathogen and the host, and disease symptoms are the consequence of immune reaction against infected cells rather than a direct damage on the infected cell itself. The cellular processes that have to be shaped in order to assure this type of fine-tuned interaction are still unknown. The secretion of a number of proteins was found to be required for the successful interaction in animal and plant pathogens. Type III and Type IV are highly conserved and specialised secretion devices that are required by plant and animal pathogens as well as by symbiotic rhizobiae for a successful interaction with the host. Both systems form supramolecular structures on the bacterial surface which are involved in protein translocation into the host cells.

1.3. Type IV Secretion System

In *A. tumefaciens* there is a collinear arrangement of genes, the *virB* operon, that shares high similarity with the *tra* genes of *E. coli* involved in conjugal transfer of DNA (Christie and Vogel, 2000). These genes share also high similarity to the *ptl* operon of *Bordetella pertussis*, the *cag* genes of *Helicobacter pylori*, the *Legionella pneumophila icm* genes and the *Rickettsia prowazekii* RP open reading frame (ORF), all known to be involved in virulence. In *A. tumefaciens*, the *virB* operon codes for most of the proteins forming the system that transfers the Ti-plasmid DNA from the bacteria to the cell nucleus of the plant cell. In this model, the transport machinery should interact with the proteins covering the DNA and should have no contact with naked DNA. It is proposed that the type IV secretion system present in some pathogenic bacteria has the role of delivering proteins into the host cell by a cell contact dependent mechanism similar to DNA conjugation.

1.4. The VirB System of *Brucella abortus*

The *virB* operon of *B. abortus* has been identified by our group (Sieira *et al.*, 2000). This operon is composed by 13 ORFs that share homology with other bacterial Type IV secretion system genes known to be involved in the intracellular trafficking of pathogens, as is the case for the *dot* and *icm* genes of *L. pneumophila*. The *B. abortus virB* operon is transcribed at the beginning of the stationary phase under vegetative culture conditions, and is rapidly turned on when the bacteria are internalised in murine macrophages (Sieira *et al.*, unpublished). Polar mutations introduced in the first gene of the operon, *virB1*, abolish the ability of *Brucella* to replicate intracellularly, indicating that this system is essential for the intracellular lifestyle of this pathogen. Mice infection performed with polar and non-polar mutants in *virB10* demonstrated that the *virB* operon is a major determinant of *Brucella* virulence (Sieira *et al.*, 2000). We have analysed the intracellular pathway of *B. abortus virB10* mutant by confocal microscopy and demonstrated that a functional *virB* operon is essential for the biogenesis of the *Brucella*-containing vacuole. A polar mutation preventing the transcription of *virB10* and downstream sequences did not allow *Brucella* to bypass the endocytic pathway. Consequently, polar mutant-

containing vacuoles fused with lysosomes resulting in bacterial degradation (Comerci *et al.,* 2001). Based on the particular phenotype displayed by *virB10* polar and non-polar mutants, we hypothesise that putative effector molecule(s) secreted by the *virB* operon modulates the maturation process of the *Brucella*-containing vacuole. This maturation is required to avoid interactions with the degradative pathway and to reach the intracellular replication niche in the ER. However no effector molecule secreted by the *B. abortus* VirB system was identified so far.

2. Cyclic β-1,2-Glucans in α Proteobacteria

The presence of cyclic β-1,2-glucans in culture fluids of *Agrobacerium* and *Rhizobium* was first described in 1942. The polysaccharide is also present in 0,2 M trichloroacetic acid (TCA) *Brucella* extracts and referred as polysaccharide B (poly-B) or second component (Diaz *et al.,* 1968). It was originally described that *B. abortus* infected animals produced antibodies which precipitated poly-B, while vaccinated animals with *B. abortus* S19 did not. It was proposed that poly-B might be used in a diagnostic test for discrimination between infected and vaccinated animals. Structural analysis of purified poly-B from *B. melitensis* 16M showed that its major component is a cyclic β-1,2-glucan with a degree of polymerization between 17 to 24 (Bundle *et al.*, 1988). Purified cyclic β-1,2-glucans are not antigenic, and serological reactions attributed to poly-B were due to the presence of contaminating LPS that forms complexes with cyclic β-1,2-glucan (L'vov *et al.*, 1987). Recently cyclic β-1,2-glucans were shown to be present mainly in the periplasm of rough and smooth *Brucella* strains (Briones *et al.,* 1997) and to be important in *Brucella-cell* interaction (Iñón de Iannino *et al.,* 1998). *Agrobacterium, Rhizobium, Sinorhizobium* and *Brucella* species contain periplasmic glucans with similar structures. These oligosaccharides belong to family II on the basis of backbone organization (Bohin, 2000). They are cyclic containing only β-1,2 linkages and a degree of polymerization (DP) ranging from 17 to 25 in *A. tumefaciens, R. leguminosarum* and *B. abortus* and up to 40 in *S. meliloti* (Breedveld and Miller, 1994).

3. Biosynthesis of Cyclic β-1,2-Glucan

Dedonder and Hassid carried out for the first time the *in vitro* synthesis of cyclic β-1,2-glucans (Dedonder and Hassid, 1964). Since that time, considerable evidence has been presented showing that in different organisms the synthesis of cyclic β-1,2-glucans proceeds using UDP-glucose as sugar donor and a membrane bound enzyme. Incubation of *A. tumefaciens* inner membranes with UDP-[^{14}C]-glucose leads to the formation of soluble cyclic β-1,2-glucan and a TCA insoluble compound. Pulse-chase experiments and chemical characterisation of the reaction products led us to conclude that cyclic β-1,2-glucan synthesis proceeds through a protein intermediate as follows (Zorreguieta *et al.*, 1985):

(1) Initiation and elongation reactions:

$$\text{UDP-glucose+protein+Mg}^{2+} \Rightarrow \text{protein-β-1,2-glucan+UDP}$$

(2) Cyclization reaction:

protein-β-1,2-glucan ⇒ protein+cyclic -β-1,2-glucan

Biochemical characterisation of the *A. tumefaciens* chromosomal virulent loci (Douglas *et al.,* 1985) demonstrated that *chvB* (chromosomal virulence B) locus is involved in the synthesis of cyclic β-1,2-glucans (Puvanesarajah *et al.,* 1985). Further studies carried out with *A. tumefaciens chvB* mutants allowed the identification of *chvB* as the structural gene of the cyclic β-1,2-glucan synthetase (Cgs). Moreover it was discovered that Cgs is itself the protein intermediate during the synthesis (Zorreguieta and Ugalde, 1986) as follows:

(1) UDP-glucose+Cgs+Mg^{2+} ⇒ Cgs-β-1,2-glucan+UDP
(2) Cgs-β-1,2-glucan ⇒ Cgs+cyclic -β-1,2-glucan

Cgs is a membrane-bound enzyme of 300 kDa that contains all the enzymatic activities required for the synthesis of cyclic β-l,2-glucan (Altabe *et al.,* 1990; Castro *et al.,* 1996). A locus highly similar to *chvB,* named *ndvB,* is present and required for nodule development in *S. meliloti* (Dylan *et al.,* 1986). *S. meliloti ndvB* mutants do not synthesise cyclic β-1,2-glucans and induce the formation of empty pseudonodules, thus suggesting that cyclic β-1,2-glucans are required for plant cell invasion (Geremia *et al.,* 1987). All members of the *Rhizobiaceae* family synthesise cyclic β-l,2-glucan through the same mechanism and the lack of cyclic glucan resulted in severe defective plant interaction (Breedveld and Miller, 1994). However some exceptions were described (Iñón de Iannino *et al.,* 1996).

Based on the stereochemistry of the donor substrate and the product, Cgs belongs to the inverting glycosyl-transferases class and contains at least three well defined enzymatic activities: (1) initiation, transference of glucose from UDP-glucose to an amino acid residue of Cgs that has not been identified yet; (2) chain elongation, UDP-glucose β-1,2-glucosyl-transferase activity, that results in the formation of a linear β-1,2-linked glucose polymer covalently bound through its reducing end to an unknown amino acid residue of Cgs (protein intermediate); and (3) glucan cyclization, formation of a β-1,2-linkage between the reducing and non reducing end of the β-1,2-oligosaccharide and release from the protein intermediate. Due to the fact that only cyclic glucan has been detected after release from the protein intermediate, cyclization and release reactions may proceed in the same reaction step (Willamson *et al.,* 1992). Cgs is thus an unusual enzyme that contains three different activities in a large membrane bound 300 kDa single polypeptide chain. The enzymatic reaction leads to the formation of cyclic β-1,2-glucan molecules with a degree of polymerisation ranging from 17 to 40 glucose residues. It was suggested that the size distribution of cyclic β-1,2-glucans depends on the competing elongation and cyclization reactions (Willamson *et al.,* 1992). The synthesis of cyclic β-1,2-glucans takes place on the cytoplasmic side of the inner membrane where UDP-glucose is available.

The membrane topology of Cgs was determined by *lacZ* and *phoA* fusions (A. Ciocchini, unpublished). These experiments revealed that the protein passes six times through the membrane forming 4 cytoplasmic and 3 periplasmic domains (Figure 1). The cytoplasmic domain expanding from amino acid 479 to amino acid 822 contains three DXD motifs (D613XD615; D636XD638 and D730XD732) and one RXXRW motif (R782XXR785W786). In other glycosyl-transferases these motifs have been described to

be required for sugar nucleotide binding/catalysis and for processive activity, respectively. Site directed mutagenesis of D636XD638 by A636XD638 and R782XXR785W786 by R782XXA785W786 resulted in inactive enzymes (A. Ciocchini *et al.,* unpublished). These results suggest that this region of the protein contains important catalytic domains (Figure 1). The C-terminal region of the *Agrobacterium, Sinorhizobium* and *Brucella* proteins shows overall identities of 27 and 28% with the complete cellobiose and cellodextrin phosphorylases of *Clostriduim stercorarium* respectively. It is remarkable that this region although highly conserved in all Cgs, seems to plays no role in cyclic glucan synthesis. Since truncated proteins lacking the C-terminal 1,000 amino acid residues are fully active in terms of synthesis of cyclic glucan and host interaction

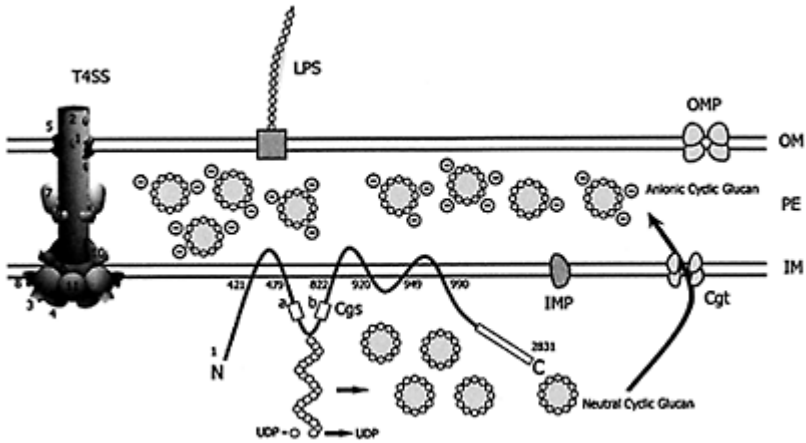

Figure 1. Schematic representation of *Brucella* cell envelope. Cgs: Cyclic β-l,2-Glucan synthetase (numbers indicate amino acid residue positions; a and b boxes indicate DXD and RXXRW motives; C-terminal box indicates the region of Cgs homologue to cellobiose/ cellodextrin phosphorylase). Cgt: cyclic β-1,2-transporter. PE: periplasmic space. IM: inner membrane. OM: outer membrane. IMP: inner membrane proteins. OMP: outer membrane proteins. T4SS: Type IV secretion system VirB (numbers indicate the VirB proteins). LPS:

lipopolysaccharide. UDP-O: uridine-diphospho-glucose.

(Iñón de Iannino *et al.,* 1998; Zorreguieta *et al.,* 1988). It is not known yet if this region of the protein is active as cellodextrin phosphorylase and if it may play a role in the utilisation of cellulose as carbon source in the soil.

4. Secretion of Cyclic β-1,2-Glucans

Cyclic β-1,2-glucans are synthesized on the cytoplasmatic side of the bacterial membrane and actively transported into the periplasmic space by an ABC-type transporter. In *A. tumefaciens chvA* and in *S. meliloti ndvA* loci were identified as the cyclic β-1,2-glucan transporters (Stanfield *et al.,* 1988; Cangelosi *et al.,* 1989; Iñon de Iannino and Ugalde, 1989). The inner membrane protein ChvA of *A. tumefaciens* or the equivalent NdvA of *R. meliloti* are responsible for the transport of non-substituted neutral cyclic glucans from the cytoplasm to the periplasm. It is postulated that once in the periplasm, neutral cyclic β-1,2-glucans are modified with non-glycosidic substituents like glycerol phosphate, succinate, methyl malonate, etc by the action of periplasmic enzymes (Breedveld and Miller, 1994). *Agrobacterium* and *Rhizobium chvA/ndvA* mutants are affected in their normal interaction with plants, thus suggesting that cyclic β-1,2-glucans must be located in the periplasm and or modified with non-glycosidic residues to exert its action.

5. *B. abortus* Cyclic β-1,2-Glucan Transporter

Brucellae posses broader transport capabilities than other sequenced intracellular pathogens. ATP-binding cassette (ABC)-transporters are probably the most common as well as the most wide-spread active transport systems (Nikaido, 2002). Over two-thirds of *Brucellae* transporters are ABC-transporters. For example *B. suis* has 22 amino acid transporters, 16 sugar transporters, and 11 peptide transporters (Paulsen *et al.,* 2002). Something similar was observed in *A. tumefaciens, S. meliloti,* and *M. loti* (Tsolis, 2002). The number of amino acid and peptide transporters could be related to the intracellular replication niche of *Brucella* in the ER, where peptides and amino acids might be plentiful. Also such a large number of ABC-transporters might indicate that *Brucella* require a complex variety of signal delivering systems to secrete molecules that interact with the host cell and suggest their importance in virulence. However little is known on the phenotype of mutants and the substrates secreted by *Brucella* ABC-transporters.

We have recently identified and located in the large chromosome I the *B. abortus* cyclic β-1,2-glucans transporter *(cgt)* (Roset *et al.,* unpublished). *B. abortus* Cgt is an ABC-transporter that complemented *S. meliloti* nodule development *(ndvA)* and *A. tumefaciens* chromosomal virulence *(chvA)* mutants. Both mutants recovered periplasmatic cyclic β-1,2-glucan transport, motility and tumours induction in *A. tumefaciens* or nodule occupancy in *S. meliloti*. All traits strictly associated with the presence of cyclic β-1,2-glucan in the periplasm (Roset *et al.,* unpublished). Nucleotide sequencing revealed that *B. abortus cgt* gene is a 1,797 bp ORF coding for a predicted

membrane protein of 599 amino acid residues (65.9 kDa), 58.5 and 59.9 % identical to *S. meliloti* NdvA and *A. tumefaciens* ChvA, respectively. Additionally, *B. abortus cgt*, like *S. meliloti* and *A. tumefaciens ndvA/ chvA*, possesses ATP-binding motifs and the ABC signature domain features of

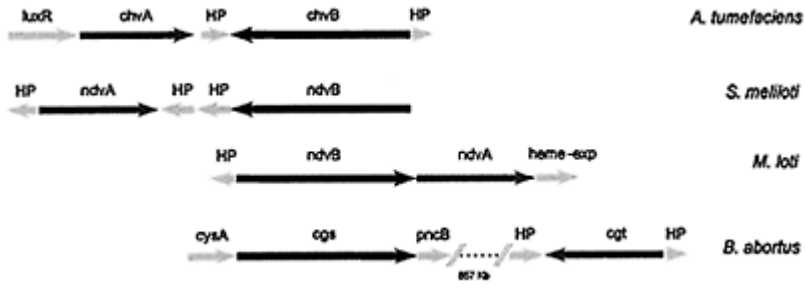

Figure 2. Genomic organisation of cyclic β-1,2-Glucan synthetase (Cgs) and cyclic β-1,2-transporter (Cgt) in different organisms. *chvB/ndvB:* chromosomal virulence/nodule development B equivalent to *Brucella cgs. chvA/ndvA:* chromosomal virulence/nodule development A equivalent to *Brucella cgt*. HP: hypothetical protein.

typical ABC-transporters. Confirmation that Cgt is indeed the *B. abortus* cyclic β-1,2-glucan transporter was obtained by constructing null mutants of *B. abortus 2308* and *819* strains. In both backgrounds the mutants do not transport cyclic β-1,2-glucan to the periplasm, display reduced virulence in mice and defective intracellular multiplication in HeLa cells. These results strongly suggest that cyclic β-1,2-glucan must be transported into the periplasmatic space to exert its action as virulence factor (Roset *et al.*, unpublished).

6. *B. abortus* Genome Organization of the Genes Encoding the Proteins Involved in Cyclic β-1,2-Glucan Synthesis and Secretion

Interestingly the structural genes encoding the proteins for the synthesis *(chvB/ ndvB)* and secretion *(chvA/ndvA)* of cyclic β-1,2-glucan in *Agrobacterium* and *Rhizobium* form a cluster in the chromosome transcribed in a convergent fashion (Figure 2). *Cgs* and *cgt* are the *Brucella* equivalent of *chvB/ndvB* and *chvA/ndvA* respectively. Complete genome sequence of *B. abortus, B. melitensis* and *B. suis* revealed that contrary to what happens in *Sinorhizobium, Mesorhizobium* and *Agrobacterium,* in which *cgs* and *cgt* are

contiguous, *Brucellae cgs* and *cgt* are both located in chromosome I but distant from each other by 858.86 kb (Figure 2). Thus *Brucellae cgs* and *cgt* genes were either acquired independently or have suffered a severe genome rearrangement during evolution.

7. Pleiotropic Phenotype of Cyclic Glucan Mutants

Besides being defective in cyclic β-l,2-glucan synthesis, *chvB* and *ndvB* mutants have also some other membrane associated phenotypes, as for example sensitivity to detergents, antibiotics and peptides, defective assembly of flagella and Type IV secretion system, etc. In *Rhizobium* and *Agrobacterium,* a strict correlation was observed between synthesis or secretion of cyclic β-1,2-glucan with flagella assembly and motility (Douglas *et al.,* 1982; Geremia *et al.,* 1987; Roset *et al.,* unpublished). In *A. tumefaciens,* export of the oncogenic T-DNA is mediated by the products of the *virB* operon. It has been reported (Fullner and Nester, 1996) that DNA transfer from *Agrobacterium* to plant cells does not occur at elevated temperatures, observation that correlates well with much earlier studies on the temperature sensitivity of crown gall tumour development on plants. It was described that steady-state levels of VirB10 are sensitive to growth temperature while levels of several other VirB proteins are considerably less affected. This temperature-dependent failure to accumulate VirB10 is exacerbated in cyclic β-l,2-glucan *chvB* mutant. Moreover, the virulence of *chvB* mutants can be partially restored by lowering the temperature at which the bacteria and the plant tissue are co-cultivated (Banta *et al.,* 1998). These results suggest that cyclic β-1,2-glucan may play a role either in the stabilisation and/or assemble of VirB membrane macromolecular complex, similar to the effect observed on flagella assembly. *Cgs* mutants were obtained from *B. abortus* strain 2308 and S19. Both mutants showed greater sensitivity to surfactants like deoxycholic acid, sodium dodecyl sulphate, and Zwittergent than the parental strains, suggesting cell surface alterations. According to these results it can not be ruled that cyclic β-1,2-glucans play a role in the stabilisation and or assemble of membrane proteins that, in turn, may be required for effective *Brucella* cell interaction.

8. Osmoregulation of Cyclic Glucan Accumulation

It has been described that, as is the case with the membrane-derived oligosaccharide in *E. coli* (Kennedy, 1982), the synthesis *in vivo* of cyclic β-1,2-glucan is osmoregulated in *Agrobacterium* and *Rhizobium* (Miller *et al.,* 1986; Breedveld *et al.,* 1990; Dylan *et al.,* 1990; Zorreguieta *et al.,* 1990). It was proposed that cyclic β-1,2-glucans may contribute to the osmolarity of the periplasmic space in hypo-osmotic environment, equilibrating the cytoplasmic and periplasmic osmotic pressure. *A. tumefaciens* and *S. meliloti* mutants unable to form cyclic β-1,2-glucan do not grow normally in low-osmolarity media (Zorreguieta *et al.,* 1990), thus suggesting that in *Rhizobiacea* periplasmic cyclic β-1,2-glucans play a general role during hypo-osmotic adaptation. Contrary to what happens in most species of the genera *Agrobacterium* and *Rhizobium,* the accumulation of cellular glucans in *Brucellae* is not osmotically regulated (Briones *et al.,* 1997).

Osmoregulation of glucan synthesis can be achieved through changing the level of gene expression and/or at the level of glucosyl-transferase activity. Cgs, the enzyme responsible for cyclic β-l,2-glucan synthesis, is present constitutively, suggesting that osmotic regulation of glucan biosynthesis occurs through modulation of the enzymatic activity. Studies *in vitro* performed with crude membrane preparations showed that *Brucellae* cyclic β-1,2-glucan synthetase activity was not inhibited by 0.5 M KCl or potassium glutamate, concentrations that completely inhibited the osmosensitive enzymes of *A. tumefaciens* A348 and *R. meliloti* 102F34 strains (Iñón de Iannino *et al.,* 2000). *B. abortus* Cgs, expressed in the *A. tumefaciens* A1011 *chvB* or R. *meliloti GRT21s ndvB* mutants backgrounds, restored the synthesis of cyclic β-1,2-glucan, but in the recombinant strains the accumulation of cyclic glucan was no longer inhibited when cultured in media of high osmolarity (0.25 M NaCl or 0.5 M mannitol), showing that osmoregulation of glucan synthesis depends on the modulation of Cgs enzymatic activity. Cgs amino acid sequence is highly conserved between *Agrobacterium*, *Rhizobium* and *Brucella;* however, subtle not yet identified differences, may account for the different behaviour under high osmolarity (Briones *et al.*, 1997; Iñón de Iannino *et al.,* 2000).

When the *A. tumefaciens* cyclic β-1,2-glucan synthetase gene was expressed in the *R. meliloti* GRT21s *ndvB* mutant background, the recombinant strain displayed marked inhibition of cyclic β-l,2-glucan synthesis when grown in high-osmolarity media. However, when this gene was introduced in a *B. abortus cgs* mutant background no inhibition of glucan synthesis at high osmolarity was observed. *In vitro* studies with crude membranes isolated from recombinant strains revealed that *Brucella* Cgs was not inhibited by high concentrations of KCl or K-glutamate even when expressed in *Agrobacterium* or *Rhizobium* backgrounds. It was concluded that in *Brucella* the regulation of cyclic β-1,2-glucan synthesis at high osmolarity is due to convergent mechanisms: i) a cyclic β-1,2-glucan synthetase not affected by high concentration of osmolytes such as KCl or K-glutamate; ii) the accumulation at high osmolarity of compatible solutes that might protect the enzyme from the inhibition by osmolytes; and iii) the accumulation of osmolytes other than KCl or K-glutamate with no effect on Cgs activity.

9. Virulence and Cyclic Glucan

A number of genes involved in virulence and symbiosis in *Agrobacterium* and *Rhizobium* are highly conserved in *Brucellae*. Interestingly a number of them are membrane bound proteins or multi-component membrane associated complexes, involved either in sensing or delivering extracellular signals. For example: i) the conservation between *Agrobacterium* and *Brucella* of almost the entire VirB operon. (Sieira *et al.,* 2000); ii) the *S. meliloti* Chvl/ExoS and *A. tumefaciens* Chvl/ChvG two-component sensor response regulators with *B. abortus* BvrR/BvrS (Sola-Landa *et al.,* 1998; see also Chapter 10), and iii) the *S. meliloti* and *B. abortus* BacA (LeVier *et al.,* 2000). All these genes play key roles in virulence and or symbiosis, thus suggesting a convergent evolution of genes and or functions for successful host interaction between a plant and an animal pathogen.

Cgs the enzyme responsible for cyclic glucan synthesis is another interesting example of a highly conserved gene between *Agrobacterium*, *Rhizobium* and *Brucella*. *B. abortus*

cgs gene expressed under its own promoter restored the synthesis of cyclic glucan of *S. meliloti ndvB* and *A. tumefaciens chvB* mutants. Moreover nodule development or tumour induction and assembly of flagella two phenotypes associated to the absence of cyclic glucan were also restored thus indicating that all the functions assigned to *S. meliloti* NdvB and *A. tumefaciens* ChvB are highly conserved in *B. abortus* Cgs. As described above, contrary to what happens in *Agrobacterium* and some *Rhizobium* species, the accumulation of cellular cyclic β-l,2-glucan in *Brucella* is not osmoregulated (Briones *et al.,* 1997). It seems that *Brucella* evolved to produce cyclic β-1,2-glucans event under high extracellular osmotic pressure.

Brucella cyclic β-1,2-glucan mutants displayed reduced virulence in mice and defective intracellular trafficking (Briones *et al.,* 2001; Briones *et al.,* unpublished). This phenotype is associated with the absence of glucan since site directed mutagenesis that yielded an inactive protein correctly inserted in the membrane displayed the same reduced virulence as the null mutants (Ciocchini *et al.,* unpublished). It remains to be established if the presence of cyclic glucan exerts its action directly or through the stabilisation and/or facilitation of the assembly of membrane macromolecular systems. Detailed analysis of the membrane architecture of *Brucella cgs* mutants would help to answer this question. It is interesting to remark that *B. abortus* strain 2308 *cgs* mutants have reduced but not abolished virulence. On the other hand, the reduction of virulence is more pronounced in the already partially avirulent vaccine strain *B. abortus* S19 (Briones *et al.,* 2001). Both mutants showed greater sensitivity to surfactants like deoxycholic acid, sodium dodecyl sulphate, and Zwittergent than the parental strains, suggesting cell surface alterations. Although not to the same extent, both mutants display reduced virulence in mice and defective intracellular multiplication in HeLa cells. The *B. abortus* S19 *cgs* mutant was completely cleared from the spleens of mice after 4 weeks, while the 2308 mutant showed a 1.5 log reduction of the number of brucellae isolated from the spleens after 12 weeks. These results suggest that cyclic β-1,2-glucans play an important role in the residual virulence of the attenuated *B. abortus* S19 strain. Although the *cgs* mutant was cleared from the spleens earlier than the wild type parental strain (*B. abortus* S19) and produced less inflammatory response, its ability to confer protection against the virulent strain *B. abortus* 2308 was fully retained. Equivalent levels of induction of spleen gamma interferon mRNA and anti-LPS immunoglobulin G2a (IgG2a) subtype antibodies were observed in mice injected with *B. abortus* S19 or the *cgs* mutant. However, the titter of anti-LPS antibodies of the IgG1 subtype induced by the *cgs* mutant was lower than that observed with the parental S19 strain, thus suggesting that the *cgs* mutant induces a relatively exclusive Th1 response (Briones *et al.,* 2001).

The intracellular behaviour and trafficking of *cgs* mutants in HeLa cell is subtly affected as it is shown in Figures 3 and 4 (Briones *et al.,* unpublished). It can be observed in Figure 3 that at different post infection time the percentage of infected cells and the number of *Brucella* per cell is diminished in the *cgs* mutant. On the other hand, co-localization of LAMP2 and cathepsin markers with *Brucella*-containing-fagosomes revealed that cells infected with the *cgs* mutant have a higher percentage of LAMP2 and cathepsin positive fagosomes 24 h post infection (Figure 4). These results suggest that cyclic glucan plays a role preventing fusion between early endosome with the phagosome and facilitates reaching the ER replication niche. If this action is exerted by periplasmic

cyclic glucan itself or indirectly by stabilising other bacterial membrane structures remains to be established.

Cgs is a 300 kDa membrane protein, encoded by a 9 kb gene, that uses UDP-glucose to produce the cyclic glucan that may account for about 15% of the cells wet weight. There is no evidence that this glucan may be used as carbon or energy storage product. Which is then the selective pressure that drives the conservation of such a large protein that diverts energy into an apparently useless storage polysaccharide? Probably the best interpretation is that *Brucella* cyclic glucan may play a role outside the host cells under field condition, as in *Agrobacterium* and *Rhizobium* in which the absence of cyclic glucan has a more stringent phenotype. It is tempting to speculate that soil bacteria like *Agrobacterium* and *Rhizobium*

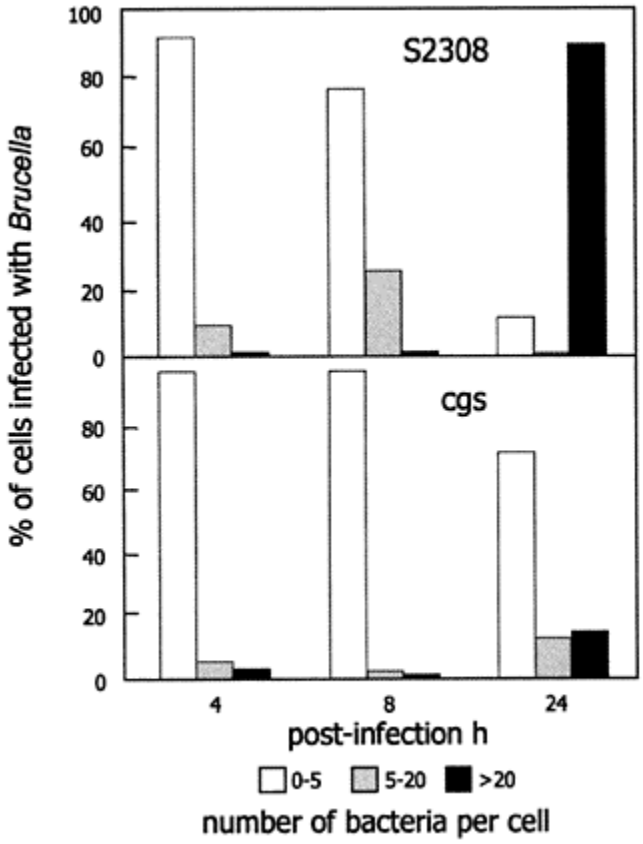

Figure 3. Infection of HeLa cells with *B. abortus* wild type strain 2308 and *cgs* mutant. Quantitation of 2308 or *cgs* mutant-infected cells with 0 to 5 bacteria (open bars), with 5 to 20 (light

grey bars), with more than 20 (solid dark bars) at 4, 8 and 24 h after infection. *B. abortus* was detected with an O-antigen monoclonal antibody developed with FICT donkey anti-mouse antibody.

are exposed to stronger environmental constriction than *Brucella* during their interaction with the host since they are always exposed to soil condition while *Brucella* may be transmitted from host to host. However no studies have been carried out so far to determine the role of cyclic glucan in the survival of *Brucella* outside the animal cells or during natural transmission between hosts.

10. Conclusions

B. abortus produces and secretes into the periplasmic space cyclic β-1,2-glucan. The enzymes involved in the synthesis (Cgs) and secretion (Cgt) are highly conserved in other members of the a Proteobacteria group such as *Sinorhizobium, Mesorhizobium* and *Agrobacterium,* in all of which periplasmatic cyclic β-1,2-glucan is required for successful bacteria plant interaction either as endosymbiont or pathogen. The conserved functions correlate with a high degree of protein sequence conservation. Complete genome sequences revealed that *Agrobacterium, Sinorhizobium, Mesorhizobium* and *Brucella* display a high degree of genome conservation and gene synteny. However, contrary to what happens

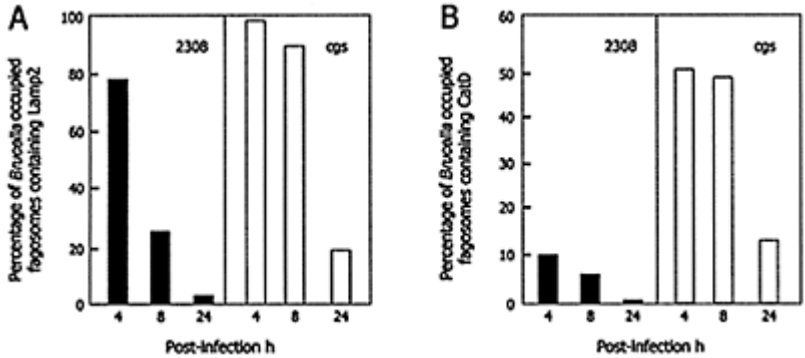

Figure 4. Kinetics of acquisition of LAMP1 (A) and cathepsin D (B) markers by vacuoles containing *Brucella*. HeLa cells were infected with the virulent wild type *B. abortus*

strain 2308 or a null *cgs* mutant. At the indicated post-infection time cells were processed for double indirect immuno-fluorescence microscopy.

in *Agrobacterium*, *Sinorhizobium* and *Mesorhizobium*, in which *cgs* and *cgt* are contiguous, *Brucella cgs* and *cgt* genes map distant from each other by more than 800 kb, thus suggesting that either they were acquired independent or that a severe genome rearrangement has occurred.

B. abortus cgs and *cgt* mutants display very similar phenotype with reduced virulence in mice and defective intracellular multiplication in HeLa cells, thus suggesting that cyclic glucan must be synthesise and secreted into the periplasm to exert its action as virulence factor. However further work is required to rule out that modification of cyclic glucan, a process that takes place in the periplasm, has any effect on virulence and intracellular multiplication. It is not known if Cgs and Cgt interact each other in the inner membrane, however indirect evidences suggested that this is the case. For example, the degree of polymerisation and the total amount of cyclic glucans produced by *A. tumefaciens cgt* mutants is different than that of wild type strain. It was also observed that the pattern of trypsin digestion of membrane Cgs changed in a *cgt* null mutant background (Iñón de Iannino and Ugalde, 1989). Further work is required however, to reveal how these two proteins interact each other during the process of synthesis and secretion of cyclic β-l,2-glucan.

The mechanism of action of cyclic glucan is not clear. However, several evidences suggest that periplasmatic cyclic β-1,2-glucan may have a role on the assembly and or stabilisation of macromolecular membrane components such as flagella, outer membrane proteins, Type IV secretion VirB complex, etc. Detailed study of membrane architecture of wild type and cyclic β-1,2-glucan mutants may shed light on the mechanism of action of this unique polysaccharide.

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Chapter 10
The *Brucella* BvrS/BvrR and Related Two-Component Regulatory Systems of the α-2 Proteobacteria: Common Regulatory Strategies of Animal and Plant Pathogens and Endosymbionts

Ignacio López-Goñi*, Lorea Manterola and Shen Q.Pan

From: *Brucella:* Molecular and Cellular Biology. Edited by: Ignacio López-Goñi and Ignacio Moriyón

Abstract

The α-2 Proteobacteria include pathogenic intracellular and extracellular bacteria of animals and plants as well as plant endosymbionts sharing conserved two-component regulatory systems. *Brucella* BvrS/BvrR is similar to the ChvG(ExoS)/ ChvI of *Agrobacterium* and *Sinorhizobium*, and to the BatS/BatR putative system of *Bartonella*. Moreover, the structure around the genes encoding these systems is essentially the same. Dysfunction of these systems alters the cell envelopes: *bvrS*/ *bvrR* mutants display increased surface hydrophobicity, sensitivity to bactericidal peptides and detergents, and altered lipid A acylation; *chvG* mutants are sensitive to detergents and acidic pH. Moreover, BvrS/BvrR regulates transcription of at least Omp25 and Omp22, ChvG/ChvI regulates the acidic pH-inducible outer membrane protein Aop (and also genes *katA*, *virB* and *virE*), and *S. meliloti* ExoS regulates the production of succinoglycan. These systems are critical in the interaction with eukaryotic cells: *bvrS* and *bvrR* mutants are avirulent in mice, sensitive to serum, and hampered in cell penetration and intracellular trafficking; *chvG* and *chvI* mutants lack tumour inducing ability; and *S. meliloti* succinoglycan-deficient mutants cannot establish symbiosis. These similarities suggest that these systems have a common ancestor that, while evolving to sense pericellular or intracellular signals, has kept the control

of cell surface characteristics. Moreover, they suggest a co-evolution of the systems and the cognate surface molecules.

1. Introduction

Bacteria experience a multitude of environmental conditions in the complex niches they inhabit. The ability to respond to environmental stimuli is especially important for plant and animal-associated bacteria that compete with other organisms both in the open environment and in the host. Many two-component sensory transduction systems operate and control complex regulatory networks in plant and animal pathogens (Hoch and Silhavy, 1995). These signalling pathways

* For correspondence email ilgoni@unav.es

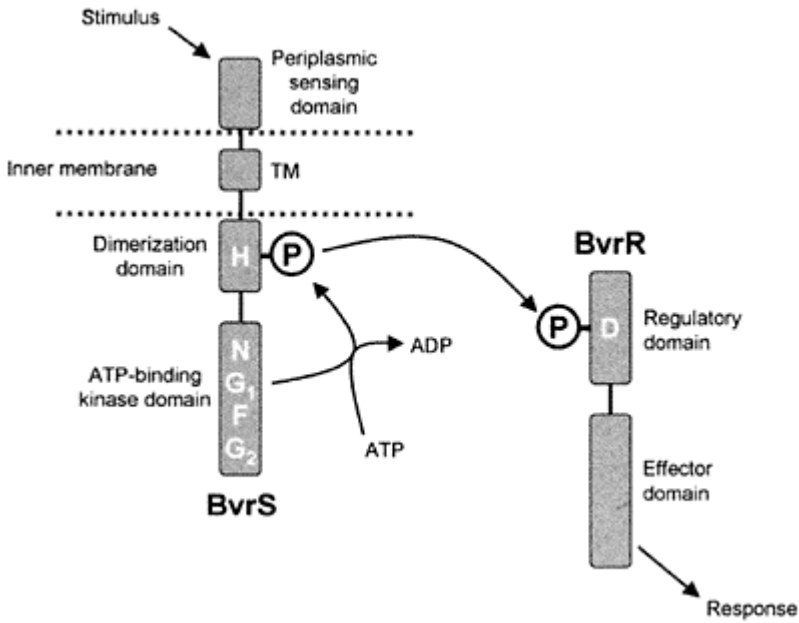

Figure 1. *Brucella* BvrS/BvrR two-component regulatory system. Like typical two-component systems, BvrS/BvrR consists of a dimeric transmembrane sensor BvrS and a cytoplasmic BvrR. A monomer of a BvrS is shown with transmembrane segments indicated as TM. Conserved

sequence motifs N, G1, F and G2 located in the ATP-binding kinase domain are shown. BvrS catalyses ATP-dependent autophosphorylation of a well conserved His residue (H, located in the dimerisation domain). The activities of BvrS are modulated by an unidentified environmental stimulus. The phosphoryl group (P) is then transferred to a specific Asp residue (D), located in the regulatory domain in BvrR. Phosphorylation of BvrR activates the effector domain (with a DNA-recognition region), which produce an specific cellular response.

are structured around two conserved proteins: a membrane bound sensor histidine protein kinase that is phosphorylated at histidine (His) residue, and a cytoplasmic response regulator protein phosphorylated at aspartate (Asp) residue. Histidine protein kinase recognises a stimulus from the environment and transfers a high-energy phosphoryl group to the response regulator protein, which is often a transcriptional factor. This results first in its activation and then in the regulation of genes necessary for several cellular functions. Many of these functions are critical including cell division, sporulation, chemotaxis, metabolism, transport, response to stress, and expression of toxins and other proteins important for pathogenesis (Hoch and Silhavy, 1995).

2. *Brucella* BvrS/BvrR Two-Component Regulatory System

Facultative intracellular bacteria such as *Brucella* must survive in varied and changing conditions ranging from the open environment to the intracellular medium. For this, the bacterium must co-ordinate an intricate network of factors to generate a suitable adaptive response to the various signals. The *B. melitensis* genome encodes 19 predicted histidine protein kinases, 21 predicted response regulator proteins and 2 hybrids (see Chapter 7). However, few of these have been characterised. At the moment BvrS/BvrR is the best characterised two-component regulatory system of *Brucella* (Sola-Landa *et al.*, 1998; Guzmán-Verri *et al.*, 2002; López-Goñi *et al.*, 2002). It was first identified in *B. abortus* as controlling the natural resistance of *Brucella* to bactericidal cationic peptides that disrupt the cell envelope of most Gram negative and Gram positive bacteria. BvrS/BvrR belongs to classical two-component regulatory systems that consist of a single His-to-Asp phosphotransfer event between a histidine kinase and a response regulator. BvrR is a cytoplasmic protein that shows significant similarity to OmpR/PhoB subfamily of

response regulator proteins with a specific Asp residue located within a conserved regulatory domain and an effector domain with DNA-binding activity (López-Goñi *et al.*, 2002). BvrS is a membrane-bound homodimeric protein that has three conserved regions with homologs in members of the histidine protein kinase superfamily: an amino-terminal periplasmic sensing domain with transmembrane segments, a cytoplasmic dimerisation domain with a specific His residue, and the carboxy-terminal ATP-binding kinase domain with a set of conserved primary sequence motifs designated the N, G1, F and G2 boxes (López-Goñi *et al.*, 2002; see also Chapter 7). Therefore, by homology with prototypical two-component systems, BvrS/BvrR should work as follows. A still unidentified environmental stimulus, detected by the sensor domain of BvrS, regulates the histidine kinase activity. Upon activation, BvrS catalyses the ATP-dependent autophosphorylation of the specific His within its dimerisation domain. BvrR then catalyses the transfer of the phosphoryl group from the phospho-His of BvrS to one of its own Asp residues. Phosphorylation of the conserved regulatory domain of BvrR activates the effector domain that elicits the specific output response (Figure 1).

After the description of the *B. abortus* BvrR/BvrS, the analysis of the genomes of *B. suis* and *B. melitensis* demonstrated its presence in these species with only few amino acid differences (López-Goñi *et al.*, 2002). In addition, the corresponding genes can be detected by PCR in all biovars of the classical *Brucella* species and in the strains isolated from marine mammals. This indicates that this two-component system is highly conserved in *Brucella* (I.López-Goñi, unpublished).

3. The Pleiotropic Control of the BvrS/BvrR and its Role in Host-Interaction

As demonstrated for other two-component systems, multiple genes should be under the control of BvrS/BvrR. *B. abortus* mutants in this system were more susceptible to bactericidal polycationic substances like polymyxin B, melittin or poly-L-lysine, and displayed a more hydrophobic outer membrane surface than the parental strain (Sola-Landa *et al.*, 1998; Sola-Landa, 2000). This evidence suggests an altered outer membrane structure. There is an additional clue on the activities in which the system may be involved. In complex media like tryptic soy broth, *B. abortus bvrS/bvrR* mutants exhibit a growth similar to that of the parental strain. However, the growth of the *bvrR* mutant is slightly inhibited in minimal media with different carbon and nitrogen sources (Table 1), suggesting a linkage between the two-component regulatory system BvrS/BvrR and nitrogen and carbon utilisation (Manterola, 2004).

As mentioned above, the first observations suggested that the BvrS/BvrR system somehow modulate the structure of the *B. abortus* outer membrane. Later

Table 1. Growth of parental strain *B. abortus* 2308 and a *bvrR* mutant in defined media with different carbon and nitrogen sources[1]

Strain	Glucose				Piruvate			
	–	$(NH_4)_2SO_4$	Glutamic	$(NH_4)_2SO_4$ Glutamic	–	$(NH4)_2SO_4$	Glutamic	$(NH_4)_2SO_4$ Glutamic
2308	++	++	+++	+++	++	+++	+++	+++
bvrR::Tn5	–	–	++	+++	–	+++	+++	++++

1. Minimal media contained 0.1% glucose or 3% piruvate as carbon source plus the proportion of mineral salts and vitamins described by Plommet (1991). Nitrogen was added as 0.05% $(NH_4)_2SO_4$ and/or as 1% glutamic acid. Growth was measured spectrophotometrically in a Labsystems Bioscreen C apparatus after 3 days of incubation at 37°C and 200 r.p.m., and expressed semi-quantitatively (++++, maximal growth; –no growth).

studies demonstrated that dysfunction of *bvrS* and *bvrR* altered the profile of *B. abortus* group 3 outer membrane proteins (Omps) (Guzmán-Verri *et al.*, 2002). Probing the surface of both mutants with monoclonal antibodies showed the presence of all Omps described so far (the lipoproteins Omp10, Omp16 and Omp19, the Omp2b porin and Omp1) but Omp25. Two-dimensional gel electrophoresis of envelope fractions confirmed the absence of Omp25 in the *bvrS/bvrR* mutants, and the *omp25* promoter activity was also dramatically decreased in both *bvrS* and *bvrR* mutants as compared to that of the wild type strain. The electrophoretic analysis also revealed reduction or absence of a second set of protein spots. By peptide mass mapping, this spots corresponded to a previously unknown Omp of 22 kDa (Omp3b or Omp22), and further studies demonstrated that expression of Omp22 was transcriptionally regulated by the two-component system BvrS/BvrR. The two-dimensional electrophoretic analysis of outer membrane fractions from the mutant in the sensor showed a decrease in one protein spot (27 kDa and pI=5.1, approximately) and an increase in other protein spots (one with 22 kDa and pI=5.1, and two with 26 kDa and pI=9.1, approximately) (Guzmán-Verri *et al.*, 2002). Using the *B. abortus* Omp25 and Omp22 sequences, a search for homologous genes in the genome of *B. melitensis* was carried out. This search revealed seven proteins with calculated mass of 22–34 kDa and with pI from 4.6 to 9.2 (Guzmán-Verri *et al.*, 2002), all of them new members of the group 3 Omps. Recently, Salhi *et al.* (2003) have classified these seven Omps in four-subgroups based on their amino acid sequence identities: Omp25, Omp22 (Omp3b), the Omp25b-Omp25c-Omp25d cluster, and the Omp31-Omp31b subgroup. Moreover, these authors have demonstrated that all these new members of group 3 Omps are expressed in *B. suis* and in other *Brucella* species. Although experimental evidence is lacking, the above-mentioned two-dimensional electrophoretic analysis suggests that BvrS/ BvrR system might also regulate the expression of some of these new Omps.

Recent results have demonstrated that *B. abortus* knock-out mutants at *omp25* or *omp22* genes are not as attenuated or sensitive to bactericidal peptides as the *bvrS* and

bvrR mutants (Manterola *et al.,* 2003a). This indicates that additional structures may be under the regulation of the BvrS/BvrR system. To test this possibility, several cell envelope fractions were analysed. No quantitative differences were found in periplasmic cyclic 1,2-β-glucans, lipopolysaccharide (LPS) or native hapten polysaccharide content, or in free lipid profiles. By SDS-PAGE and ^{13}C-NMR, no differences were found in LPS O-polysaccharide polymerisation and in the sugar linkage pattern. Despite this, there might be some qualitative differences in the LPS, as suggested by three sets of observations. First, *bvrS* mutant cells bearing the parental LPS artificially inserted in the their outer membranes showed increased polymyxin B resistance. Conversely, parental cells bearing the LPS of the *bvrS* mutant displayed increased sensitivity, and by electron microscopy, the envelope morphological alterations associated with the action of this peptide (Manterola *et al.,* 2003b; E.Moreno, unpublished). Second, probing the LPS of *bvrR* and *bvrS* mutants with dansylated polymyxin suggested increased (or anomalous) binding of the polycation. Third, polymyxin B had an acyl-chain fluidifying effect on the LPS of the two-component system mutants which is stronger than on the wild type LPS (Manterola *et al.,* 2003b; I.López-Goñi, unpublished). Consistent with these three pieces of evidence, there was an increase in underacylated forms in the lipid A of the *bvrR* and *bvrS* mutants (Manterola *et al.,* 2003b; I.López-Goñi, unpublished). A study of the degree of acyl-chain fluidity of the LPS of the mutants and the parental stains performed by Fourier-transformed infrared spectroscopy was also performed (Manterola *et al.,* 2003b). The results showed that the vibration of the lipid A acyl-chains was increased in the LPS of the mutants, as expected for LPS with underacylated lipid A.

Modification of the LPS molecules appears to be a common mechanism used by Gram negative bacteria for adapting to different environments. For instance, *Salmonella* spp. modify the LPS in response to host signals (Soncini and Groisman, 1996). This phenomenon is mediated by the PhoP/PhoQ and PmrA/PmrB systems, a two-component regulatory network that controls over 40 genes, some of which have been implicated in virulence (Miller *et al.,* 1989, 1990). Among the most relevant are the lipid A and LPS core modifications, including the addition of amino-arabinose or phosphoethanolamine to the diglucosamine backbone, replacement of myristate with 2-OH myristate, and the formation of hepta-acylated lipid A by addition of palmitate (Vaara *et al.,* 1979; Helander *et al.,* 1994; Gunn and Miller, 1996; Guo *et al.,* 1997, 1998; Gunn *et al.,* 1998; Zhou *et al.,* 2001; and see also Chapter 8). Guo *et al.* (1998) have proposed that an increased acylation of lipid A alters the fluidity of the outer membrane by augmenting the hydrophobic interactions among the lipid A acyl tails. A consequence of this might be an increased resistance to bactericidal cationic peptides, as these elements of the innate immune have to penetrate through the outer membrane to act. Indeed, the *B. abortus bvrS/bvrR* mutants are more sensitive to bactericidal peptides and surfactants (Sola-Landa *et al.,* 1998), and increase lipid A underacylation and LPS acyl-chain fluidity. Presently, it is not known whether such LPS changes result from a true regulation by BvrS/BvrR or from a pleiotropic effect caused by the absence of some group 3 Omps. However, these alterations in lipid A could contribute to the lack of virulence of the *bvrS/bvrR* mutants.

Activation of the serum complement cascade by bacteria and their components in the absence of specific antibodies is part of the innate immune system. In contrast to the parental strain, the *bvrR* and *bvrS* mutants were highly sensitive

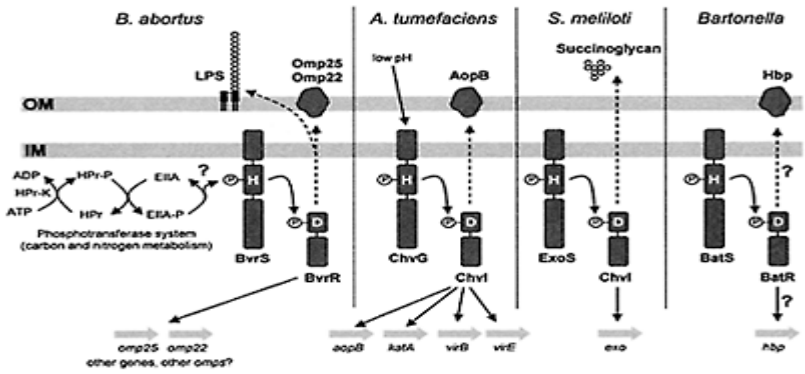

Figure 2. Model proposed to describe the BvrS/BvrR-homologous two-component regulatory systems in animal and plant-associated bacteria. In *B. abortus*, BvrS/BvrR regulates the transcription of at least two Omps and the structure of the lipid A of the LPS. Based on the comparison of the gene arrangements, a link between the phosphotransferase system and the two-component regulatory system is proposed (*see also* Chapter 7). In *A. tumefaciens*, ChvG is a global pH sensor and ChvG/ChvI regulates some acid pH-inducible genes like *aopB*, *katA*, *virB* and *virE*. AopB is an Omp involved in tumorigenesis of *A. tumefaciens*. In *S. meliloti*, ExoS/ChvI controls the production of succinoglycan, an exopolysaccharide which plays a crucial role in the establishment of the symbiosis with alfalfa. The model proposes that the *Bartonella* BatS/BatR system probably regulates the hemin-binding proteins (Hbp), a family of outer membrane

proteins. OM, outer membrane; IM, inner membrane.

to bovine and human normal serum (Manterola, *et al.,* 2003b) and eliminated by post infection day 12 from mouse spleens (Sola-Landa *et al.,* 1998). In addition, whereas the parental strain replicates within professional and non-professional phagocytes, none of these mutants multiplies in murine macrophages (peritoneal and Raw264.7 cells) or in HeLa cells. In agreement with this observations, *B. suis bvrR* mutants have been isolated in a human macrophage THP-1 survival screening test (Köhler *et al.,* 2002).

Electron and fluorescent microscopy clearly showed that mutation on *bvrR* or *bvrS* genes hampered both the penetration of *B. abortus* into cells and the control by the bacterium of intracellular trafficking and replication (Sola-Landa *et al.,* 1998). Further studies have contributed to clarify this picture. For instance, in contrast to wild type *B. abortus,* at least the *bvrS* mutant did not recruit the small GTPases of the Rho subfamily required for actin polymerisation and penetration to cells (Guzmán-Verri *et al.,* 2001; see also Chapter 14). Likewise, although the *bvrS* mutant bound to cells in higher numbers, the Cdc42 GTPase was directly activated only by the parental strain. This observation indicates that the BvrS/BvrR system was required for recruiting those molecular determinants necessary for invasion (Guzmán-Verri *et al.,* 2001). Mutants in the two-component system internalised by macrophages readily destroyed within phagolysosomes (Sola-Landa *et al.,* 1998). Similarly, when they were ingested by epithelial cells displaying phagocytic phenotype by intoxication with CNF, these mutants were also destroyed within lysosomal compartments (Guzmán-Verri *et al.,* 2001). In contrast, wild type *Brucella* pursued its intracellular trafficking from early vacuolar compartments to autophagosomes and finally to the endoplasmic reticulum, where it extensively replicates (see Chapter 14). This means that, once inside the cells, mutants in the BvrS/BvrR system are incapable of inhibiting lysosome fusion, indicating that this two-component system is involved not only in cell invasion but also in controlling vacuole maturation and intracellular trafficking.

4. ChvG/ChvI in Plant-Associated *Agrobacterium* and *Sinorhizobium*

The *Brucella* BvrS/BvrR proteins possess a high level of identity with other two-component systems present in cell associated α-2 Proteobacteria which are also necessary for endosymbiosis and bacterial parasitism (Figure 2). For instance, BvrS/BvrR has strong homology to the *Agrobacterium tumefaciens* ChvI/ChvG system. *A. tumefaciens* is a Gram negative plant pathogen that infects a wide range of plants by causing crown gall tumours. The bacterial infection occurs at plant wound sites and involves the transfer of oncogenic DNA (T-DNA) from the bacterium into the plant nucleus. The T-DNA and the virulence *(vir)* genes required for T-DNA transfer are located on a large plasmid called tumour-inducing (Ti) plasmid. In addition, some chromosomal genes are involved in the infection process (for reviews see Gelvin, 2000; Zhu *et al.,* 2000; Zupan *et al.,* 2000). Sequencing of the *A. tumefaciens* genome has revealed at least 25 two-component pathways (Goodner *et al.* 2001; Wood *et al.,* 2001). The two best studied are VirA/VirG and ChvI/ChvG (Charles *et al.,* 1992; Winans, 1992; Winans *et al.,* 1994). The

VirA/VirG system controls the induction of the Ti-plasmid-harbored *vir* genes, which in turn are responsible for the processing and transfer of the T-DNA. In an attempt to identify additional chromosomal virulence genes, Cangelosi *et al.* (1991) used *TnphoA* to generate insertion mutations in genes that encode proteins with extracytoplasmic domains, because certain virulence determinants might be associated with the cell envelope. The resulting insertion mutants were screened for virulence and one of the avirulent mutants was selected for further characterisation. Charles and Nester (1993) found that the mutation occurred at a gene encoding a putative histidine sensor protein kinase which was designated *chvG* and its cognate response regulator *chvI* was identified through sequencing of the region. The same locus encoding the two-component regulatory system *chvG/chvI* was identified independently by Mantis and Winans (1993) when an *E. coli phoB* mutant was complemented with members of an *Agrobacterium* clone bank.

It was previously observed that *A. tumefaciens chvG* mutants were much more sensitive to detergents, antibiotics and low pH than the wild type strain (Charles and Nester, 1993). This suggested that the permeability of the cell envelope was altered in the *chvG/chvI* mutants. Since the lack of the membrane component of the ChvG/ChvI signal transduction apparatus alone was unlikely to result in gross cell envelope alterations, it was assumed that this system was involved in the regulation of at least one other cell envelope gene. Experimental data gathered so far supported this hypothesis (see below).

It is known that pH plays an important role in *Agrobacterium*-plant interactions. Expression of *vir* genes, controlled primarily through the VirA/VirG system, depends on external acidification (Winans, 1992), and is directly responsible for the virulence of *A. tumefaciens* to cause tumours on plants. In addition, there are other acid inducible genes residing on *A. tumefaciens* chromosomes. Two chromosomal genes involved in *Agrobacterium* tumourigenesis and inducible by acidic pH have been identified: *aopB,* located on the circular chromosome, and *katA* located on the linear chromosome (Xu and Pan, 2000; Jia et al., 2002). The latter gene encodes a catalase involved in detoxification of H_2O_2 released during *Agrobacterium*-plant interaction, and *aopB* encodes an Omp exposed on the bacterial cell surface. Since ChvG was a sensor protein and *chvG* mutants were highly sensitive to acidic pH (Charles and Nester, 1993), Li *et al.* (2002) suspected that ChvG/ChvI might be involved in the regulation of acidic pH-inducible genes. Based on their results (see below), they proposed that ChvG is a global sensor protein in *Agrobacterium* that can directly or indirectly sense acidity (Li *et al.,* 2002).

To determine whether ChvG is involved in the regulation of *aopB* and *katA,* the expression of *aopB::gfp* and *katA::gfp* fusions was measured in the presence and absence of ChvG (Li *et al.,* 2002). The *aopB::gfp* expression in the *chvG*$^+$ strain was induced about 8-fold by an acid pH, which is consistent with the previous observation that *aopB* was inducible by acid pH (Jia *et al.,* 2002). However, in a chvG mutant the *aopB::gfp* expression was undetectable at pH 7.0 or pH 5.5, suggesting that ChvG is absolutely required for the expression and consequently acidic pH induction of *aopB.* To study the effect of *chvG* on *katA*, the *katA::gfp* expression was examined in the absence of any functional *katA* gene (Li *et al.* 2002), since a functional KatA could repress the *katA::gfp* expression (Xu *et al.,* 2001) and thus interfere with the studies. It was found that the *katA:: gfp* expression in the *chvG*$^+$ background was induced about 9-fold by acidic pH (Li *et al.,* 2002), which is also consistent with the previous observation that *katA* was

inducible by acidic pH (Xu *et al.,* 2001). However, in the *chvG* mutant, the *katA:: gfp* was expressed at a level similar at both pH 7.0 and pH 5.5. These data suggest that *chvG* was required for the responsiveness of *katA* gene expression to the pH change. In addition, the *katA::gfp* expression level in the *chvG* mutants grown at pH 7.0 or pH 5.5 was comparable to that in the *chvG*$^+$ cells grown at pH 5.5. These results suggested that *katA* expression is repressed at neutral pH and an acid pH can derepress the ChvG-mediated repression of *katA* expression.

Since the expression of all the Ti plasmid harbored *vir* genes requires an acidic pH environment in addition to the plant phenolic compounds, such as acetosyringone (Winans, 1992), the *virB::lacZ* or *virE::lacZ* gene expression was measured in the presence of 100 µM acetosyringone at pH 7.0 or pH 5.5 (Li *et al.,* 2002). The *virB::lacZ* and *virE::lacZ* gene expressions in the *chvG*$^+$ strain were induced about 15-fold and 10-fold, respectively, by an acid pH. However, the *virB: :lacZ* and *virE::lacZ* expression in the *chvG* mutant was much lower than that in the *chvG*$^+$ strain, and the induction of both *virB::lacZ* and *virE::lacZ* expression by acidic pH was marginal. These results indicated that ChvG is also involved in the induction of *virB* and *virE* by an acid pH, and suggests that ChvG may regulate all the *vir* genes harbored on the Ti-plasmid, as they are regulated as one regulon.

Nodulation of the leguminous plant alfalfa by *S. meliloti* is a complex developmental process that requires a series of signal exchanges between the host and bacteria (Sessitsch *et al.,* 2002). Plant flavonoids and bacterial Nod factors are among the best-characterised signals involved in this communication process. Plant flavonoids secreted from the roots induce Nod factor production by the bacteria. The Nod factor then induces root hair curling and initiates nodule development. In order for *S. meliloti* cells to successfully invade the nodules they elicit on alfalfa roots, they must be able to synthesise at least one of the following polysaccharides: succinoglycan, exopolysaccharide II, or a particular 3-deoxy-D-manno-2-octulosonic acid-containing capsular polysaccharide (Reuber and Walker 1993; Reuhs *et al.,* 1995; González *et al.,* 1996). Succinoglycan is a polymer composed of repeating octasaccharide subunits containing seven glucose molecules and one galactose molecule (Reinhold *et al.,* 1994). A tetramer of succinoglycan subunits was reported to partially restore nodule invasion by *exo* mutants when added with the *exo* mutant to the roots of alfalfa plants (Battisti *et al.,* 1992). These suggest that succinoglycan molecules function as bacterially derived signalling molecules that ensure the successful entry of the bacteria into developing nodules. In the free-living stage, succinoglycan production by *S. meliloti* is sensitive to the concentration of ammonia, phosphate, and sulphate present in the growth media (Leigh *et al.,* 1985). In the symbiotic stage, the *exo* genes are expressed only in the invasion zone inside the nodule, not in the more mature regions of the nodule (Reuber *et al.,* 1991). This suggests that succinoglycan production is turned off after the bacteria invade the plant cells but before they differentiate into bacteroids.

Two succinoglycan-overproducing mutants, *exoR95*::Tn*5* and *exoS96*::Tn*5,* in which the transcription of succinoglycan biosynthesis genes was increased have been identified (Doherty *et al.,* 1988; Reed *et al.,* 1991). Contrary to the *exoR* mutant, *exoS* mutant induced nitrogen-fixing nodules on alfalfa roots and synthesised less succinoglycan in the presence of ammonia. Both genes *exoS* and *exoR* were located on the *S. meliloti* chromosome and therefore they were not

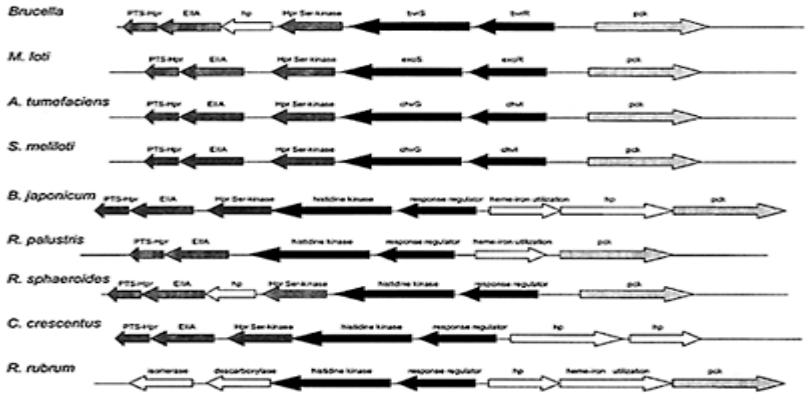

Figure 3. Genetic map of the region encoding the BvrS/BvrR-homologous two-component regulatory systems of several members of the α-2 Proteobacteria (*Brucella*, Agrobacterium *tumefaciens*, *Mesorhizobium loti*, *Sinorhizobium meliloti*, *Bradyrhizobium japonicum*, *Rhodopseudomonas palustris*, *Rhodobacter sphaeroides*, *Caulobacter crescentus*, *Rhodospirillum rubrum* and *Novosphingobium aromaticivorans*). The sensor histidine kinase and the response regulator genes are shown in black. Except in *C. crescentus*, *pck* is located upstream the two-component regulatory genes, and the genes downstream relate to the phosphotransferase system (shown in grey). The genetic organisation in *B. melitensis* and *B. suis* is the same. The organisation of these genes is also conserved in *Bartonella*, except *pck* (S.Andersson, personal communication). The genetic arrangement in *N. aromaticivorans* is the same as in *R. sphaeroides*.

Sequences were retrieved from either NCBI (http://www.ncbi.nlm.nih.gov/) or the *B. melitensis* database at the Molecular Biology Research Unit, University of Namur, Belgium (http://serine.urbm.fundp.ac.be/~seqbruce/GENOMES/B_MELITENSIS/).

linked to other *exo* genes located on the symbiotic megaplasmid and previously described (Doherty *et al.* 1988). Through cloning and sequencing, Cheng and Walker (1998) found that the ExoS was highly homologous to the *Agrobacterium* sensor protein ChvG, and revealed the existence of a *chvI* homologue located just upstream of the *S. meliloti exoS* gene. They also demonstrated that the *S. meliloti* ExoS cytoplasmic domain fragment was possesses the kinase activity. It would be of significance to determine if *S. meliloti* ExoS is responsible also for sensing the acidity of the environment during its symbiosis with the plants. It remains to be determined if the pH plays a role in this process and if, like ChvG, ExoS is also involved in regulating the expression of any acidic pH-inducible genes in *S. meliloti*.

5. Common Regulatory Strategies in α-2 Proteobacteria

The fact that *B. abortus* BvrS/BvrR, *A. tumefaciens* ChvG/ChvI, and *S. meliloti* ExoS/ChvI systems are involved in symbiosis and parasitism (Charles and Nester, 1993; Cheng and Walker, 1998; Sola-Landa *et al.*, 1998) suggests that they are crucial for the adaptation to the pericellular or intracellular environment (Moreno *et al.*, 1990). Several similarities between these two-component regulatory systems in *Brucella* and in other α-2 of the *Proteobacteria* are evident.

A search for BvrS/BvrR homologs in the available genomes of α-2 Proteobacteria yielded homologs not only in *A. tumefaciens* and *S. meliloti* but also in *Rhizobium* spp., *Mesorhizobium loti, Bradyrhizobium japonicum, Bartonella quintana, Bartonella bacilliformis, Bartonella taylorii, Caulobacter crescentus, Rhodopseudomonas palustris, Rhodobacter sphaeroides, Rhodospirillum rubrum* and *Novosphingobium aromaticivorans*. However, the comparison of the protein sequences showed that the percentages of identity of both BvrS and BvrR are higher in cell associated bacteria like *Mesorhizobium, Sinorhizobium, Agrobacterium* and *Bartonella*, than in the other α-2 Proteobacteria (BvrR had 80–93 % of identity to the regulators ChvI and BatR, and BvrS had 64–67 % of identity to the sensors ChvG and BatS). In this regard, Guzmán-Verri *et al.* (2002) have demonstrated that anti *S. meliloti* ExoS antibodies cross-react with *B. abortus* wild type cell lysates and no with *bvrS* and *bvrR* mutants. Recently, they have also reported that *B. abortus bvrS* mutant complemented with the ExoS protein recuperated the ability to invade and reach the endoplasmic reticulum avoiding lysosomes and

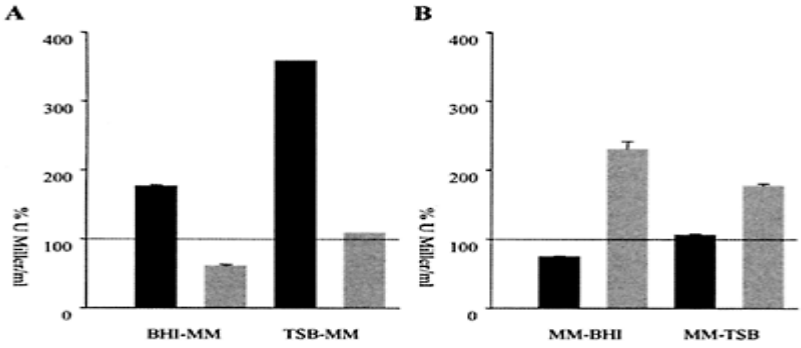

Figure 4. Transcriptional analysis of *omp25* (in black) and *omp22* (in grey) after a nutritional shift. *B. abortus omp25::lacZ* and *omp22::lacZ* were grown in either complex (BHI or TSB) or minimal media (MM) up to the exponential phase, centrifuged and washed with saline. Then the cells were transferred to fresh media, and incubated for 4 h before measuring β-galactosidase activity. (A), β-galactosidase activity of cells transferred from complex to minimal media; (B) β-galactosidase activity of cells transferred from minimal to complex media. Results are expressed as the percentage activity with respect to that obtained with cells treated as in the above-described experiments but transferred to the same fresh medium used in the first incubation (no nutritional shift).

replicated successfully in HeLa and macrophage cells (Chaves-Olarte *et al.*, 2003). These results suggest that the BvrS/BvrR system is functionally interchangeable with the ExoS/ChvG system from *S. meliloti*.

Comparative genome analysis also revealed that in addition to the *bvrS*/*bvrR* genes, the genome structure around these genes is essentially the same for all the α-2 Proteobacteria (Hu and Saier, 2002; Figure 3). Genes encoding proteins related to the

phosphotransferase system, including a HPr Ser-kinase, an EIIA permease of the mannose family and a HPr homologue precede those of the two-component regulatory system. In most of these loci, upstream of the regulatory gene a phosphoenolpyruvate carboxykinase structural gene *(pck)* is divergently expressed. Based on this gene arrangement, it has been elegantly suggested by Letesson and De Bolle (see Chapter 7) that the phosphotransferase could interact with the BvrS sensor kinase, which in turn phosphorylates the response regulator. Then, the BvrR could control transcription of the *pck* gene, which encodes an essential enzyme of gluconeogenesis and Krebs cycle. This hypothesis could explain the observation that mutants in the regulatory gene *bvrR* were inhibited in some minimal media suggesting a mechanism linking the two-component regulatory system BvrS/BvrR and metabolism (Figure 2).

B. abortus BvrS/BvrR and *A. tumefaciens* ChvI/ChvG systems control the expression of at least Omp25-Omp22 and Aop, respectively. *Agrobacterium* Aop is inducible by acidic pH and, as mentioned before, ChvG is a global pH sensor protein that regulates the expression of other acidic pH-inducible genes. Although

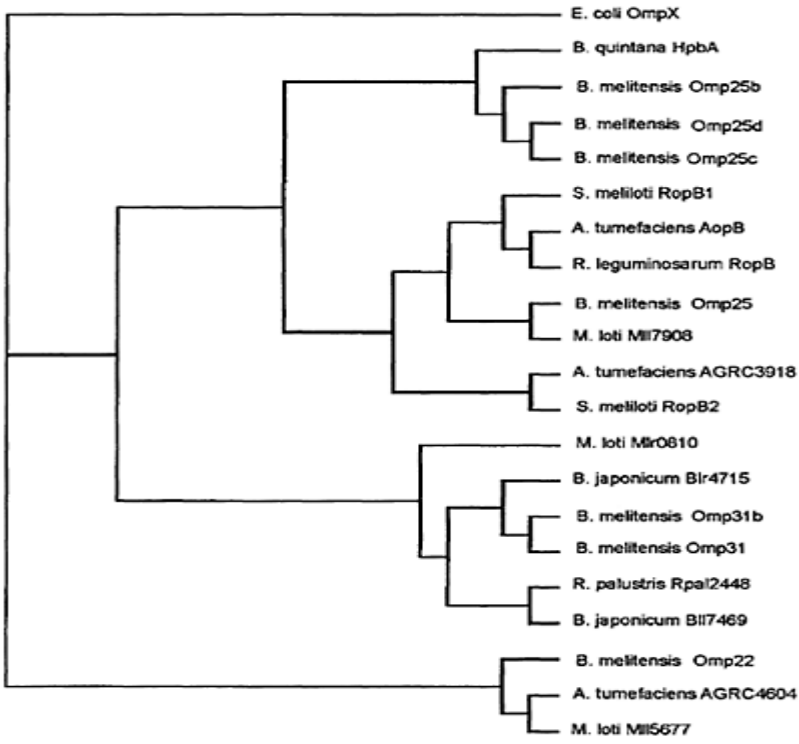

Figure 5. Neighbour-joining tree derived from the distance-matrix analysis of 22–31 kDa Omps sequences of several members of the α-2 Proteobacteria *(B. melitensis,*

Bartonella quintana, Agrobacterium tumefaciens, Sinorhizobium meliloti, Rhizobium leguminosarum, Mesorhizobium loti, Bradyrhizobium japonicum, and Rhodopseudomonas palustris). The five-member gene family corresponding to Hbp proteins in *B. quintana* (Minninck *et al.*, 2003) are all grouped together in the same position that HbpA. Protein designations are shown as they appear in GenBank or in the corresponding references. Omp25/Omp22 and Aop are regulated by BvrS/ BvrR and ChvG/ChvI systems, respectively. The sequence of the *E. coli* 0157:H7 OmpX protein was included as control. Protein sequences were aligned using the ClustalW 1.8 program and the tree was generated by the method of Neighbor-Joining/UPGMA 3.6a3. (http://www.infobiogen.fr/services/menuserv.html).

there is no evidence on the environmental stimulus activating the *Brucella* BvrS, preliminary results obtained in the laboratory of one of us show that *B. abortus* Omp25 and Omp22 are not inducible by pH changes. Using *omp25::lacZ* and *omp22::lacZ* transcriptional fusions, the level of expression of *omp25* and *omp22* promoters was measured by the β-galactosidase assay under different growth conditions. It was found that nutritional shifts significantly affected the levels of *omp25* and *omp22* transcriptions. For example, the transcription of *omp25* increased when cells grown in rich complex media were transferred to minimal media, and decreased when cells grown in minimal media were incubated in complex media. On the contrary, the transcription level of *omp22* increased upon transferring from minimal to complex media, and decreased upon the reverse transfer (Figure 4). Acidic, heat or osmotic shocks, or exposure to anaerobic atmosphere did not change the transcriptional expression of either *omp25* or *omp22*.

Interestingly, BLAST searches revealed that Omps regulated by BvrS/BvrR and ChvI/ChvG were highly homologous. Using the protein sequences we searched also for homologous genes in the α-2 Proteobacteria (Guzmán-Verri *et al.*, 2002). This search yielded a significant number of Omps, some of them related to the adaptation of the bacteria to eukaryotic pericellular or intracellular habitats. They included *R. leguminosarum* RopB and RopA (expressed in ex plant bacteria cells and severely

decreased during bacteroid formation), *S. meliloti* RopB2 (induced only during infection) and *Bartonella* Hbp family (hemin-binding proteins) (Figure 5). On this basis, it can be predicted that expression of these and other proteins homologous to *Brucella* group 3 Omps and Aop is under the control of systems homologous to BvrS/BvrR like ChvI/ChvG. Specifically, it seems very likely that the five-member outer membrane hemin-binding protein (Hbp) of *Bartonella* are under the control of the two-component regulatory system BatS/BatR homologous to BvrS/BvrR and ChvI/ChvG (Figure 2).

6. Acknowledgements

Research at the Department of Microbiology (University of Navarra) is supported by the European Commission (Research Contract QLK2-CT-2002–00918), Ministerio de Ciencia y Tecnología of Spain (AGL2000–0305-C02), Redes Temáticas de Investigación Cooperativa del FIS—Promoción de las Regiones (objetivo 2, 2000–2006, Red Temática de Investigación en Brucellosis, G03/204) and PIUNA from University of Navarra.

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Chapter 11
Erythritol Metabolism and Virulence in *Brucella*

Juan M.García-Lobo* and Felix J.Sangari García

From: *Brucella:* Molecular and Cellular Biology. Edited by: Ignacio López-Goñi and Ignacio Moriyón

Abstract

Erythritol metabolism by bacteria in the genus *Brucella* has been recognised from long time ago as a peculiar trait related to the capability of the members in this genus to produce abortions in ruminants. This association has been explained by the high concentration of erythritol in foetal tissues linked to the capability of *Brucella* to use erythritol preferentially over other nutrients. The pathway for erythritol degradation in *Brucella* is well known and more recently the genes encoding an operon for erythritol catabolism have been identified and characterised. This finding, along with the availability of genomic data and genetic tools, allows a closer analysis of the mechanisms underlying the role of erythritol metabolism in the virulence of *Brucella*.

1. Introduction

Erythritol is a four carbon atom sugar alcohol (polyol), small amounts of which are found in some fruits, and remarkably, in high concentration in foetal tissues in ruminants. A recent report showed that the concentration of erythritol in foetal sheep blood was 60 times higher than in the maternal blood (Teng *et al.*, 2002). Even though erythritol is structurally similar to very central metabolites such as glycerol or erythrose, it is unusually inert from a metabolic point of view. It is not normally metabolised by mammals, a property which makes erythritol useful as a no caloric edulcorant often used as a food additive. Erythritol ingested by humans is either excreted in unaltered form or metabolised by intestinal microbiota. This shows that some microorganisms have the ability to utilise this compound. However exactly which organisms can is not known the moment. It has been reported that some pseudomonas, *Propionobacterium*

(Wawszkiewicz and Barker, 1968) and many members of the *Rhizobiazeae* can utilise erythritol, therefore many other microorganisms can probably too.

Among all the bacterial species, members of the genus *Brucella* are the most closely associated with erythritol. The *Brucellae* are not only able to use erythritol, but they use it in preference to other sugars. In addition it has been described that erythritol promotes growth of the *Brucella* in some conditions. Furthermore a relationship between the ability of utilise erythritol and the pathogenicity of *Brucella* has long been established. The characteristic reproductive alterations induced by *Brucella* infection in ungulates such as abortions and reproductive

* For correspondence email jmglobo@unican.es

organ colonisation, were correlated to the presence of high erythritol levels in these organs. Accordingly it was hypothesised that the specific colonisation by *Brucella* organisms resulted from a positive tropism for erythritol (Smith *et al.,* 1962) due to the capability of *Brucella* to use erythritol as a growth factor and carbon source (Williams *et al.,* 1962). As a result of this relationship, the biochemical characterization of erythritol metabolism and the study of the genetic organisation of the genes encoding enzymes involved in erythritol catabolism was first (and almost exclusively) performed in *Brucella*.

2. The Biochemistry of Erythritol Catabolism in *Brucella*

The pathway for erythritol catabolism in *Brucella* was elucidated nearly years ago by Sperry and Robertson (1975a). Using classical radiolabelling experiments these authors demonstrated that the pathway started with the activation of erythritol by phosphorylation with ATP, and was followed by the oxidation of erythritol phosphate to D-erythrulose-1 phosphate, (this step was linked to NADH production). Two further dehydrogenation steps transform this product initially into 3-keto erythrose 4-phosphate and then into 3-keto erythronate 4-phosphate. This last compound undergoes a final decarboxilation step to create dihydroxyacetone phosphate, which subsequently feeds the central carbohydrate metabolism (Sperry and Robertson, 1975a). Enzymes involved in this pathway were described at the time—mostly from their catalytic properties—without many molecular details. Erythritol kinase and the decarboxylase involved in the last step were described as soluble enzymes whereas the three dehydrogenase activities were to some extent bound to membranes.

2.1. Erythritol Metabolism in the S19 Vaccine Strain

For many years the only vaccine used to prevent bovine brucellosis was the *Brucella abortus* strain 19 (S19, also known as B19). This is a smooth live attenuated vaccine, that became attenuated spontaneously. S19 was isolated in 1923 by John M.Buck and was widely used in the control of bovine brucellosis (Nicoletti, 1990). The cultures provided by the USDA before 1956 showed differences both in growth in the presence of erythritol and in erythritol oxidation rates. At that time erythritol sensitive cultures were selected

and used to substitute the previous batches of vaccine. This new vaccine called US19 or just S19 provided further support to the existence of a connection between erythritol metabolism and pathogenicity in *Brucella*. Sperry and Robertson also analysed the metabolism of erythritol in the S19 vaccine strain. They detected all of the erythritol degradation enzymes in S19, except the D-erythrulose 1-phosphate dehydrogenase. Furthermore, they described that the level of ATP dropped 10 fold in 30 minutes after the addition of erythritol to exponentially growing S19 cells (Sperry and Robertson, 1975b). These results led to the conclusion that the inhibition of growth produced by erythritol in the S19 vaccine was due to a drop in ATP level plus the accumulation of erythrulose 4-P which could be toxic for *Brucella*.

2.2. The *ery* Operon in *Brucella*

To localise and study the genes encoding the enzymes involved in the erythritol catabolic pathway, Tn5 transposon mutagenesis was performed on the virulent *B. abortus* strain 2308, and mutants with the same erythritol inhibition phenotype observed in the vaccine S19 were selected. The chromosomal region around the site on Tn5 insertion in one of these mutants (called mutant 227) was cloned and sequenced. The analysis of the nucleotide sequence data revealed the presence of an operon containing four genes *(eryABCD)*, essential for erythritol degradation in *Brucella*. The four *ery* genes were separated by short intergenic regions and a putative transcription terminator was also found after *eryD*. Furthermore primer extension assays were used to identify a single promoter upstream of *eryA*. All these data suggested that the four *ery* genes in *Brucella* were organised as an operon (Sangari *et al.*, 2000).

The *ery* operon was also analysed in the vaccine strain S19. A deletion of 702 bp was detected in the *ery* operon of this strain (Sangari *et al.*, 1994). The limits of this deletion were flanked by 13 bp direct repeats, probably involved in the genesis of the deletion. Flanking directed repeats approximately 15 bp long are frequently observed in spontaneously occurring deletions in *Brucella*. In S19, the deletion affected two genes, *eryC* and *eryD*. The 3′ end of *eryC* and the 5′ region of *eryD* are deleted leading to the production of a fused EryC-D polypeptide in S19. This fused polypeptide had 150 aa missing from the carboxy half of EryC and 80 aa missing from the amino end of EryD. This deletion should make the two genes inactive and the non-functional fused polypeptide produced in S19 will either degrade or accumulate in bacterial cells.

The gene *eryA* encodes a 517 aa polypeptide. Homology searches showed that EryA belongs to a family of sugar kinases, very similar to several bacterial xylulose kinases. EryA has been overproduced and shown to catalyse phosphorylation of erythritol in the presence of ATP (Lillo *et al.*, 2003). Accordingly *eryA* was predicted to encode erythritol kinase in *Brucella* spp. *EryB* encodes a 502 aa polypeptide with a high identity (53%) with glycerol 3-P dehydrogenases. Due to the structural similitude between glycerol 3-P and erythritol 4-P, it was assumed that the product of *eryB* in *Brucella* was the erythritol 4-P dehydrogenase, identified previously as the enzyme that catalyses the second step in erythritol degradation. In addition *eryB* was the gene inactivated by Tn5 in the erythritol sensitive mutant 227. *EryC* encodes a 310 aa polypeptide containing no previously identified protein domains or close relatives in the databases other than its putative ortologs. Short regions of homology in *eryC* predict a role for the corresponding enzyme

in sugar metabolism. The stronger argument to assign a function to *eryC* was provided by the vaccine strain S19. As mentioned before the *eryC* gene was partially deleted in S19 and at the same time it had been described that erythrulose 4-phosphate dehydrogenase was missing in this strain (Sperry and Robertson, 1975b). The deletion in S19 also affects *eryD*, but as we mention below, a regulatory function has been assigned to EryD, making it more feasible that the product of *eryC* in *Brucella* was erythrulose 4-P dehydrogenase. *EryD* encodes a 316 aa polypeptide belonging to the DeoR family of transcriptional regulators and contains a sugar binding domain, as well as a HTH motif, present in DNA-binding proteins. Mutants with an inactive *eryD* gene presented an elevated expression of the *ery* genes as measured from the RNA levels. Both homology search and experimental studies fully support the assignation of a repressor function for EryD. Experimental data showed that transcription of the *ery* operon was induced by erythritol only in the presence of EryD. This result further indicates the capability of EryD to respond to the presence of erythritol probably in the way classical repressors do i.e. the binding of sugar to the repressor will affect repressor-DNA interactions resulting in variations in the level of transcription.

In addition to regulating the expression of its own degradative genes, there is some evidence suggesting that erythritol may also regulate other genes in *Brucella*. On the one hand it is well known that erythritol is used by *Brucella* in preference to glucose or other sugars, suggesting the existence of some kind of repression activity such as is found in the classical catabolite repression system in *E. coli*. On the other hand, some sequences were described with promoter activity dependent on the presence of erythritol (GenBank accession numbers AF072569-AF072580). The existence of multiple promoters regulated by erythritol would suggest a broader role for erythritol in the regulation of sugar metabolism or other processes in *Brucella*. Finally the absence of homologues of genes encoding an adenylate cyclase activity in *Brucella* is remarkable. This absence can be interpreted as a clue that points to a mechanism for regulation of sugar metabolism in *Brucella* that could be build from activities of several independent regulators, responding to sugars. Such a model has already been described in *B. subtilis* and other Gram-positive bacteria lacking a global control mechanism that uses cAMP as an intermediary (Titgemeyer and Hillen, 2002). If it exists, such a model would reinforce the possible role of erythritol as a regulator of sugar metabolism in *Brucella*.

2.3. Transport of Erythritol

The question of how erythritol is transported into the bacterial cell is also extremely important. From studies with other sugars it would be expected that diffusion through the lipid bilayer would not provide sufficient movement of erythritol into the cell. Therefore it is thought that a specific transport system is required to transfer the molecule across the inner membrane of *Brucella*. Since the first step in erythritol degradation is phosphorylation with ATP, it can be assumed that erythritol has to be transported into *Brucella* in the non-phosphorylated form. This excluded a transport mechanism linked to phosphorylation such as the phosphotransferase system. Glycerol, a molecule similar to erythritol, is transported into many bacteria by means of a transporter protein known as the glycerol facilitator or GlpF. This protein forms a pore in the cytoplasmic membrane supporting the flow of glycerol driven by the chemical gradient. Once in the cytoplasm,

glycerol is quickly phosphorylated, keeping the inward glycerol flux active. It has been shown that the *E. coli* GlpF facilitator is able to transport erythritol as well as glycerol (Heller *et al.*, 1980). GlpF belongs to the MIP (major intrinsic protein) family of proteins that have the ability to form transmembrane channels. The amino acid sequences of the MIP proteins contain a number of well conserved domains, which were used for the design of degenerate oligonucleotides primers. These primers were used in PCR reactions to amplify *Brucella* genomic fragments encoding putative MIP

Table 1. Summary of the results of Blast comparisons for each of the translated *ery* genes. Only the obvious ortologs for each translated gene are shown in the table as well as some related genes of similar functions relevant for the discussion used to assign functions to the *ery* genes. The score and E value of the hits are as described by Altschul *et al.*, 1997

	Score	**E value**
EryA		
NP_386341.1 Putative Erythritol Kinase Protein *[Sinorhizobium meliloti]*	459	e-128
NP_107624.1 Xylulose kinase *[Mesorhizobium loti]*	458	e-128
NP_349219.1 Xylulose kinase *[Clostridium acetobutylicum]*	108	3e-22
NP_437019 putative L-xylulose kinase protein *[Sinorhizobium meliloti]*.	105	2e-21
NP_356760.1 AGR_L_1942p *[Agrobacterium tumefaciens]*.	95	3e-18
NP_5 34367. 1 L-xylulose kinase *[Agrobacterium tumefaciens]*	92	2e-17
EryB		
NP_107626 glycerol-3-phosphate dehydrogenase *[Mesorhizobium loti]*	664	0
NP_386338 Putative Erythritol Phosphate Dehydrogenase Protein *[Sinorhizobium meliloti]*	639	0
NP_ 102461 glycerol-3-phosphate dehydrogenase *[Mesorhizobium loti]*	497	e-139
EryC		
NP_386334.1 Putative D-Erythrulose-1-Phosphate Dehydrogenase Protein *[Sinorhizobium meliloti]*	443	e-123
NP_107628.1 Hypothetical protein *[Mesorhizobium loti]*	394	e-108
ZP_00015385.1 Hypothetical protein *[Rhodospirillum rubrum]*	79	1e-13
P33374 Uptake hydrogenase large subunit *[Alcaligenes hydrogenophilus]*	40	0.059
EryD		

NP_386347 Putative *ERY* operon repressor transcription regulator protein *[Sinorhizobium meliloti]*	351	7e-96
NP_107618 Transcriptional regulator *[Mesorhizobium loti]*	342	6e-93
AAC45765.1 operon regulator; SmoC *[Rho dobacter sphaeroides]*	173	4e-42

proteins. This approach allowed us to isolate a gene encoding an aquaporin, another member of the MIP family of proteins specialised in water transport (Rodriguez *et al.*, 2000). The *Brucella* aquaporin was shown to be a functional water channel, but it was unable to mediate glycerol transport. Analysis of *aqpZ* mutants also demonstrated that they were unaffected in erythritol transport.

In silico analysis of the complete sequence of the *B. melitensis* chromosomes confirmed the absence of a *glpT* ortolog in the genome of this species. Analysis of the *Brucella* genome revealed the presence of many putative sugar transporters, among them, a putative polyol ABC-transporter was proposed to be the erythritol transporter (DelVecchio *et al.*, 2002). However a knock out mutant for this transporter failed to confirm this hypothesis (unpublished results), and erythritol remains an orphan substrate awaiting a specific carrier.

Table 2. Relationship between genes in the *ery* regions of *S. meliloti, M. loti* and *Brucella*. Numbering of genes for *S. meliloti* and *M. loti* was taken from the KEGG database, and *Brucella* gene number corresponds to the annotated *B. melitensis* sequence deposited in GenBank under accession numbers NC 03317 and NC 03318. Gene order is identical in *B. abortus and B. suis*. Genes in a row are ortologs. Consecutive numbering usually means that the genes are adjacent in the genomes. All the *S. meliloti* genes are in the chromosome except Smb20199, the *fba* ortolog, which is located in the plasmid pSymB. See the text and Figure 1 for further explanation

S. meliloti	***M. loti***	***Brucella***
SMc01629	mlr7259	BMEII0427 *eryD*
SMc01628	mlr7261	–
SMc01627	mlr7264	–
SMc01626	mlr7265	–
SMc01625	mlr7266	–
SMc01624	mlr7267	–

SMc01623	mlr7268	BMEII0430 *eryA*
SMc01622	mlr7269	–
SMc01621	–	–
SMc01620	mlr7270	BMEII0429 *eryB*
SMc01619	mlr7271	–
SMc01618	mlr3603	–
SMc01617	mlr3604	–
SMc01616	mlr7272	BMEII0428 *eryC*
Smb20199	mlr7273	BMEII0423 *fba*
SMc01615	mlr7274	BMEII0426 *deoR*
SMc01614	mlr7275	BMEII0425 *tpi*
SMc01613	mlr7276	BMEII0424 *rpi*

3. Erythritol Catabolism Genes in Other Bacteria

The availability of the genomic sequences of many different bacteria makes it possible the search for the presence of *ery* operon genes in a variety of different organisms. Using the BLAST comparison tool in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) we performed searches with all of the genes in the *ery* operon. The flanking regions and gene order were analysed on line in the Kioto Encyclopaedia of Genes and Genomes (KEGG) at the server http://www.genome.ad.jp/kegg/kegg2.html.

In addition to *Brucella*, the genes constituting the *ery* operon are found in *Mesorhizobium loti* and *Sinorhizobium meliloti*. Blast comparisons of the four *ery* genes indicated that ortologs existed only in the two above mentioned bacteria.

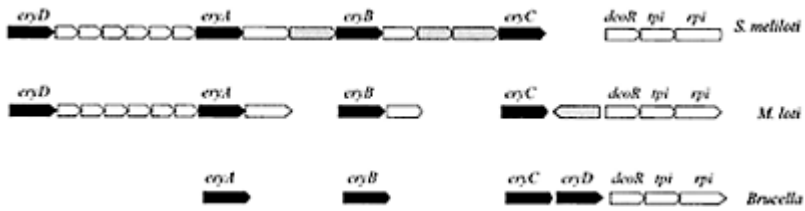

Figure 1. Graphic representation of the genomic regions containing the *ery* genes in *S. meliloti*, *M. loti* and *Brucella*. The genes are located in the circular chromosomes of *S. meliloti*

and *M. loti* and in the small chromosome of *Brucella*. Blank spaces have been introduced to preserve the alignment of the genes, but they do not correspond to any DNA. *Ery* genes are represented as black solid arrows, white arrows represent genes that are syntenic at least in two species, while the grey arrows represent genes that only appear in one of the species in that genomic region. Arrow sizes may not represent actual gene sizes.

A high degree of homology was found between EryB and glycerol phosphate dehydrogenase. A summary of this analysis is shown in Table 1.

When we analysed the gene order, we found that the four *ery* ortologs in the chromosome of *S. meliloti* were not organised in an operon as in *Brucella*. Instead They were interspersed in a large cluster of 17 genes (from SMc01629 to SMc01613) involved in sugar metabolism and transcribed in the same orientation. Following the direction of the transcription, *eryD* (SMc01629) is the first gene, followed by a group of 5 genes (SMc01628-SMc01624) encoding a putative glycerol phosphate ABC-transport system not found in *Brucella*. Then ortologs of *Brucella eryA* (SMc01623), *eryB* (SMc01620) and *eryC* (SMC01616) are found intercalated with other genes encoding products involved in sugar metabolism. Three genes encoding a transcriptional regulator (SMc01615), triose phosphate isomerase (SMc01614) and ribose phosphate isomerase (SMc01613) respectively are found immediately downstream of *eryC*. Ortologs of these three genes conserving the gene order are also found downstream to the *ery* operon in *Brucella*. This gene organisation is somehow related with the gene order observed in *M. loti*. In this bacteria there are also clear ortologs of the four *ery* genes. In this case they are included in a cluster of 15 genes (mlr7259–7276), also involved in sugar metabolism. The cluster starts with *eryD* (mlr7259) followed by an ABC-transport system very similar to the one described in *S. meliloti*. Then *eryA* (mlr7268), *eryB* (mlr7270) and *eryC* (mlr7272) are found in this order but also with some intercalated genes among them. The cluster ends with a similar group of genes containing a transcriptional regulator (mlr7274), triose phosphate isomerase (mlr7275) and ribose phosphate isomerase (mlr7276). (See Table 2 and Figure 1).

Comparison of the chromosomal regions of the three organisms containing the *ery* genes may indicate an evolutionary relationship among them. A reductionist process and some gene shuffling would be operating from *S. meliloti* (the longer gene region) down to *Brucella*. This bacterial genus shows the shorter gene region, which is in agreement with its smaller genomic size, and as is expected for a bacterium adapted to an intracellular lifestyle. On the other hand, the *ery* genes in *Brucella* are clustered together and organised as a transcriptional unit, revealing that they probably play an essential function in the biology of this bacterial genus. Whether *ery* genes are functional in *S. meliloti* and

in *M. loti* is not actually known. Both bacterial species seem to be able to grow on erythritol as sole carbon source, and this would at least indicate that the *ery* genes are functional in spite of their dispersed location in the genomes of the two bacteria (Figure 1).

4. Erythritol Metabolism and Virulence

The identification of the genes involved in the degradation of erythritol, the publication of genomic data and the development of new gene manipulation tools allowed the construction of defined mutants to investigate the relevance of the metabolism of erythritol in the *Brucella* pathogenic process. In addition to the S19 vaccine (a natural Δ*ery*CD) mutant and the mutant 227 (*eryB::*Tn*5*), we constructed the strain 2308 Δ*eryCD*, a *B. abortus* 2308 derivative carrying the same deletion as the S19 vaccine, and strain FJS19, a derivative of S19 with a wild type *ery* operon reconstructed by means of homologous recombination. This set of strains was assayed in a murine model and in both cultured (J774) and peritoneal murine macrophages (Sangari *et al.,* 1998). The results of this set of experiments did not reveal a significant correlation between the capability to catabolise erythritol and the growth properties of the mutants. In the mouse model the *B. abortus* 2308 strain and their Δ*eryCD* derivative persisted for the same length of time, while the S19 vaccine and their *ery* reconstructed derivative FJS19 both persisted for the same, shorter period of time. An independent survey for the identification of genes involved in survival inside macrophages showed that Tn5 insertions in *eryB* and *eryC* genes resulted in a diminished capacity for intracellular growth (Kohler *et al.,* 2002; see also Chapter 15). This study was performed with *B. suis* mutants growing inside human derived cell line THP-1. In conclusion, these results argue against a role for the erythritol metabolism in the *in vivo* growth capability of *Brucella* in mice. This would indicate that attenuation of S19 is not due to the deletion of the *ery* genes but to a mutation elsewhere.

On the other hand, it is also possible that the murine model is not appropriate to test the relevance of erythritol metabolism, since erythritol is not as abundant in rodents as it is in ruminants. According to this, the role of erythritol in virulence should be tested in a ruminant animal model, preferentially in a pregnant ruminant model, since this is the natural environment in which erythritol is present at high concentrations.

4.1. The Iron Connection

As happens with many other bacteria, the competition for available iron seems to be an important aspect of *Brucella* pathogenicity. Apparently *Brucella* is able to take iron from the environment through several independent mechanisms. Both iron heme transport (Almiron *et al.,* 2001) and siderophore production (Lopez-Goni *et al.,* 1992; Gonzalea Carrero *et al.,* 2002) have been described to be operative in *Brucella*. Several reports indicate that a connection exists between iron uptake by siderophores and erythritol metabolism in *Brucella* (see also Chapter 12). The role of the production of dihydroxibenzoate (DHBA) in *Brucella* virulence was

Table 3. Identification of methyl-erythritol 4-phosphate pathway genes and their gene products in the *B. melitensis* genome. The gene for the methyl-erythritol 4-phosphate synthase is absent in the genome of the three sequenced *Brucella* species. The gene number corresponds to the annotated *B. melitensis* sequence deposited in GenBank under accession numbers NC 03317 and NC 03318

Gene number	Product
BMEI1498	1-deoxyxylulose-5-phosphate synthase [EC:2.2.1.7]
ABSENT	2-C-methyl-D-erythritol 4-phosphate synthase [EC: 1.1.1.267]
BMEI0863	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase/2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase [EC:2.7.7.60/4.6.1.12]
BMEI1537	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase [EC:2.7.1.148]
BMEI0269	4-hydroxy-3-methylbut-2-en-l-yl diphosphate synthase [EC:1.17.4.3]
BMEI1459	4-hydroxy-3-methylbut-2-enyl diphosphate reductase [EC:1.17.1.2]

investigated with the use of a mutant *(entC)* unable to synthesise this compound. It was found that production of DHBA was not essential for virulence in a mouse infection model (Bellaire *et al.,* 1999), but on the contrary it was found to be important in a pregnant cattle model (Bellaire *et al.,* 2003b), and also in bovine trophoblasts grown in the presence of erythritol (Parent *et al.,* 2002), suggesting a connection between erythritol metabolism and virulence by means of iron uptake or siderophore production. Furthermore, it has also been shown that *in vitro* growth of *Brucella abortus* in low iron conditions in the presence of erythritol requires the production of DHBA siderophores (Bellaire *et al.,* 2003a), demonstrating once again a connection between erythritol an iron uptake. This may indicate that *Brucella* uses different iron uptake mechanisms under different conditions, and that DHBA based siderophores may be needed in the presence of erythritol i.e. for ruminant foetal colonisation.

The nature of this hypothetical connection between erythritol metabolism and virulence is unknown by the moment. However there is a possible biochemical way to connect both systems, erythritol catabolism and DHBA biosynthesis through the precursor of the DHB A biosynthetic pathway, erythrose 4-P. This sugar phosphate could be easily derived from erythritol catabolism intermediates. The product of EryB is erythrulose 4-P, that would easily convert into erythrose 4-P by an isomerization reaction common in sugar metabolism. Other possible connections exist between the two pathways from downstream intermediates in the erythritol degradative pathway. In support of this hypothesis, we should mention here that the function of EryC is not well supported from the genomic or sequence data available up to this date.

4.2. Erythritol and Mevalonate: The γδ-T Cell Connection

Another hypothetical and highly speculative connection between erythritol metabolism and *Brucella* virulence could be the activation of γδ-T cells by an intermediate derived from erythritol metabolism. It is well known that peripheral blood of brucellosis patients shows an elevated content of γδ-T cells. These are also elevated in blood from patients of other infectious diseases such as erlichiosis, salmonellosis or tularemia. It has also been reported that γδ-T cells play an important role in the control of *B. suis* by human monocytes (Ottones *et al.*, 2000a), and that activation of this T cell subset was mediated by a diffusible non-peptidic substance derived from *Brucella* metabolism (Ottones *et al.*, 2000b). Recently a metabolite 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) has been identified as an extremely potent inducer of the expansion of these cells. This compound is an intermediate of the methyl-erythritol 4-phosphate (MEP) pathway for mevalonate biosynthesis. In general, it is believed that most microorganisms able to induce strong γδ-T cell responses posses the MEP pathway (Eberl *et al.*, 2003). Genomic analysis has revealed that all the enzymes of the pathway were present in the genome of *B. melitensis* except the enzyme that produces methyl erythritol phosphate from deoxyxylulose 5-P (Table 3).

There is an apparent contradiction between the ability of *Brucella* to induce a γδ-T cell response and the lack of a key enzyme in the MEP pathway. There is obviously the possibility that the reaction is catalysed by an alternative unknown enzyme or that there is an alternative method to produce methyl erythritol in *Brucella* connected in some way with erythritol metabolism in this bacteria. This would be supported again by the loose function attribution made for EryC, and of course by the ability of *Brucella* to induce a strong γδ-T cell response.

5. Acknowledgements

Work in the authors laboratory was supported by contracts QLRT 1999–0014 and QLRT-2000–01200 with the European Union, and by Redes Temáticas de Investigación Cooperativa del FIS—Promoción de las Regiones (objetivo 2, 2000–2006, Red Temática de Investigación en Brucellosis, G03/204).

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Chapter 12
Iron Metabolism in *Brucella*

R.Martin Roop II*, Bryan H.Bellaire, Eric Anderson and James T.Paulley

From: *Brucella:* Molecular and Cellular Biology. Edited by: Ignacio López-Goñi and Ignacio Moriyón

Abstract

Like most pathogenic bacteria, the *Brucella* spp. encounter extreme iron deprivation in their mammalian hosts. Two siderophores have been described for these bacteria, the simple catechol 2,3-dihydroxybenzoic acid (2,3-DHBA) and the more complex 2,3-DHBA-based siderophore brucebactin. Experimental evidence has clearly shown that 2,3-DHBA production is required for the wild type virulence of *Brucella abortus* 2308 in the reproductive tract of pregnant ruminants. In contrast, lack of catechol siderophore production does not compromise the capacity of this bacterium to establish and maintain chronic spleen infection in the mouse model. Surveys of the genome sequences of *Brucella melitensis* 16M and *Brucella suis* 1330 and experimental evidence obtained in the laboratory suggest that transport systems allowing the brucellae to utilise heme and ferric dicitrate as iron sources may be important for sustaining the intracellular lifestyle of the brucellae in host macrophages. The accumulation of excess intracellular iron can enhance oxidative damage to bacterial cells as a consequence of Fenton chemistry. Iron toxicity could be particularly problematic for the brucellae, as oxidative killing appears to be one of the primary mechanisms employed by host phagocytes to control the intracellular replication of these bacteria. Accordingly, experimental evidence and analysis of genome sequence data indicate that the *Brucella* spp. employ multiple transcriptional regulators including a ferric uptake regulator (Fur) to strictly control iron uptake in response to intracellular iron levels.

1. Introduction

As is typical of most bacteria, the *Brucella* spp. require iron as a micronutrient (Waring *et al.,* 1953; Evenson and Gerhardt, 1955). The acquisition of sufficient iron to meet their physiologic needs is a particular challenge for the brucellae, as these bacteria are found in nature exclusively in association with mammalian hosts. Iron that is not incorporated into host tissue components is tightly bound to host iron-binding proteins such as transferrin and lactoferrin in the extracellular spaces, and by ferritin within host cells (Griffiths, 1999). This tight sequestration of iron in mammals serves not only to prevent iron toxicity in the host, but it also limits the availability of this nutrient to invading microbes. In fact, iron restriction plays an important role in both the innate and acquired immune responses in the host. The hypoferremic response elicited during the initial stages of the host response to infection further exacerbates the iron deprivation that pathogens face in the extracellular environment (Weinberg, 1995). Upon the induction of acquired immunity, IFN-γ activation of host macrophages enhances iron restriction in the phagosomal compartment, in part through the reduced production of transferrin receptors on the surface of activated macrophages (Weinberg, 2000). Another possible mechanism is through the proposed capacity of the natural resistance-associated macrophage protein 1 (Nramp1) to mediate active efflux of iron from the phagosomal compartment (Wyllie *et al.,* 2002).

The capacity of the brucellae to the gain access to the host's iron stores is critical to virulence, since these bacteria maintain primarily an intracellular existence in infected mammals. Within their natural hosts, the *Brucella* spp. predominantly occupy two host cell types, macrophages and placental trophoblasts (Anderson *et al.,* 1986b; Meador and Deyoe, 1989; Baldwin and Roop, 1999). Both of these cell types play central roles in iron metabolism in the host. Macrophages scavenge aged erythrocytes and haptoglobin-hemoglobin complexes from the circulation and in this manner perform a critical role in the recycling of iron (Bratosin *et al.,* 1998). Placental trophoblasts serve as a gateway for shuttling iron and other nutrients from the mother to the developing foetus (Schroder, 1995). Experimental evidence suggests that the iron needs of the brucellae differ during their survival and replication in these two host cell types and accordingly, different iron acquisition systems appear to be used by the brucellae to meet their iron requirements within these host cells (Bellaire *et al.,* 1999; González-Carreró *et al.,* 2002; Parent *et al.,* 2002; Bellaire *et al.,* 2003a; 2003b). Whether these differences are a reflection of the physiologic state and growth kinetics of the brucellae during residence in these host cells, or the types of iron available in these two host cell types, is presently unknown.

* For correspondence email roopr@mail.ecu.edu

2. Central Role of 2,3-Dihydroxybenzoic Acid in Iron Acquisition by the *Brucella* spp.

Studies by López-Goñi *et al.* (1992) showed that *B. abortus* 2308 secreted the simple catechol 2,3-dihydrobenzoic acid (2,3-DHBA) in response to iron limitation *in vitro*. More importantly, biochemical assays performed by these investigators clearly showed that 2,3-DHBA and the related monocatechols 2,3-dihydroxybenzoyl-serine (2,3-DHBA-Ser) and 2,3-dihydroxybenzoyl-glycine (2,3-DHB A-Gly) could serve as siderophores and mediate iron acquisition in *B. abortus* 2308. No evidence was obtained for the utilisation of more complex polycatechol siderophores or any type of hydroxamate siderophore by this bacterium in response to iron limitation. Experimental evidence also indicated that *B. abortus* 2308 was unable to utilise more complex polycatechols such as enterochelin or hydroxamate siderophores when these compounds were added to iron-deprived cultures. Subsequent studies by these investigators showed that 2,3-DHBA was the sole catechol-type siderophore produced by a wide variety of *B. abortus, B. melitensis, B. suis, B. ovis* and *B. neotomae* strains in response to iron deprivation (López-Goñi and Moriyón, 1995). It is important to note, however, that these latter studies also showed that 2,3-DHBA production was not detected in all of the *Brucella* strains

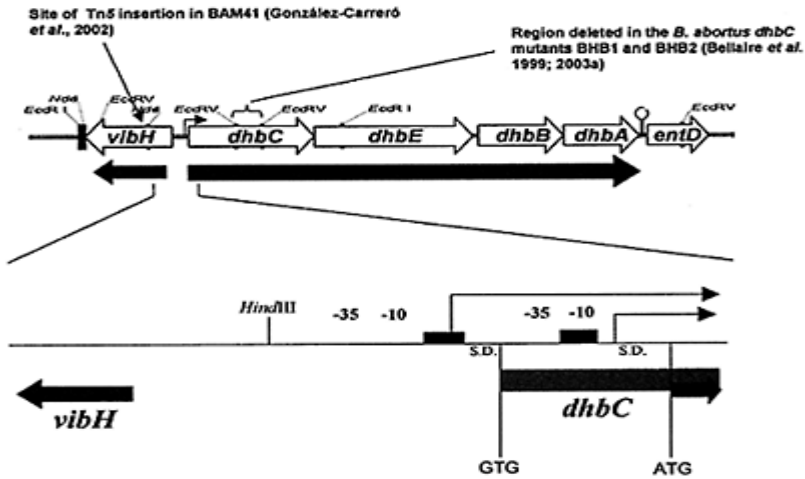

Figure 1. Schematic representation of the *B. obortus* 2308 2,3-DHBA and brucebactin biosynthesis locus. Sites where mutation results in loss of brucebactin biosynthesis *(vibH)* or 2,3-DHB A and brucebactin biosynthesis

(dhbC) are noted. The *vibH* and *entD* genes are transcribed independently of the *dhbCEBA* operon. The *dhbC* promoter regions are identified by the "−35, −10" designations, black boxes represent putative Fur-binding sites, and GTG and ATG represent the alternative start codons predicted for DhbC (Bellaire *et al.*, 2003a).

tested in response to iron restriction, and that failure to produce this catechol did not always correlate with growth restriction in iron-deprived cultures. These experimental findings provided the first clue that the brucellae utilise multiple iron acquisition systems to meet their physiologic needs.

To gain a better understanding of the genetic basis for 2,3-DHBA production by the brucellae, genetic complementation of the *Escherichia coli entC* mutant SAB11 (Barghouthi *et al.*, 1991) was used as a strategy to identify and clone the 2,3-DHBA biosynthesis genes from *B. abortus* 2308 (Bellaire *et al.*, 1999). Further characterization of the *Brucella* 2,3-DHBA biosynthesis locus revealed that it is comprised of four genes, *dhbC, E, B* and *A*, organised in an operon (Figure 1). The predicted products of three of these genes, isochorismate synthase (DhbC), isochorismate lyase (DhbB) and 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (DhbA) are homologous of the enzymes that convert chorismate to 2,3-dihydroxybenzoic acid in other bacteria (Massad *et al.*, 1994). The *dhbE* gene, on the other hand, is a homologue of the *E. coli entE*, a gene encoding an enzyme involved in the conversion of 2,3-DHBA into the more complex catechol siderophore enterochelin (Gehring *et al.*, 1987). Two iron-responsive promoters have been identified upstream of the *B. abortus dhbCEBA* operon and both of these promoter sequences contain canonical Fur-boxes (Bellaire *et al.*, 2003a). Transcripts arising from both of these promoters have been detected in response to iron limitation in *B. abortus* 2308, but differential expression from either promoter has not been observed under any of the experimental conditions examined to date (B. Bellaire, unpublished).

Initial studies suggested that 2,3-DHBA might play an important role in iron acquisition in the experimental murine host. Specifically, the addition of exogenous 2,3-DHBA to monolayers of murine macrophages infected with *B. abortus* 2308 led to enhanced intracellular replication of the brucellae within these phagocytes (Leonard *et al.*, 1997). To better define the role of 2,3-DHBA in iron acquisition and virulence, gene replacement was used to construct an isogenic *dhbC* mutant from virulent *B. abortus* 2308 (Bellaire *et al.*, 1999). The resulting strain, designated BHB1, failed to produce 2,3-DHBA in response to iron deprivation as determined by both the Arnow (1937) and liquid CAS (Schwyn and Neilands, 1987) assays. Studies with this mutant showed that 2,3-DHBA production is not required for the wild type replication of *B. abortus* 2308 in cultured naive and IFN-γ activated peritoneal macrophages from BALB/c mice (Bellaire *et al.*, 1999). Likewise, only a minimal defect in intracellular survival was observed when the *B. abortus dhbC* mutant was compared to the parent strain in cultured macrophages obtained from C57BL6 mice (Parent *et al.*, 2002). More importantly, however, the *dhbC*

mutant BHB1 established and maintained chronic spleen infections in both BALB/c (*Brucella* "sensitive") and C57BL6 (*Brucella* "resistant") mice with the same efficiency as the parental 2308 strain (Bellaire *et al.,* 1990; Parent *et al.,* 2002).

A much different picture emerged when the *B. abortus dhbC* mutant BHB1 was evaluated in pregnant cattle (Bellaire *et al.,* 2003a). Specifically, the *dhbC* mutant displays a remarkable degree of attenuation in these animals when compared to the parental 2308 strain. A possible clue to the basis for the differential virulence of the *B. abortus dhbC* mutant in mice versus pregnant ruminants was revealed during an evaluation of the *in vitro* properties of this mutant (Bellaire *et al.,* 2003b). The *dhbC* mutant exhibits a marked growth restriction compared to 2308 when these strains are cultivated in the presence of erythritol under low iron conditions. This growth restriction is not observed when these two strains are grown in the presence of erythritol under iron replete conditions, nor when these two strains are grown under low iron conditions in the presence of other readily utilisable carbon and energy sources such as glucose, galactose, arabinose, xylose or maltose. The precise basis for this growth restriction is presently unknown, but experimental evidence strongly suggests that the parental strain *B. abortus* 2308 has a greater physiologic need for iron during growth in the presence of erythritol than it does during growth in the presence of other readily usable carbon and energy sources and that 2,3-DHBA production helps meet this increased need for iron (Bellaire *et al.,* 2003b). Regardless of the precise physiologic basis for the inability of the *B. abortus dhbC* mutant to grow efficiently in the presence of erythritol when subjected to iron restriction *in vitro,* this phenotype could have major implications in the *in vivo* setting. Erythritol is the favoured carbon source of *B. abortus* (Anderson and Smith, 1965; Sperry and Robertson, 1975) and placental trophoblasts produce copious amounts of this four-carbon sugar alcohol during the third trimester of pregnancy (Smith *et al.,* 1962; Enright, 1990). This is the stage of gestation at which pregnant ruminants are most susceptible to *Brucella* induced abortions (Enright, 1990), and it has been postulated that the high levels of erythritol produced by placental trophoblasts fuels the extensive replication of the brucellae in the gravid ruminant reproductive tract, ultimately leading to abortion (Smith *et al.,* 1962). Consequently, it is possible that the basis for the attenuation of the *B. abortus dhbC* mutant in pregnant ruminants is the inability of this strain to exhibit wild type replication in the ruminant reproductive tract during the last trimester of pregnancy (Bellaire *et al.,* 2003b). A detailed comparison of the *in vitro* and *in vivo* phenotypes of *B. abortus dhb* mutants and strains with defined mutations in the erythritol catabolism *(ery)* genes (Sangari *et al.,* 2000) will be necessary, however, to more accurately define the relationship between 2,3-DHBA production, erythritol catabolism and virulence in the natural ruminant host.

3. Evidence For the Production of a More Complex 2,3-DHBA-Based Siderophore by *B. abortus* 2308

In their report describing the siderophore properties of 2,3-DHBA, López-Goñi *et al.* (1992) also described the production of a second catechol by *B. abortus* 2308 in response to iron limitation *in vitro*. This compound displayed slower migration on thin layer chromatographs than 2,3-DHBA and was produced in much smaller amounts, but was not

further characterised. Recently, González-Carreró *et al.* (2002) described the isolation of a Tn5 mutant of *B. abortus* 2308 that does make this second catechol in response to iron deprivation, but overproduces 2,3-DHBA under these same growth conditions. This mutant, designated BAM41 by the authors, also displayed growth restriction in an iron deprived medium that could be overcome by the addition of the slower migrating catechol to these cultures but not by the addition of 2,3-DHBA. The site of the Tn5 insertion in BAM41 is within a gene residing proximal to the *dhbCEBA* operon but predicted to be transcribed in the opposite direction (Figure 1). This gene is a homologue of *vibH*, a gene involved in the conversion of 2,3-DHBA to the more complex siderophore vibriobactin by *Vibrio cholerae* (Keating *et al.*, 2000a). Based on this finding and the phenotype of BAM41, González-Carreró *et al.* (2002) postulated that the *Brucella* VibH homologue participates in the conversion of 2,3-DHBA into a more complex siderophore represented by the slower migrating catechol detected by thin layer chromatography. They proposed the name brucebactin for this 2,3-DHBA based siderophore. Attempts to elucidate the structure of brucebactin have been complicated by the instability of this catechol in culture supernatants (González-Carreró *et al.*, 2002). However, the biochemical properties of VibH may provide some important insights in this regard. VibH is a freestanding non-ribosomal peptide synthetase condensation domain responsible for the fusion of 2,3-DHBA to the polyamine norspermidine during the biosynthesis of vibriobactin (Keating *et al.*, 2000). Consequently, it is conceivable that the *Brucella* VibH works in concert with the products of *dhbB, dhbE* and *entD* [which lies just 3′ of the *dhbCEBA* operon, but in a different operon (Figure 1)] to produce a monocatechol derivative of 2,3-DHBA (Bellaire *et al.*, 2003a). Such a function would be consistent with the observation that *B. abortus* has the ability to utilise monocatechols such as 2,3-DHBA-Gly or 2,3-DHBA-Ser as siderophores. Although both the *Vibrio* and *Brucella* VibHs share extensive amino acid identity with the N-terminal 1/3 of bacterial EntF proteins, enzymes involved in the condensation of 2,3-DHBA molecules into more complex polycatechols such as enterochelin (Gehring *et al.*, 1998), the VibH proteins lacks the appropriate thioesterase, peptidyl carrier and aryl carrier protein domains that allow the full-length EntF homologues to catalyse the assembly of 2,3-DHBA and amino acids into the more complex polycatechol siderophores. In fact, it is important to note that *V. cholerae* requires an additional protein (VibF) to complete the synthesis of vibriobactin, employing the product of the VibH reaction, the monocatechol 2,3-DHBA-norspermidine, as one of the intermediates (Keating *et al.*, 2000b). Further study will be required to define the structure of brucebactin as well as the role of the *Brucella dhbE, dhbB, vibH* and *entD* genes in the conversion of 2,3-DHBA to brucebactin.

Predictably, the *B. abortus vibH* mutant BAM41 displayed no significant attenuation in the murine macrophage-like cell line J774 (González-Carreró, 2002), which corroborates earlier results obtained with the *B. abortus dhbC* mutant BHB1 in cultured murine macrophages (Bellaire *et al.*, 1999; Parent *et al.*, 2002) indicating that production of catechol siderophores contributes little, if any, to the virulence of *B. abortus* 2308 in the experimental murine host. As noted in the previous section, however, the relationship between catechol siderophore production and virulence in the natural ruminant host appears to be much different for this bacterium. Consequently, it will be imperative to use genetic approaches to establish the individual contributions of 2,3-DHBA and

brucebactin production to the virulence of *B. abortus* 2308 in pregnant cattle, and to more precisely define the roles that these catechol siderophores play in erythritol metabolism.

4. Transport of Fe^{+++}-2,3-DHBA and Fe^{+++}-Brucebactin Into *Brucella*

Fe^{+++}-siderophore complexes, also known as ferrisiderophores, are typically transported across the inner and outer membranes of Gram negative bacteria by two separate energy-dependent transport systems (Earhardt 1996). For instance, the Fe^{+++}-enterochelin complex is transported across the outer membrane of *E. coli* via the enterochelin receptor FepA (Lundigran and Kadner, 1986). The TonB-ExbB-ExbD complex provides energy for this process. This periplasm-spanning complex allows outer membrane receptors to utilise energy from the proton motive force generated at the cytoplasmic membrane as a conseqnence of respiratory metabolism (Letain and Postle, 1997). Once in the periplasm, the Fe^{+++}-enterochelin complex is bound by the periplasmic binding protein FepB (Stephens *et al.*, 1995). This protein, in turn, transfers the ferrisiderophore complex to the inner membrane ABC-type transport system composed of FepC, D and G, which mediates its transport into the cytoplasm, where the iron is subsequently released from the siderophore (Shea and McIntosh, 1991). This combination of periplasmic binding protein and cognate ABC transporter is typical of the systems that transport ferrisiderophores across the cytoplasmic membrane (Braun *et al.*, 1998).

Although the *B. abortus* 2,3-DHBA biosynthesis genes have been characterised (Bellaire *et al.* 2003a), the genes encoding the machinery that transports the Fe^{+++}-2,3-DHBA complex into the bacterial cell have yet to be identified. Employing respiration inhibitors, López-Goñi *et al.* (1992) demonstrated that transport of $^{55}Fe^{+++}$-2,3-DHBA into *B. abortus* is an energy-dependent process. These studies

Table 1. Putative iron transport genes located in the *Brucella melitensis* 16M and *Brucella suis* 1330 genome sequences

Predicted gene product[1]	***B. melitensis/B. suis* gene designations**
TonB-dependent outer membrane ferrisiderophore transporters	
CirA	BMEI0657/BR1347
Fiu	BMEII0297/BRA0999
Periplasmic binding protein-dependent cytoplasmic membrane ferrisiderophore transporters	
FepB	BMEI0658/BR1346
FepD	BMEI0659/BR1345
FepC	BMEI0660/BR1344
FatB	BMEII0607/BRA0675
FatC	BMEII0605/BRA0677
FatD	BMEII0606/BRA0676

FatE	BMEII0604/BRA0678
TonB-dependent outer membrane heme transporter	
BhuA	BMEII0105/BRA1190
Periplasmic binding protein-dependent cytoplasmic membrane heme transporter	
ShuT	BMEII0535/BRA0756
ShuU	BMEII0536/BRA0755
ShuV	BMEII0537/BRA0754
Periplasmic binding protein-dependent cytoplasmic membrane ferric iron (Fe^{+++}) transporters	
SfuA	BMEII0565/BRA0720
SfuB	BMEII0566/BRA0719
SfuC	BMEII0567/BRA0718
SfuA	BMEII0584/BRA0700
SfuB	BMEII0585/BRA0699
SfuC	BMEII0583/BRA0701
Cytoplasmic membrane ferrous iron (Fe^{++}) transporters	
CorA	BMEI1374/BR0559
MntH	BMEI0569/BR1441
Cytoplasmic membrane-outer membrane energy transduction system	
ExbB	BMEI0365/BR1666
ExbD	BMEI0364/BR1667
TonB	BMEI0363/BR1668

[1] Based on genome annotation and/or amino acid identity and the presence of conserved domains detected by BLAST analysis against sequences in the NCBI database.

also showed that transport of Fe^{+++}-2,3-DHBA into *B. abortus* has some unusual properties in comparison with other bacterial ferrisiderophore transport systems. First of all, these investigators were unable to detect the presence of iron-repressible outer membrane proteins in *B. abortus*. They also noted that *B. abortus* cultures took up $^{55}Fe^{+++}$-2,3-DHB A at equivalent rates regardless of whether they were previously grown in iron-deprived or iron-replete conditions. These findings led López-Goñi *et al.* (1992) to postulate that Fe^{+++}-DHBA might cross the outer membrane of *B. abortus* via a non-specific transport system (e.g. a porin) or by means of the receptor-independent "hydrophobic pathway" (Hancock, 1984), with subsequent transport into the cytoplasm via a typical energy-dependent ferrisiderophore transporter in the cytoplasmic membrane. Such a scenario certainly appears to be plausible based on the small size of 2,3-DHBA, its hydrophobic properties and the unusual hydrophobicity profile of the *B. abortus* outer

membrane (Moriyón and López-Goñi, 1998). The recent demonstration that a *B. melitensis exbB* mutant cannot use 2,3-DHBA as a siderophore (Danese, 2002), however, indicates that transport of this catechol across the outer membrane requires energy. More importantly, this finding argues for the participation of a typical TonB-linked outer membrane ferric siderophore receptor (van der Helm, 1998) in this process.

Surveys of the *B. melitensis* 16M and *B. suis* 1330 genomes reveal two strong candidates for genes encoding TonB-dependent outer membrane transporters for Fe^{+++}-2,3-DHBA. These genes encode homologues of the CirA (Griggs *et al.,* 1987) and Fiu (Hantke 1983) proteins of *E. coli* (Table 1). Both CirA and Fiu are members of the TonB-dependent ferrisiderophore receptor family of proteins (van der Helm 1998), but more importantly, CirA and Fiu are responsible for the transport of Fe^{+++}-2,3-DHBA across the outer membrane of *E. coli* (Hantke 1990). The *Brucella cirA* homologue also appears to be organised in an operon with a set of genes predicted to encode members of the FepB, FecCD and FepC families of proteins, which make up the periplasmic-binding protein (FecB) and associated ABC transporters (FecCD and FepC) for a variety of bacterial cytoplasmic membrane ferric siderophore transport systems (Braun *et al.,* 1998). A second set of genes in the *B. melitensis* 16M and *B. suis* 1330 genomes whose products may be involved in binding the Fe^{+++}-2,3-DHBA in the periplasm and transporting this complex across the cytoplasmic membrane are homologous of the *Vibrio anguillarum fatB, C, D* and *E* (Table 1). The products of these latter genes transport Fe^{+++} bound to the 2,3-DHBA-based catechol siderophore anguibactin across the cytoplasmic membrane of *Vibrio anguillarum* (Actis *et al.,* 1988).

The *cirA* and *fiu* homologues represent two of the three genes in the *B. melitensis* 16M and *B. suis* 1330 genomes predicted to encode TonB-dependent outer membrane receptors. The third gene in this group most likely encodes a heme receptor (see next section). This raises an interesting question with regard to the transport of Fe^{+++}-brucebactin across the outer membrane. Specifically, if brucebactin turns out to be a monocatechol, then it is quite possible that the same TonB-dependent outer membrane transporters that shuttle Fe^{+++}-2,3-DHBA across the outer membrane will also transport Fe^{+++}-brucebactin. This is the case in *E. coli*, where CirA and Fiu have the capacity to transport both Fe^{+++}-2,3-DHBA and Fe^{+++}-2,3-DHBA-Ser (Earhardt, 1996). If brucebactin is a polycatechol, then it becomes more difficult to find a good candidate for a gene encoding an outer membrane receptor for Fe^{+++}-brucebactin in the *B. melitensis* and *B. suis* genomes. Transport of the polycatechol ferrisiderophores across the outer membrane of Gram negative bacteria typically requires the presence of a highly specific outer membrane transporter (Braun *et al.* 1998), and these proteins usually display significant levels of amino acid identity with the *E. coli* enterochelin receptor FepA (Lundrigan and Kadner, 1986). BLAST analyses aimed at identifying *fepA* homologues in the *B. melitensis* 16M and *B. suis* 1330 genomes yields only one good candidate in each genome, the *Brucella cirA* homologues BMEI0657 and BR1347.

5. Evidence for Other Iron Acquisition Systems in The *Brucella* spp.

5.1. Utilisation of Heme as an Iron Source

Experimental evidence indicates that *B. abortus* 2308 and *B. melitensis* 16M can both use heme as an iron source *in vitro* (Bellaire, 2001; Danese, 2002). The capacity of these organisms to utilise heme could be especially beneficial to their intracellular lifestyle, since this compound is thought to a readily available iron source for microbes that survive and replicate within mammalian cells (Genco and Dixon, 2001). Moreover, macrophages, the primary cells that the brucellae inhabit within their mammalians hosts, are responsible for the phagocytosis and degradation of senescent erythrocytes, a process that liberates heme (Bratosin *et al.*, 1998). Ruminant placental trophoblasts also take up erythrocytes from the maternal circulation and degrade these cells and in doing so provide iron to the developing foetus (Anderson *et al.*, 1986a). Consequently, heme may be a relevant iron source for *B. abortus* and *B. melitensis* strains during replication in the ruminant reproductive tract.

Open reading frame (ORF) BMEII0105 in the *B. melitensis* 16M genome and ORF BRA1190 in the *B. suis* 1330 genome are predicted to encode homologues of ShuA and ChuA, TonB-dependent heme transporters that reside in the outer membranes of *Shigella dysenteriae* and *Escherichia coli*, respectively (Torres and Payne, 1997; Wyckoff *et al.*, 1998) (Table 1). When the corresponding gene is deleted from *B. abortus* 2308 by gene replacement, the resulting mutant cannot use heme as an iron source when grown in the presence of the chelator EDDA (J. T.Paulley, unpublished). This mutant retains its capacity to use 2,3-DHBA, ferric dicitrate and $FeCl_3$ as iron sources under these conditions, however. Based on these experimental findings we have given the *Brucella shuA* homologue the designation *bhuA* (for *Brucella* heme uptake). Experiments designed to confirm the role of *Brucella bhuA* in heme transport and to assess the potential role of this gene in virulence are underway.

Once heme is transported across the outer membrane of Gram negative bacteria, this compound is bound by a periplasmic binding protein and transported across the cytoplasmic membrane by a transport system closely resembling that used for the transport of ferrisiderophores (Braun *et al.*, 1998). In some bacteria, the genes encoding the heme receptor and cognate periplasmic binding protein-dependent transport system reside in the same operon, but in others these genes reside in different locations. The latter situation would appear to be the case with the *Brucella* spp. as homologues of the *Shigella* heme uptake genes *shuT, U* and *V* (Wyckoff *et al.*, 1998) are also present in *B. melitensis* 16M and *B. suis* 1330 (Table 1), but they do not lie in close proximity to *bhuA* in either genome.

There is also indirect evidence suggesting that members of the Omp25/31 class of outer membrane proteins (Salhi *et al.*, 2003) could potentially play a role in heme utilisation by the *Brucella* spp. (Table 1). The homologous HbpA protein in *Bartonella quintana* and *Bartonella henselae* binds heme directly in *in vitro* assays (Carroll *et al.*, 2000; Zimmerman *et al.*, 2003), and expression of the *B. henselae hbpA* gene in the *E. coli hemA* mutant EB53 allows this strain to transport heme and relieve its heme

auxotrophy (Zimmerman *et al.*, 2003). Studies with *B. abortus, B. melitensis* and *B. ovis omp25* mutants in experimental animals indicate that the Omp25/Omp31 proteins are important for virulence (Edmonds *et al.*, 2001; 2002a; 2002b), but a specific role for these proteins in heme utilisation has not been described.

5.2. Utilisation of Ferric Dicitrate as an Iron Source

Ferric dicitrate serves as an efficient iron source for *B. abortus* 2308 and B. *melitensis* 16M *in vitro* (Bellaire, 2001; Danese, 2002). The capacity of the brucellae to use ferric dicitrate as an iron source may have important implications with regard to the intracellular lifestyle of these strains. While much remains unknown about the composition of the labile iron pool in host cells, it is believed that citrate is an important transitional chelator of internalised iron (Olakanmi *et al.*, 2002; Petrat *et al.*, 2002), and thus, ferric dicitrate could serve as a readily available source of iron for intracellular pathogens. In *E. coli*, ferric dicitrate is transported across the outer membrane by the TonB-dependent transporter FecA (Pressler *et al.*, 1988). Unlike the iron transport systems that transport heme and ferrisiderophores across both membranes of Gram negative bacteria as intact complexes, the ferric dicitrate transport system in *E. coli* removes the Fe^{+++} from the Fe^{+++}-dicitrate complex in the periplasm and transports free Fe^{+++} across the cytoplasmic membrane, with the citrate being released into the periplasm (Braun *et al.*, 1998).

Although no gene predicted to encode a clear cut FecA homologue could be found in the *B. melitensis* 16M and *B. suis* 1330 genomes, these genomes do contain two sets of genes encoding homologues of the SfuA, B and C class of iron transporters (Table 1). This transport system was originally described in *Serratia marcescens*, where SfuA binds free Fe^{+++} in the periplasm and SfuB and C transport this cation across the cytoplasmic membrane (Angerer *et al.*, 1992). More importantly, recent experimental evidence suggests that Fe^{+++} released from ferric dicitrate in the periplasmic compartment of Gram negative bacteria, and possibly the ferric dicitrate complex itself, represent important substrates for the SfuABC type transporters (Braun *et al.*, 1998; Shouldice *et al.*, 2003). Based on the potential relevance of ferric dicitrate as an iron source for the brucellae *in vivo*, it is particularly notable that one of the *Brucella sfuA* homologues was identified by Eskra *et al.* (2001) in a screen for genes that are differentially expressed by *B. abortus* 2308 during residence in host macrophages.

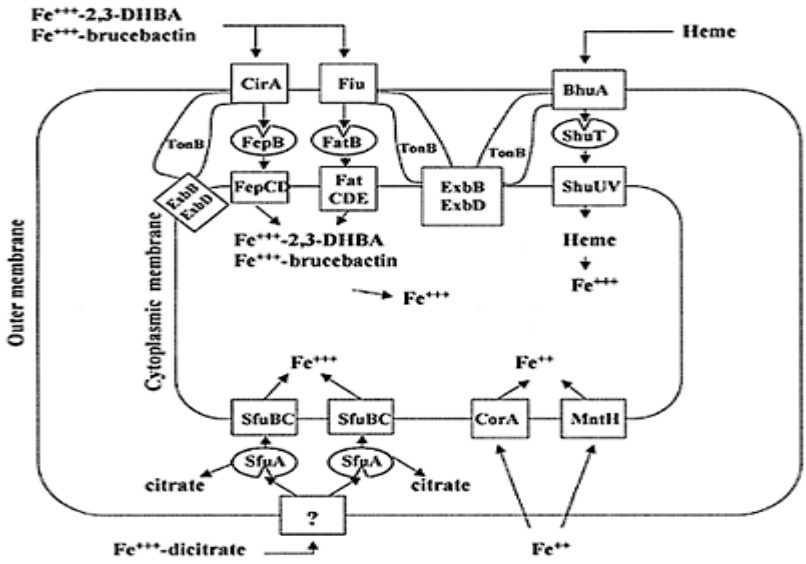

Figure 2. Hypothetical iron transport systems for Fe^{++}-brucebactin, Fe^{+++}-2,3-DHBA, heme, Fe^{+++}-dicitrate and Fe^{++} in the *Brucella* spp.

5.3. Utilisation of Ferrous Iron

Although iron exists primarily in the relatively insoluble ferric form, Fe^{+++}, under aerobic conditions at physiologic pH (e.g. ≈7.0), as the oxidation/reduction potential of the surrounding medium drops or the medium becomes acidic, the more soluble ferrous form of this cation, Fe^{++}, becomes predominant. Since the brucellae reside within an acidified phagosome, at least during the initial stages of infection (Porte *et al.*, 1999; Köhler *et al.*, 2003), Fe^{++} may represent a biologically relevant iron source for these bacteria *in vivo*. Although the *B. melitensis* 16M and *B. suis* 1330 genomes do not contain homologues of *feoAB*, the genes encoding the prototypic ferrous iron transport system described in the enterics and other bacteria (Kammler *et al.*, 1993), these genomes do contain genes homologous to those encoding divalent cation transporters that reportedly have the capacity to transport Fe^{++} across the cytoplasmic membrane (Table 1). The first of these is a *corA* homologue. CorA is a relatively non-specific divalent cation transporter that resides in the cytoplasmic membrane of *E. coli* and *Salmonella typhimurium* and is involved in the uptake of Mg^{++} Mn^{++}, Co^{++} and Fe^{++} (Hantke 1997). The other *Brucella* gene that may function in Fe^{++} transport is a homologue of the *Salmonella mntH*, which encodes a cytoplasmic membrane transporter that reportedly participates in the uptake of both Mn^{++} and Fe^{++} (Makui *et al.*, 2000).

A diagram depicting the iron transport systems predicted to be present in the *Brucella* spp. is shown in Figure 2.

6. Regulation of *Brucella* Iron Acquisition Genes

Although the brucellae require iron for growth and replication, experimental evidence suggests that like most organisms they strictly regulate their iron acquisition genes in response to cellular iron levels. For instance, the initial studies described by López-Goñi *et al.* (1992) indicated that *B. abortus* 2308 only produces 2,3-DHBA in response to iron deprivation *in vitro,* and subsequent studies have shown that transcription of the *dhbCEBA* operon is repressed during growth under iron-replete conditions (Bellaire *et al.,* 2003a). Tight regulation of iron acquisition genes is observed in both eukaryotes and prokaryotes (Eisenstein and Blemings, 1998; Ratledge and Dover, 2000; Fillebeen and Pantopoulos, 2002; Andrews *et al.,* 2003), and this serves to diminish the potential for iron toxicity that can result from intracellular Fenton chemistry and the subsequent production of hydroxyl radicals. Preventing iron overload may be especially important for the *Brucella* spp. in the host, as experimental evidence indicates that oxidative killing is one of the primary brucellacidal mechanisms employed by macrophages (Jiang and Baldwin, 1993).

6.1. Fur

In most bacteria, the iron responsive ferric uptake regulator (Fur) (Escolar *et al.,* 1999) or a functional homologue of this protein [e.g. DtxR in *Corynebacterium diphtheriae* (Tao *et al.,* 1994) or IdeR in *Mycobacterium tuberculosis* (Rodriguez and Smith, 2003)] plays a central role in ensuring that siderophore biosynthesis and iron transport genes are only expressed when the bacterium experiences iron deprivation. When intracellular iron levels reach a threshold, Fe^{++} binds to Fur, DtxR and IdeR and these proteins serve as transcriptional repressors, binding in or near the promoter regions of iron acquisition genes, preventing their transcription. When intracellular iron levels become limiting, Fur, DtxR and IdeR are inactive. The *B. abortus fur* (GenBank accession no. AF023177) (Phillips *et al.,* 1996) has been cloned and characterised, and the corresponding genes have been given the designations BMEI0375 in the *B. melitensis* 16M genome and BR1654 in the *B. suis* 1330 genome. The *Brucella* Fur functions in an iron-responsive manner in the *E. coli fur* mutant H1780 (Hantke, 1987), but surprisingly, an isogenic *fur* mutant constructed from *B. abortus* 2308 displays wild type repression of both *dhbCEBA* transcription and 2,3-DHBA production during growth under iron replete conditions (Figure 3). These experimental findings are particularly intriguing considering the presence of the putative Fur boxes in the *dhbCEBA* promoter region and the fact that cloned copies of the *dhbCEBA* promoter region are positive in the Fur titration assay (Stojiljkovic *et al.,* 1994). It seems clear from these observations, however, that Fur is not the predominant iron-responsive

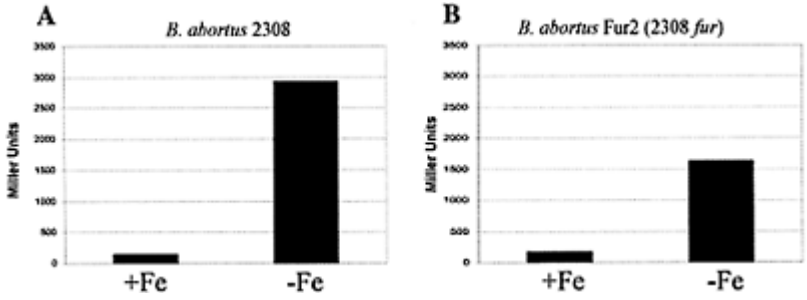

Figure 3. Transcription of *dhbC* in *B. abortus* 2308 (A) and the isogenic *fur* mutant Fur2 (B) following growth under iron-deprived (−Fe) and iron replete (+Fe) conditions. −Fe represents growth in the low iron minimal medium described by López-Goñi *et al.* (1992) and +Fe represents growth in this same medium supplemented with 50 µM $FeCl_3$.

regulator of the 2,3-DHBA biosynthesis operon in *B. abortus* 2308. What role, if any, Fur plays in the iron-responsive regulation of *dhbCEBA* and other *Brucella* iron acquisition genes remains to be determined.

6.2. RirA and Irr

The *Brucella* spp. are α-proteobacteria, and they are closely related phylogenetically to the rhizobia (Moreno *et al.*, 1990). Homologues of the *Rhizobium leguminosarum rirA* (Todd *et al.*, 2002) and *Bradyrhizobium japonicum irr* (Hamza *et al.*, 2000) genes can be found in the genomes of *B. melitensis* 16M and *B. suis* 1330, and it is possible that the products of one or both of these genes participate in the tight iron-responsive regulation of the *Brucella dhbCEBA* operon in a *fur* background. RirA (rhizobial iron regulator A) is a 17 kDa protein that displays no sequence homology with Fur, DtxR or IdeR, yet this protein demonstrates Fur-like repression of a myriad of iron uptake genes in response to cellular iron levels in *R. leguminosarum* (Todd *et al.*, 2002). Interestingly, *R. leguminosarum* possesses an authentic Fur homologue that functionally restores wild type iron-responsive gene regulation in an *E. coli fur* mutant. Genetic analysis of *R. leguminosarum fur* and *rirA* mutants, however, indicates that RirA is the predominant iron-responsive regulator of the iron acquisition genes in this bacterium, while Fur plays a minimal, if any, role in this process (Wexler *et al.*, 2003). The precise biochemical mechanism by which RirA accomplishes iron-responsive regulation of gene expression in *R. leguminosarum* is presently unknown.

The *B. japonicum* Irr (iron responsive regulator) is a transcriptional repressor that regulates the expression of *hemB*, a gene involved in heme biosynthesis, in response to cellular iron levels (Hamza *et al.*, 1998). The accumulation of protoporphyrins lacking iron can be toxic for cells, and it is thought that Irr serves to maintain the appropriate balance between protoporphyrin synthesis and iron availability in *B. japonicum*. Irr is of approximately the same size and shares significant amino acid identity with Fur, but Irr responds to cellular iron levels in a manner different than that of Fur. Specifically, Irr binds to the *hemB* promoter and represses transcription when cellular iron levels are low (as opposed to Fur binding its cognate promoters when cellular iron levels are high). In fact, when cellular iron levels exceed a threshold, Irr is inactivated and degraded. Notably, in addition to its role in regulating expression of the *hemB* gene, there is also evidence that Irr regulates the production of an uncharacterised iron transport system in *B. japonicum* (Hamza *et al.*, 1998).

As more studies describing the nature of iron-responsive gene regulation in the α-proteobacteria are being reported, it is becoming apparent that multiple transcriptional repressors can participate in this regulation. This is in contrast to the predominant role played by Fur or Fur homologues such as DtxR or IdeR in iron-responsive gene expression in other bacteria. The hierarchy of these multiple iron-responsive gene regulators also appears to differ considerably between the two α-proteobacteria that have been best studied in this regard. In *B. japonicum*, Fur and Irr both appear to play major roles in controlling gene expression in response to cellular iron levels (Hamza *et al.*, 1999), while in *R. leguminosarum* RirA appears to be the major regulator of iron-responsive gene expression (Wexler *et al.*, 2003). The wild type iron-responsive regulation of the *B. abortus dhbCEBA* operon in a *fur* mutant also suggests Fur is not the dominant iron-responsive regulator in the *Brucella* spp. Obviously, it will be informative to determine the relative contributions of Fur, RirA and Irr in regulating the iron metabolism genes in *Brucella*. The results of such studies will not only provide us with important information regarding the regulatory mechanisms employed by the brucellae to prevent iron toxicity, but they may also provide use with some interesting insights regarding the parallels between the intracellular lifestyles of the brucellae and the rhizobia in their respective mammalian and plant hosts.

6.3. DhbR

AlcR (Beaumont *et al.*, 1998; Pradel *et al.*, 1998), PchR (Heinrichs and Poole, 1993) and YbtA (Fetherston *et al.*, 1996) are AraC-like transcriptional activators that stimulate the transcription of siderophore biosynthesis and uptake genes in *Bordetella, Pseudomonas* and *Yersinia*. The cognate siderophore serves as a co-activator for AlcR (alcaligin), PchR (pyochelin) and YbtR (yersiniabactin) and expression of the corresponding genes is regulated Fur. This latter property provides a mechanism for regulating the expression of the genes encoding these transcriptional activators in response to cellular iron levels. ORFs BMEII0104 in the *B. melitensis* 16M genome and BRA1192 in the *B. suis* genome are predicted to encode AlcR homologues. The corresponding gene has been cloned from *B. abortus* 2308 (E.Anderson, unpublished), and genetic evidence indicates that the product of this gene is required for optimal *dhbCEBA* expression and 2,3-DHBA production in response to iron limitation *in vitro*. Based on these findings, this gene has

been given the designation *dhbR* (*dhbCEBA* regulator) to conform to the nomenclature used for its counterparts in *Bordetella, Pseudomonas* and *Yersinia*. Wild type repression of *dhbCEBA* transcription is still seen in a *B. abortus dhbR* mutant under iron-replete culture conditions (E.Anderson, unpublished), indicating that iron-responsive regulators other than DhbR and Fur (see above) coordinate the expression of the 2,3-DHBA biosynthesis genes in accordance with cellular iron levels.

7. Conclusions

Experimental evidence and surveys of the genome sequences of *B. melitensis* 16M and *B. suis* 1330 indicate that the brucellae are well-equipped to meet their physiologic needs for iron during residence in their mammalian hosts. Different sets of iron acquisition genes appear to be important during the chronic stage of the infection, when macrophages are the predominant host cell, and during the acute stage of the infection in the natural host, when placental trophoblasts become a major site of intracellular residence for the brucellae. The *Brucella* spp. also appear to utilise iron responsive regulators other than, or in addition to, Fur to control expression of their iron acquisition genes, thereby reducing the potential for oxidative damage resulting from iron overload. The availability of genome sequence data has allowed us to make reasonable predictions regarding the nature of the iron acquisition pathways used by the brucellae during their residence in the mammalian host, but establishing the functionality of these pathways and their role in virulence will require experimental verification.

8. Acknowledgements

The authors thank our collaborators on the *Brucella* iron studies, Cynthia Baldwin and Philip Elzer, for making these studies possible, and the United States Department of Agriculture's National Research Initiative Competitive Grants Program for providing financial support for this work. We also thank Ignacio López-Goñi for his advice and encouragement during the course of these studies, and the members of the García-Lobo and Letesson laboratories for their willingness to maintain open dialogs between our research groups as our independent studies in this area progress. We also thank the many other investigators who have helped us in our efforts to elucidate the iron acquisition strategies employed by the brucellae, a list of which is too extensive to document here.

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Chapter 13
Brucella Interaction with Membrane Lipids of the Host Cell

Masahisa Watarai*

From: *Brucella:* Molecular and Cellular Biology. Edited by: Ignacio López-Goñi and Ignacio Moriyón

Abstract

Brucella species are facultative intracellular bacteria capable of surviving inside macrophages and are thought to actively modify their phagosomes to avoid lysosomal fusion for intracellular survival. Both entry and intracellular growth of *Brucella* are dependent on interaction with microdomains of the cellular membranes. These microdomains, commonly referred to as lipid rafts, are enriched in glycosylphosphatidylinositol (GPI)-anchored proteins, glycosphingolipids and cholesterol. Lipid raft-associated molecules are selectively incorporated into phagosomes containing *Brucella* and treatment of raft-disrupting agents inhibits bacterial internalisation and intracellular replication. As lipid rafts participate in the signalling pathway in immune cells, entry processes associated with lipid rafts may lead *Brucella* into compartments that avoid fusion with the lysosomal network in the early stage of infection. Plasma membrane cholesterol of macrophages is also required for bacterial proliferation in mice. Thus, lipid raft microdomains not only influence bacterial internalisation and intracellular replication, but also contribute to the establishment of *Brucella* infection.

1. Introduction

Phagocytosis by professional phagocytes, such as macrophages and neutrophils, is the initial step in the degradation of dying cells, inert particles and live infectious agents. It has a critical role in essential biological functions, including inflammation, immunity and development (Lopes *et al.,* 2000). Inert particles are captured by macrophages by using Fc receptors for immunoglobulin-opsonized particles, complement receptors for

complement-opsonized particles, mannose receptor, scavenger receptor and integrins. Several intracellular pathogens can use these surface receptors to internalise into phagocytic host cells, either as a means to avoid the host immune system, or as an integral part of their replicative cycle (Virji, 1996). For many bacterial pathogens, adherence host tissue is believed to be essential for virulence, and the microbial characteristics that promote adherence to receptors on a host cell surface are considered to be attributes of virulence (Casadevall and Pirofski, 2001). After uptake by macrophages, inert particles are found in a membrane-derived phagosome, which undergoes a maturation process

* For correspondence email watarai@obihiro.ac.jp

into a hydrolase-rich phagolysosome. In contrast, intracellular pathogens control the membrane-derived phagosomes, circumventing host defences and further degradation, thus transforming their compartments into replicative phagosomes (Meresse *et al.,* 1999).

Brucella species are facultative intracellular pathogens that survive in a variety of cells, including macrophages, and their virulence and chronic infections are thought to be due to their ability to avoid the killing mechanisms within macrophages (Baldwin and Winter, 1994; Sangari and Aguero, 1996). Inhibition of phagosome-ly sosome fusion has been proposed as a mechanism for intracellular survival of *Brucella* in professional and non-professional phagocytes (Frenchick *et al.,* 1985; Pizarro-Cerdá *et al.,* 1998a; 1998b; Arenas et al., 2000). The mechanisms of entry into host cells and signal transduction, especially interaction between cellular receptors and bacterial ligands, may include these events. However, little is known about bacterial effectors and cellular targets that control *Brucella* phagosome maturation.

Several pathogens, including *Brucella,* use cholesterol-enriched microdomains, so-call lipid rafts, to infect host cells (Duncan *et al.,* 2002). The following sections of this chapter review selected aspects of interaction between *Brucella* and lipid raft-associated molecules on the plasma membrane when bacteria infect into macrophages, and then discuss the correlates of proliferation in mice that have been used in virulence assay.

2. Cholesterol Metabolism in Macrophages

Cells maintain a cholesterol gradient across the secretory system, with the lowest concentrations in the endoplasmic reticulum (ER) and the highest in the plasma membrane. Cholesterol is heterogeneously distributed in the endocytic pathway. Little is known about how this heterogeneous distribution of cholesterol is maintained despite continuous vesicular traffic between organelles (Liscum and Munn, 1999).

2.1. Intracellular Cholesterol Transport Pathway

Low density lipoprotein (LDL) is internalised by the LDL receptor and is delivered to late endosomes, where cholesterol esters are hydrolysed. Studies of cellular trafficking of cholesterol derived from the metabolism of LDL show that after hydrolysis of LDL cholesteryl ester in lysosomes, most LDL-derived cholesterol traffics to the plasma

membrane (Brasaemle and Attie, 1990; Liscum and Munn, 1999). The molecular basis of these transport processes is not fully known, but the protein that is defective in Niemann-Pick C disease, called NPC1, clearly has a role (Pentchev *et al.*, 1995; 1997; Kobayashi *et al.*, 1999; Cruz *et al.*, 2000). Recent studies have suggested that free cholesterol is first transported to the plasma membrane and then to a type of endocytic vesicle in an NPC1-independent process, followed by NPC1-dependent trafficking either back to the plasma membrane or to other peripheral organelles (Cruz *et al.*, 2000). Other molecules that may facilitate free cholesterol trafficking include lysophosphatidic acid (Kobayashi *et al.*, 1999), a protein called NPC2 that has not yet been cloned but is defective in a rare type of Niemann-Pick C disease (Pentchev *et al.*, 1995; 1997).

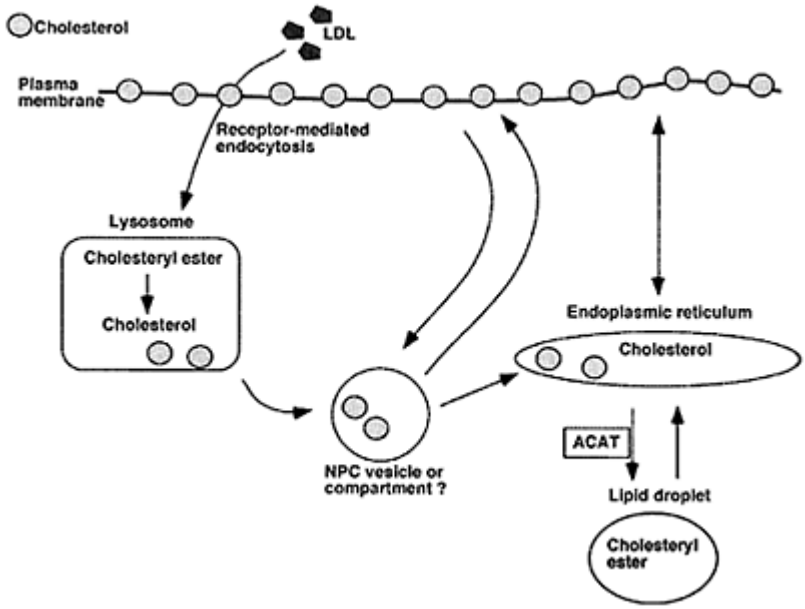

Figure 1. Schema of cholesterol trafficking in macrophages.

Excess plasma membrane cholesterol then enters the ER, where the cholesterol is re-esterified by acyl coenzyme A cholesterol acyltransferase (ACAT) and is stored in cellular lipid droplets (Brown *et al.*, 1980) (Figure 1). Trafficking of free cholesterol to ACAT is energy-dependent, requires an intact actin cytoskeleton, and probably includes vesicular transport (Tabas, 1997). ACAT is stimulated when cellular-free cholesterol levels reach a certain threshold level above the ambient cellular cholesterol concentration (Xu and Tabas, 1991; Okwu *et al.*, 1994).

2.2. Cholesterol-rich Microdomains

Lipid rafts are dynamic assemblies of proteins and lipids that float freely within the liquid-disordered bilayer of cellular membranes, but they can also cluster to form larger, ordered platforms (London and Brown, 2000; Simons and Toomre, 2000). Cholesterol and lipids are not homogeneously distributed within membrane bilayers. Cholesterol-rich and sphingolipid-rich microdomains, lipid rafts, resistant to solubilisation at low temperature by non-ionic detergents such as Triton X-100, may have an important role in cholesterol transport (Simons and Ikonen, 1997; Ikonen, 2001). The association with detergent-resistant membranes (DRMs) is a useful criterion to estimate if a protein associates with lipid rafts (London and Brown, 2000). Constitutive raft residents include glycophosphatidylinositol (GPI)-anchored proteins, doubly acylated proteins (such as tyrosine kinases of the Src family, heterotrimeric G proteins, and endothelial nitric oxide synthase [eNOS]), cholesterol-linked and palmitate-anchored proteins (Simons and Toomre, 2000). A model for the organisation of lipid rafts associates sphingolipids laterally with one another through weak interaction between the carbohydrate heads of the glycosphingolipids (Simons and Ikonen, 1997). In this model, any space between associating sphingolipids is filled by cholesterol, which is present in both membrane leaflets, and functions as a spacer. The close-packed sphingolipid-cholesterol clusters behave as assemblies within the exoplasmic leaflet, where the intervening fluid regions are occupied by unsaturated phosphatidylcholine molecules (Simons and Ikonen, 1997). The function of lipid rafts could be to select and concentrate molecules in a micro-environment of the membrane as kinases and their substrates for signal transduction or as cargo molecules and in a transport mechanism for trafficking. This implies that determinants must exist that allow the specific inclusion or exclusion of proteins from the microdomains (London and Brown, 2000; Simons and Toomre, 2000). Such compartmentalisation may help the signals achieve the required threshold at the physiological concentrations of the stimuli.

3. Lipid Rafts on *Brucella* Phagosomes

3.1. *B. abortus* Induces Macropinocytosis of Macrophages

Time-lapse videomicroscopy has been used to follow the internalisation of *B. abortus* strains by mouse bone marrow-derived macrophages (Watarai *et al.,* 2002b). After contact of macrophages with wild type *B. abortus,* the bacteria move round from the site of initial bacterial contact with the macrophage (Kim *et al.,* 2002; Watarai *et al.,* 2002b). Bacterial swimming on the macrophage surface often lasts for up to several minutes with generalised plasma membrane ruffling before eventual enclosure in large vacuoles. Contact of the *virB4* mutant of *B. abortus* with the target macrophage, in contrast, results in much smaller ruffling restricted to the area near the bacteria. The ruffles associated with internalisation of the *virB4* mutant results in a more rapid uptake than for the wild type strain. If the bacteria are deposited onto macrophages by centrifugation, and are stained with phalloidin to detect actin filament formation by using fluorescence

microscopy, the wild type strain shows generalised actin polymerisation around the site of bacterial binding; this can also be observed by phase-contrast microscopy. The *virB4* mutant shows primarily small regions of phalloidin staining at the sites of binding. Therefore, *B. abortus* appears to promote events on the macrophage cell surface that are dependent on the presence of the VirB system.

Time-lapse videomicroscopy has shown that the bacteria appear to internalise into large vacuoles after induction of generalised membrane ruffling. Further analysis of mouse bone marrow-derived macrophages incubated briefly with *B. abortus* and fixing, has shown that the wild type strain of bacteria is in large concentric vacuoles that are similar in morphology to fluid-filled macropinosomes (Swanson, 1989). Macrophages incubated simultaneously with *B. abortus* and the fluid phase marker tetramethyl rhodamine isothiocyanate (TRITC)-dextran accumulate the marker in large vacuoles containing the wild type strain but little or no marker accumulates in phagosomes containing the *virB4* mutant. Similarly, phase contrast micrographs have shown the wild type strain in large phase-transparent compartments, but the *virB4* mutant is in much smaller compartments (Kim *et al.*, 2002; Watarai *et al.*, 2002b).

The differences in rate of phagocytosis and the formation of macropinosomes for the wild type and mutant strain have been quantify microscopically at various times of incubation. In macropinosome formation, by 15 min after deposition of the wild type strain, more than 50% of the bacteria are in large compartments containing the fluid phase marker that can be detected by phase contrast microscopy. Later, macropinosomes are difficult to see with few bacteria co-staining with the fluid phase marker at 35 min after infection. Phagosomes containing the *virB4* mutant, however, are relatively devoid of the fluid phase marker (Kim *et al.*, 2002; Watarai *et al.*, 2002b).

Salmonella typhimurium internalised by mouse bone marrow-derived macrophages is accompanied by macropinosome formation, and the persistence of these phagosomes, which are spacious, correlates with the ability of the microorganism to survive intracellular killing mechanisms (Alpuche-Aranda *et al.*, 1994). These spacious phagosomes dilute toxic compounds, thus allowing *Salmonella* to initially survive. When combined with a delay in acidification of this phagosome, the bacterium may create a tolerable intracellular environment (Alpuche-Aranda *et al.*, 1994). In the case of *B. abortus*, macropinosomes are induced transiently and shrink rapidly, with the majority of vacuoles appearing tightly opposed against the bacterial surface within 20 min after their initial appearance (Kim *et al.*, 2002; Watarai *et al.*, 2002b). Macropinocytosis occurs within minutes of attachment to bacteria on the surface of the macrophage. Presumably, effector molecule(s) are translocated by the VirB system to the target cell during bacterial contact, initiating the process that leads to formation of the macropinosome.

3.2. *B. abortus* Macropinosomes Avoid Fusion with Other Macropinosomes

To examine the intracellular trafficking of macropinosomes containing *B. abortus*, macrophages are preloaded for 30 min with fluid phase marker before adding *B. abortus*, and infection proceeds in the presence or absence of the marker. As expected, if the bacteria are added in the continued presence of the fluid phase marker, co-localisation of the marker with the wild type strain is similar to no pre-loading the marker before

infection. The behaviour of the *virB4* mutant, however, is different than in the previous method. At 15 min after infection, the fluid phase marker transiently co-localises with the *virB4* mutant, suggesting that the marker accumulates in an intracellular compartment that fuses with the *virB4* mutant but not with the wild type strain. This can be confirmed by washing out the fluid phase marker from the medium before adding the bacteria: co-localisation of the marker with wild type strain is almost completely lost. In contrast, removing the fluid phase marker before adding the *virB4* mutant give results similar to continued incubation of the fluid phase marker during infection, with transient co-localisation of the marker (Watarai *et al.,* 2002b). These results are consistent with the wild type strain being simultaneously ingested with the marker into a fluid-filled phagosome that resists fusion with marker-filled compartments. In contrast, phagosomes containing the *virB4* mutant form without simultaneous ingestion of the fluid phase marker, but can fuse with marker-filled compartments after internalisation. These results suggest that macropinosomes containing wild type *B. abortus* avoid fusion with other macropinosomes in macrophages.

3.3. Accumulation of Lipid Rafts on *B. abortus* Macropinosomes

Aerolysin from *Aeromonas hydrophila*, which binds to the GPI moiety of GPI-anchored proteins on the cell surface (Abrami *et al.,* 1998; Wang *et al.,* 1999), affect the replicative phagosomes containing *B. abortus* (Pizarro-Cerdá *et al.,* 1998a). To find if GPI-anchored proteins are incorporated into macropinosomes containing *B. abortus*, the macropinosomes are probed with aerolysin. The kinetics and degree of association of aerolysin-labeled GPI-anchored proteins with the internalised wild type strain show maximal association after 15 min incubation at 37°C (Watarai *et al.,* 2002b). The association of these GPI-anchored proteins with the internalised wild type strain shows markedly high efficiency in macropinosomes (more than 70%). In contrast, co-localisation of these GPI-anchored proteins with the *virB4* mutant was much less pronounced. Consistent with these results, GPI-anchored protein CD48 incorporated into macropinosomes containing the wild type strain gave similar kinetics for the aerolysin-labeled GPI-anchored proteins. However, transmembrane protein CD44 associates with ruffles in the plasma membrane above the vacuole, but is excluded from macropinosomes containing the wild type strain. These results suggest that sphingolipid-rich, cholesterol-rich microdomains, lipid rafts, might be incorporated into the macropinosome containing *B. abortus*.

To investigate this possibility, macropinosomes are probed with other components associated with lipid rafts, such as GM1 gangliosides and cholesterol, by incubating *B. abortus* and biotin-labelled cholera toxin B subunit (CTB), which binds GM1-gangliosides, simultaneously with macrophages. CTB localises around the internalised wild type strain with kinetics of association similar to those for aerolysin-labeled GPI-anchored proteins. In contrast, co-localisation of CTB with the *virB4* mutant was much less pronounced (Watarai *et al.,* 2002b). As for aerolysin-labelled GPI-anchored proteins, if only a subset of bacteria found in macropinosomes is analysed, more than 75% of the macropinosomes are found to co-internalise CTB and wild type strain. To probe for raft-associated cholesterol, macrophages infected with *B. abortus* are fixed and probed with the fluorescent cholesterol-binding drug filipin (Mukherjee *et al.,* 1998). The kinetics of

association of cholesterol by using this assay, are almost identical to those for CTB-labelled GM1-gangliosides co-localisation, with the staining of the vacuoles being remarkably intense. As for aerolysin and CTB, if only macropinosomes of wild type strain are analysed, co-localisation is abundant, with close to 72% of the vacuoles staining with filipin. These results indicate that formation of the VirB-dependent macropinosome includes a sorting process that allows transient association of lipid raft-associated components with macropinosomes containing *B. abortus*.

3.4. Macropinosomes Containing *B. abortus* are Replicative Phagosomes

B. abortus alters phagosomes into a specialised organelle allowing bacterial replication by specific actions of the VirB system. To investigate if lipid-enriched phagosomes formed by wild type *B. abortus* support intracellular replication of the *virB* mutant, bone marrow-derived macrophages are co-infected simultaneously with the wild type (GFP-negative) and the Δ*virB4* mutant (GFP-positive) to identify macropinosomes containing both bacteria (Figure 2). After 15 min infection,

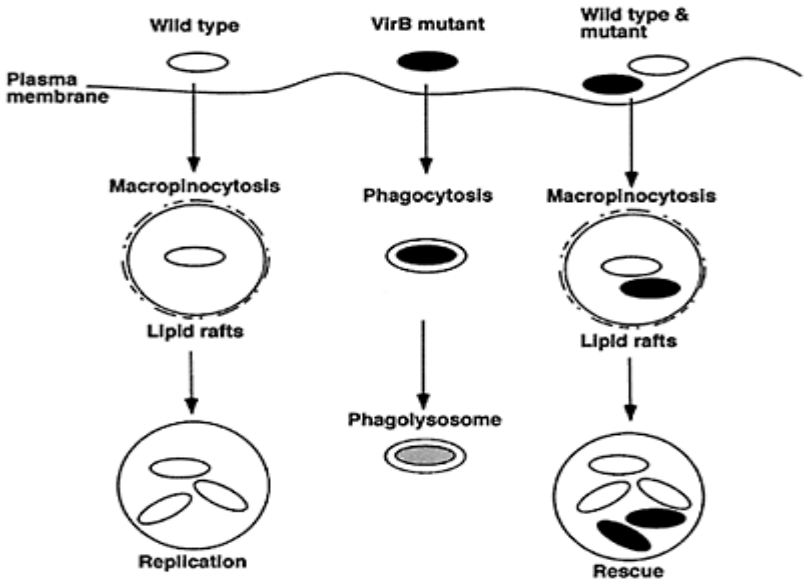

Figure 2. Macropinosomes formed by wild type *B. abortus* support intracellular replication of *virB* mutant.

shown by analysis of macropinosomes containing bacteria by fluorescence microscopy, 79% of macropinosomes contain wild type (GFP-negative), and 18% macropinosome contain both wild type (GFP-negative) and Δ*virB4* mutant (GFP-positive) (Kim *et al.*, 2002). To examine in detail the distribution of bacteria inside macropinosomes

containing both strains, the number of bacteria inside macropinosomes are scored: 52% of the macropinosomes contain single bacteria of the wild type (GFP-negative) and the Δ*virB4* mutant (GFP-positive) and 21–25% of the macropinosomes contain single or double bacteria of these strains. Macropinosomes containing more than double bacteria of both strains are hardly detectable by using this procedure.

To find if synchronous uptake of wild type and mutant strains rescues the replication of the mutant strain, macrophages are coinfected simultaneously with the wild type (GFP-negative) and the Δ*virB4* mutant (GFP-positive) and then replicative phagosomes containing both strains are identified. After 24 h infection, macrophages containing bacteria are analysed by fluorescence microscopy: 35% of macrophages contain no bacteria and 25–27% of macrophages contain the wild type (GFP-negative) or/and the Δ*virB4* mutant (GFP-positive). Macrophages containing more than six bacteria of both strains (18%) indicate that the replicating mutant strain is rescued. Replication of the *virB* mutant is only supported in macropinosomes containing the wild type strain, because replication of *virB* mutant only is not observed.

3.5. Effect of Raft-binding or Disrupting Agents on *Brucella* Infection Into Macrophages

3.5.1. Cholera Toxin B Subunit

Cholera toxin B subunit (CTB) of *Vibrio cholerae* comprises five identical polypeptides that assemble into a highly stable pentameric ring. The assembled pentameric B subunit binds stoichiometrically to five GM1 molecules on the host cell plasma membrane with high affinity and specificity (Sixma *et al.,* 1991; Zhang *et al.,* 1995a; 1995b; Merritt *et al.,* 1998). After pre-treatment with increasing concentrations of CTB, internalisation of non-opsonized *B. suis* into J774 macrophage-like cell line is strongly inhibited, but internalisation of opsonized *B. suis* is not affected by CTB treatment. Thus, internalisation of non-opsonized *B. suis* into J774 macrophages requires GM1 ganglioside, but internalisation by means of opsonic receptors does not (Naroeni and Porte, 2002).

After treatment with increasing concentrations of CTB, intracellular survival of non-opsonized *B. suis* is strongly inhibited. These results directly correlate to a decrease in internalisation. When *B. suis* is internalised by means of opsonic receptors, intracellular survival of bacteria is strongly inhibited by CTB treatment, in contrast to the results of internalisation (Naroeni and Porte, 2002).

3.5.2. Phosphatidylinositol Phosopholipase C

The use of phosphatidylinositol phosopholipase C (PI-PLC) to remove GPI-anchored molecules from the cell surface is a well-established approach. PI-PLC greatly diminishes the internalisation of wild type *B. abortus* by bone marrow-derived macrophages as the PI-PLC concentration increases, and macropinocytosis induced by *B. abortus* is also inhibited (Watarai *et al.,* 2002b). Under the same conditions, PI-PLC does not block internalisation of the *virB4* mutant. However, internalisation of wild type *B. abortus* is not inhibited completely by PI-PLC treatment.

To find if GPI-anchored proteins have a role in *B. abortus* replication in bone marrow-derived macrophages, the macrophages are treated with PI-PLC and then are infected with the wild type strain. The wild type strain replicates in macrophages without PI-PLC treatment, but it fails to replicate in PI-PLC -treated macrophages (Watarai *et al.*, 2002b). Under the same conditions, the intracellular growth of the *virB4* mutant is not affected.

3.5.3. Cholesterol-scavenging or Cholesterol-binding Drugs

Treatment by cholesterol-scavenging or cholesterol-binding drugs, β-cyclodextrin, nystatin and filipin, affects the function of lipid rafts. After pre-treatment with increasing concentrations of filipin or β-cyclodextrin, internalisation of non-opsonized *B. suis* into J774 macrophages is strongly inhibited, but internalisation of opsonized bacteria is not affected by these treatments (Naroeni and Porte, 2002). Similar results occur with filipin and β-cyclodextrin, despite their different modes of action. Thus, internalisation of non-opsonized *B. suis* into J774 macrophages requires cholesterol, but internalisation by means of opsonic receptors does not (Naroeni and Porte, 2002). Internalisation of *B. suis* rough mutants, which fail to express O antigen, is not affected by these drugs treatment (Porte *et al.*, 2003). Thus, O side chain in lipopolysaccharide (LPS) may have an important role on bacterial internalisation via lipid rafts.

After treatment with increasing concentrations of filipin and β-cyclodextrin, intracellular survival of non-opsonized *B. suis* is strongly inhibited. These results directly correlate to the decrease in internalisation. When *B. suis* is internalised by means of opsonic receptors, intracellular survival of bacteria is strongly inhibited by filipin and β-cyclodextrin treatment, in contrast to the results of internalisation (Naroeni and Porte, 2002).

These observations are confirmed by the internalisation of *B. abortus* into mouse bone marrow-derived macrophages. Filipin, β-cyclodextrin and nystatin greatly diminishes the uptake of wild type *B. abortus* by macrophages as their concentration increases (Watarai *et al.*, 2002b).

4. Role of Intracellular Cholesterol Trafficking in *Brucella* Infection

4.1. Acceleration of Bacterial Internalisation by Pre-loading Cholesterol Into Macrophages

A prominent biological property of acetylated low-density lipoprotein (acLDL) is its ability to induce lipid loading of macrophages in culture, and has been a useful model of the formation of lipid-laden macrophages (Figure 1) (Murakami *et al.*, 1995; 1996; Sawamura *et al.*, 2001). To investigate if intracellular cholesterol affects *B. abortus* internalisation into mouse bone marrow-derived macrophages, the bacteria are deposited onto macrophages preloaded with acLDL, and the intracellular bacteria are quantify microscopically at various times of incubation. Internalisation of the wild type strain accelerates by pre-loading with acLDL into macrophages, but internalisation of the *virB4* mutant is not affected (Watarai *et al.*, 2002c). In a study of cellular trafficking of cholesterol derived from the metabolism of LDL showed that after hydrolysis of LDL

cholesteryl ester in lysosomes, most LDL-derived cholesterol traffics to the plasma membrane (Liscum and Nunn, 1999). The internalisation of *B. abortus* into macrophages is accelerated by pre-loading with acLDL, indicating that cholesterol on the plasma membrane of macrophages contributes to the internalisation of *B. abortus*.

4.2. Effects of Agents That Modulate Cholesterol Trafficking on Bacterial Internalisation and Intracellular Replication

Modified lipoproteins taken up by macrophages by receptor-mediated endocytosis are delivered to lysosomes for degradation. This cholesterol is believed to mix with the bulk of cholesterol in the plasma membrane. Excess plasma membrane cholesterol then enters the cytoplasm, where the cholesterol is re-esterified by ACAT and is stored in cellular lipid droplets (Figure 1) (Murakami *et al.*, 1996). To investigate if intracellular cholesterol trafficking affects *B. abortus* internalisation into macrophages, the bacteria are deposited onto macrophages that are pre-treated with ACAT inhibitor HL-004, and the intracellular bacteria are quantify microscopically at various times of incubation. HL-004 treatment accelerates the internalisation of the wild type strain into macrophages preloaded with acLDL. Under the same conditions, it does not accelerate internalisation of the *virB4* mutant (Watarai *et al.*, 2002c). These results suggest that intracellular cholesterol transport contributes to VirB-dependent internalisation of *B. abortus*.

To confirm if plasma membrane cholesterol contributes to *B. abortus* internalisation, the effect of ketoconazole, which inhibits cholesterol transport from lysosomes to the cell surface, can be tested (Figure 1). Ketoconazole greatly diminishes internalisation of the wild type strain into macrophages preloaded with or without acLDL, but under the same conditions, it does not block internalisation of the *virB4* mutant (Watarai *et al.*, 2002c).

To find if intracellular cholesterol trafficking has a role in bacterial replication in macrophages, the macrophages are treated with acLDL, ketoconazole, or HL-004 and then are infected with the wild type strain. The wild type strain replicates in macrophages without ketoconazole treatment, but it fails to replicate in macrophages treated with ketoconazole. Although 12±2.0% of internalised wild type strain can be seen with ketoconazole treatment, the internalised bacteria do not replicate in the macrophages. Intracellular replication is not affected by acLDL and HL-004 (Watarai *et al.*, 2002c). Approximately 15% of the internalised wild type strain in untreated macrophages target improperly into a LAMP-1 positive compartment (Watarai *et al.*, 2002b). These results suggest that other uptake pathways of *B. abortus* by macrophages exist, but replicative phagosome formation requires the uptake pathway associated with plasma membrane cholesterol.

Macrophages incorporate modified LDL by means of scavenger receptors, which is not down regulated by cellular sterol levels (Goldstein *et al.*, 1979). The incorporated cholesteryl ester is delivered to lysosomes, and is hydrolysed to free cholesterol, which forms an intracellular free cholesterol pool. Excess free cholesterol is esterified by ACAT to cholesteryl ester that is stored in cytoplasmic inclusions. This cholesteryl ester is the substrate for neutral cholesteryl ester hydrolase. Thus, a cholesteryl ester cycle of de-esterification exists because of the hydrolase and re-esterification by ACAT. Free cholesterol can move between intracellular pools and the plasma membrane (Brown *et al.*, 1980). Internalisation and intracellular replication of *B. abortus* in macrophages are

modulated by cholesterol-rich microdomains, “lipid rafts”, on plasma membrane surfaces (Watarai *et al.,* 2002b). As the cholesterol microdomains are induced in macrophages when esterification of excess LDL-derived cholesterol is blocked by an ACAT inhibitor (Kruth *et al.,* 2001), internalisation of *B. abortus* by an uptake pathway associated with lipid rafts into macrophages increases by ACAT inhibitor treatment. In contrast, ketoconazole treatment greatly diminishes internalisation of *B. abortus* into macrophages. Ketoconazole interferes with trafficking of cholesterol from lysosomes to the plasma membrane (Liscum, 1990). As the appearance of cholesterol microdomains is inhibited by ketoconazole (Kruth *et al.,* 2001), internalisation of *B. abortus* by an uptake pathway associated with lipid rafts into macrophages decreases by ketoconazole treatment. These results suggest that plasma membrane cholesterol should influence the internalisation of *B. abortus*. Fewer *B. abortus* internalise into macrophages treated with ketoconazole, but the internalised bacteria do not replicate, suggesting that replicative phagosome formation require suitable intracellular cholesterol trafficking and plasma membrane cholesterol.

4.3. Role of Cholesterol Trafficking in the Establishment of *B. abortus* Infection

The most prominent cellular feature of Niemann-Pick Type C disease is lysosomal accumulation of free cholesterol, caused by impaired relocation of cholesterol derived from LDL from the lysosome to other cellular sites, such as the plasma membrane and endoplasmic reticulum (Figure 1) (Pentchev *et al.,* 1995). To investigate if NPC1 contributes to the recruitment of lipid raft-associated molecules, fluorescence-labelled lipid raft-associated molecules, such as cholesterol, GM1 gangliosides and GPI-anchored proteins, have been seen by microscopy in the plasma membrane and intracellular vesicles of macrophages of wild type mice. In contrast, these molecules accumulate only in intracellular vesicles in macrophages from NPC1-deficient mice. Localisation of the transmembrane protein CD44, not associated with lipid rafts, is not affected by NPC1 (Watarai *et al.,* 2002c). These results suggest that NPC1 influences lipid raft formation on the macrophage surface.

Whether NPC1 contributes to internalisation and intracellular replication of *B. abortus* has been seen investigated (Watarai *et al.,* 2002c). Bone marrow derived-macrophages from wild type or NPC1-deficient BALB/c mice are infected with *B. abortus*, and the intracellular bacteria are quantify microscopically at various times of incubation. Macrophages from wild type mice support internalisation and intracellular replication of *B. abortus*, but not macrophages from NPC1-deficient mice in which fewer bacteria are internalised in macrophages, but they do not replicate in the macrophages. Macrophages from wild type and NPC1-deficient mice show no marked difference in the internalisation of the *virB4* mutant (Watarai *et al.,* 2002c). In NPC1-deficient mice, *B. abortus* fails to block phagosome maturation, as shown by co-localisation of phagosomes containing the bacteria and the late endocytic marker, LAMP-1, at 1 h after infection. In contrast, *B. abortus* prevents phagosome-lysosome fusion, and therefore phagosomes containing *B. abortus* do not have endocytic and lysosomal marker proteins in macrophages from wild type mice (Watarai *et al.,* 2002c). These results suggest that replicative phagosome formation requires an uptake pathway associated with NPC1.

To find if this defect in internalisation and intracellular replication of *B. abortus* correlates with an inability to establish infection in the host, wild type or NPC1-deficient mice have been experimentally infected with *B. abortus*. Many bacteria are recovered from the spleen of wild type mice infected with *B. abortus* at 10 days after infection, but fewer bacteria are recovered from NPC1-deficient mice by counting the number of colony forming units (CFU) in each spleen (Watarai *et al.,* 2002c). These results indicate that *B. abortus* proliferation is promoted by NPC1.

The gene for NPC, referred to as *NPC1,* has been mapped on chromosome 18 in both human and mice and has been cloned (Loftus *et al.,* 1997). Although the function of NPC1 remains undefined, this protein has a crucial role in cholesterol metabolism (Liscum and Klansek, 1998). NPC1-deficient mice share many pathophysiological abnormalities of human patients with NPC, including accumulation of cholesterol in tissues (Loftus *et al.,* 1997). Lipid raft-associated molecules accumulate in intracellular vesicles in macrophages from NPC1-deficient mice (Watarai *et al.,* 2002c). NPC1 is recruited to the site of free cholesterol accumulation by enrichment of cellular cholesterol or by pharmacological intervention of cholesterol egress from the lysosomes (Zhang *et al.,* 2001). Intracellular trafficking of GM1 ganglioside in NPC1-deficient Chinese hamster ovary cells has been shown by using CTB as a probe (Sugimoto *et al.,* 2001). CTB-labelled vesicles contain the early endosome marker Rab5 but not LAMP-2, indicating that they represent early endosomes. Similarly, CTB accumulates in intracellular vesicles of human NPC fibroblasts that contain both Rab5 and early endosomal antigen 1 (Sugimoto *et al.,* 2001). Presumably, all these results indicate that cholesterol or GM1 gangloside accumulates in lysosomes or in early endosomes in macrophages from NPC1-deficient mice. Therefore, internalisation of *B. abortus* by an uptake pathway associated with lipid rafts is inhibited in macrophages from NPC1-deficient mice.

Mouse macrophages mediate resistance or susceptibility among mouse strains to some intracellular pathogens, as has been shown by studies of the *Ity/Lsh/Bcg* resistance model; resistance to *Salmonella typhimurium, Leishmania donovani* and mycobacterial species is regulated by polymorphism of the *Nramp1* gene that controls macrophage function (Forbes and Gross, 2001). Bovine Nramp1 is a major candidate for controlling the *in vivo* resistant phenotype against *B. abortus* infection (Barthel *et al.,* 2001). NPC1 promotes internalisation and intracellular replication of *B. abortus* and also contributes to bacterial proliferation in mice (Watarai *et al.,* 2002c). However, control of *B. abortus* infections is a multigenic trait (Ho and Cheers, 1982), and further investigation is needed to clarify the genetic control of *B. abortus* infection.

5. Interaction Between *Brucella* and Lipid Raft-Associated Molecules

5.1. Tail Formation of Cellular Prion Protein With Internalisation of *B. abortus*

GPI-anchored proteins can be selectively incorporated into macropinosomes containing *B. abortus* as described in a previous section. To investigate further the membrane sorting process, the distribution of GPI-anchored proteins during internalisation of *B. abortus* has

been analysed (Watarai *et al.*, 2003). Aerolysin from *A. hydrophila*, which binds to the GPI moiety of GPI-anchored proteins on the cell surface (Abrami *et al.*, 1998), has been used as a probe to detect GPI-anchored proteins. At 5 min after infection, aggregation of aerolysin-labelled GPI-anchored proteins showing tail-like formation co-localises with swimming bacteria on the macrophage surface. In contrast, CD48, a GPI-anchored protein, does not aggregate at 5 min after infection. Similar results have been obtained for other GPI-anchored proteins, such as CD55. However, when one GPI-anchored protein, cellular prion protein (PrP^C), was tested, co-localisation of aggregated PrP^C tail and swimming bacteria was observed. Sometimes, a single bacterium can have several PrP^C tails. PrP^C is also incorporated into macropinosomes containing the wild type strain, but not the *virB4* mutant, after 15 min incubation (Watarai *et al.*, 2003).

The ratio of PrP^C tail formation, co-localisation of PrP^C tail and internalised bacteria has quantify microscopically at various times of incubation. The *virB4* mutant rapidly internalises, with most bacteria internalised before further incubation, but the internalisation of the wild type strain is delayed. The wild type strain, but not the *virB4* mutant, is in macropinosomes transiently. The kinetics and degree of association of the PrP^C tail with the internalised wild type strain show maximal association after 5 min incubation. A maximal association of PrP^C with phagosomes containing the wild type strain is after 15 min incubation. In contrast, co-localisation of PrP^C with the *virB4* mutant is much less pronounced. These results suggest that bacterial products secreted by the type IV system might aggregate PrP^C specifically and form tail structures during internalisation of *B. abortus*.

5.2. Surface Exposure of Hsp60 on *B. abortus*

Bacterial factors associated with PrP^C tail formation, immunodominant proteins can be examined by using immunoblotting with human brucellosis sera, during which a major protein (60 KDa) and two minor proteins (30~25 KDa) are recognised. As immunodominant Hsp60 reacts with sera from mice experimentally infected with *B. abortus* (Roop II *et al.*, 1992), the 60 KDa protein is expected to be Hsp60. To confirm this, purified Hsp60 of *B. abortus* is analysed by immunoblotting with sera from human and animal brucellosis. As expected, Hsp60 reacts with serum from humans, cattle and sheep with naturally acquired brucellosis. Mutant strains (Δ*virB2* and Δ*virB4*) also have immunoreactive Hsp60. To examine if Hsp60 is secreted into the external medium, the culture supernatant of *B. abortus* can be analysed by immunoblotting, but immunoreactive proteins are not detected. However, surface-exposed Hsp60 on the wild type strain, but not on the *virB2* and *virB4* mutants, cannot be detected by immunofluorescence staining with anti-Hsp60 antibody. As introduction of complementing plasmid into each mutant restores the surface expression of Hsp60, the expression of Hsp60 on the bacterial surface associates with the type IV secretion system.

In a control experiment, the presence of Hsp60 on the bacterial surface does not result from wholesale re-localisation of cytoplasmic leakage (Watarai *et al.*, 2003). Surface exposure of glucose-6-phosphate dehydrogenase (G6PDH) can be found by immunofluorescence microscopy. Antibodies against G6PDH fail to react with bacterial cell surfaces. Whether the control antibody reacts with bacterial cells in the immunofluorescence experiment is uncertain. The antibody is used to probe bacteria in

the presence or absence of permeabilisation by hypotonic lysozyme treatment (Watarai *et al.,* 2001a). Antibodies against G6PDH react with permeabilised bacteria, but fail to react with the bacterial cell surface. Therefore, the surface exposure of Hsp60 is not caused by cytoplasmic leakage.

Hsp60, a member of the GroEL family of chaperonins in *E. coli*, is widely distributed and is conserved between prokaryotes and mammals (Bukau and Horwich, 1998). Hsp60 proteins are immunodominant antigens of many microbial pathogens, including *B. abortus* (Kaufmann, 1990; Roop II *et al.,* 1992). Hsp60 is believed to reside in the cytoplasm (Craig *et al.,* 1993). However, surface-exposed Hsp60 occurs in *Legionella pneumophila*, and participates in the pathogenicity (Hoffman and Garduno, 1999). Presumably, Hsp60 of *L. pneumophila* binds to unknown receptors on non-professional phagocyte HeLa cells, initiating actin polymerisation and endocytosis of the bacterium into an early endosome (Garduno *et al.,* 1998), but the role of surface-exposed Hsp60 in professional phagocytes, such as macrophages, is still unclear. As *L. pneumophila* has a type IV secretion system, the surface expression of Hsp60 of *L. pneunophila* may be a similar mechanism to that of *B. abortus*. Effector proteins secreted by the type IV system of *B. abortus* have not been identified and this may be the first to describe a candidate effector-like protein secreted by the type IV system of *B. abortus* associated manner. Hsp60 is a major antigen that elicits strong antibody responses in many bacteria (Kaufmann, 1990), including bacteria that lack the type IV secretion system. Therefore, Hsp60 may be released by other secretion system and bind to a denatured part of an effector protein of the type IV secretion system that may carry the Hsp60 to the bacterial surface.

5.3. Interaction of PrP^C With Hsp60 of *B. abortus*

As Hsp60 expressed on the bacterial surface by the type IV secretion system most likely interacts with the target cell, Hsp60 has been tested for its ability to bind to PrP^C on macrophages by using a pull-down assay with Hsp60- or PrP^C—beads (Watarai *et al.,* 2003). Analysis of the precipitated proteins by immunoblotting with anti-PrP^C or Hsp60 antibody shows that a 29 KDa PrP^C is associated with Hsp60, but not the beads alone. To confirm this association, Hsp60 is added to macrophage lysate and the proteins in the mixture are then immunoprecipitated with anti-PrP^C antibody. The precipitated proteins, analysed by immunoblotting with anti-Hsp60 antibody, contains Hsp60. As the anti-Hsp60 antibody does not recognise macrophage Hsp60, the antibody is specific for bacterial Hsp60. This Hsp60 and PrP^C association can be inhibited by adding anti-Hsp60 polyclonal antibody, purified Hsp60 or PrP^C. These results indicate that interaction between Hsp60 and PrP^C is specific. The precipitated proteins can be also analysed by silver staining, resulting in the precipitates containing two major bands (60 and 29 KDa) and two weak minor bands (74 and 27 KDa). These results suggest that Hsp60 binds mostly to PrP^C, but that Hsp60 may interact indirectly with PrP^C mediated by other cellular components (Watarai *et al.,* 2003).

To further characterise Hsp60, distribution of Hsp60 in *B. abortus*-infected macrophages can be analysed by immunofluorescence microscopy. At 5 or 15 min after infection, Hsp60 co-localises with only the bacterial surface and is not detected in the macrophage membrane or cytoplasm. To investigate if Hsp60 exposed on a bacterial

surface aggregates PrP^C on macrophages, the macrophages are infected with *Lactococcus lactis* expressing Hsp60 of *B. abortus* on its surface, and then PrP^C is detected by immunofluorescence microscopy. After 5 min incubation, PrP^C accumulates around internalised Hsp60-positive *L. lactis*, but not Hsp60-negative *L. lactis*. Quantitative data have shown that more than 70% of *L. lactis* expressing Hsp60 co-localises with PrP^C. PrP^C does not form tails with either Hsp60-positive or -negative *L. lactis. L. lactis* is seeded in the wells of a microtiter plate, macrophage lysate is added, and then the binding activity is measured by using enzyme-linked immunoabsorbent assay (ELISA) with anti-PrP^C antibody. The binding of PrP^C to Hsp60 on the *L. lactis* surface is detected, but not with Hsp60 negative *L. lactis*. *L. lactis* expressing Hsp60 of *E. coli* also co-localises with PrP^C, but the percentage of co-localisation is lower than Hsp60 of *B. abortus*. These results suggested that Hsp60 expressed on the bacterial surface promotes accumulation of PrP^C, but is not sufficient for PrP^C tail formation.

A *Saccharomyces cerevisiae* two-hybrid screening sy stem has been used to show that PrP^C interacts with Hsp60 (Edenhofer *et al.,* 1996). The PrP is the causative agent of neurodegenerative diseases, such as Creutzfeld-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) and scrapie in sheep (Prusiner, 1998). The pathological infectious form, PrP^{Sc}, is a β-sheet aggregate, whereas the normal cellular isoform, PrP^C, consists of a largely α-helical, autonomously folded C-terminal domain and an N-terminal segment that is unstructured in solution (Jackson and Clarke, 2000). Conformational conversion of PrP^C into PrP^{Sc} may include a chaperone-like factor. GroEL of *E. coli* can catalyse the aggregation of chemically denatured and of folded recombinant PrP in a model reaction *in vitro* (Stockel and Hartl, 2001). These several studies suggested that surface-exposed Hsp60 of *B. abortus* could bind to PrP^C and catalyse the aggregation of PrP^C on macrophages (Edenhofer *et al.,* 1996; DebBurman *et al.,* 1997; Guerin *et al.,* 2001; Stockel and Hartl, 2001). Consistent with this hypothesis, Hsp60 expressed on *L. lactis* catalyses the aggregation of PrP^C on macrophages. However, as PrP^C tail formation was not observed in macrophages infected with Hsp60-positive *L. lactis*, Hsp60 is not sufficient for PrP^C tail formation. Macropinosomes are not formed in macrophages infected with Hsp60-positive *L. lactis.* PrP^C tail formation is required for bacterial swimming on macrophages, and another bacterial factor, secreted by the type IV system, appears to be required for PrP^C tail formation.

B. abortus internalises into macrophages by swimming on the cell surface for several minutes, with membrane sorting during this period (Kim *et al.,* 2002; Watarai *et al.,* 2002b). PrP^C tail formation participates in the signalling pathway for *B. abortus* internalisation, because the PrP^C tail co-localises with Grb2 (unpublished results). Evidence that PrP^C interacts with Grb2 has been provided by the two-hybrid screening system (Spielhaupter and Schatzl, 2001). Grb2 is an adapter protein participating in intracellular signalling from extracellular or transmembrane receptors to intracellular signalling molecules (Koch *et al.,* 1991). The structure of Grb2 consists of a central SH2 domain flanked by two SH3 domains. The SH2 domain is responsible for interaction with tyrosine kinase, but the SH3 domains bind to proline-rich motifs (Anderson, 1990). Grb2 interacts through its SH3 domains with the Wiskott-Aldrich syndrome protein (WASP), which plays a role in regulation of the actin cytoskeleton (She *et al.,* 1997). WASP is a 64 kDa protein expressed exclusively in hematopoietic cells (Stewart *et al.,* 1996). The carboxyl terminal portion of WASP contains regions that show homology to several

actin-binding proteins, such as verprolin and cofilin, which may allow binding of WASP to filamentous actin (Derry, *et al.*, 1994). In the internalisation of *B. abortus*, surface-exposed Hsp60 of *B. abortus* promotes aggregation of PrP^C, and PrP^C tail formation is induced by unidentified factor(s) secreted by the type IV system. The interaction of PrP^C tail with Grb2 initiates cytoskeletal rearrangement and induces generalised membrane ruffling. Bacteria may obtain a driving force for swimming internalisation from membrane ruffling, like riding the wave of membrane until it is enclosed in macropinosomes. Consistent with this hypothesis, Grb2, which interacts with the PrP^C tail, is excluded in macropinosomes containing *B. abortus* (unpublished results). Presumably, the signal mediated by Grb2 is not required for replicative phagosome formation after macropinosome formation. Instead, a signal mediated by lipid rafts is needed for replicative phagosome formation (Watarai *et al.*, 2002b).

5.4. Effect of PrP^C Deficiency on *B. abortus* Infection

To investigate the role of PrP^C on *B. abortus* infection, several phenotypes of *B. abortus* virulence have been tested by using macrophages from Ngsk PrP^C-deficient mice (Sakaguchi *et al.*, 1996). Time-lapse videomicroscopy can follow the internalisation of *B. abortus* by macrophages from parent or Ngsk PrP^C-deficient C57BL/6 mice. After contact of macrophages with *B. abortus*, bacteria show swimming internalisation in macrophages from parent mice. The swimming of the bacteria on the macrophage surface lasts for several minutes with generalised plasma membrane ruffling before eventual enclosure in macropinosomes. Contact of *B. abortus* with macrophages from Ngsk PrP^C-deficient mice, in contrast, results in much smaller ruffling restricted to the area near the bacteria. The ruffles associated with internalisation of bacteria result in a more rapid uptake than for macrophages from parent mice. Five minutes after deposition on the macrophages from parent mice, *B. abortus* show generalised actin polymerisation around the site of bacterial binding by either phalloidin staining or phase contrast microscopy. Macrophages from Ngsk PrP^C-deficient mice show primarily small regions of phalloidin staining at sites of bacterial binding (Watarai *et al.*, 2003).

The differences in rate of phagocytosis and macropinosome formation for parent or Ngsk PrP^C-deficient mice can be quantified microscopically at various times of incubation. The kinetics of bacterial internalisation and macropinosome formation in macrophages from parent C57BL/6 mice are almost identical to those for macrophages from BALB/c mice. Internalisation of wild type *B abortus* into macrophages from Ngsk PrP^C-deficient mice, in contrast, is much quicker and macropinosome formation is hardly detectable. The internalised wild type strain does not replicate in macrophages from Ngsk PrP^C-deficient mice. Macrophages from parent and Ngsk PrP^C-deficient mice show no marked difference in internalisation, macropinosome formation and intracellular replication of *virB4* mutant. In macrophages from Ngsk PrP^C-deficient mice, the wild type strain fails to block phagosome maturation, as shown by co-localisation of phagosomes containing the bacteria and the late endocytic marker, LAMP-1, at 35 min after infection. In contrast, the wild type strain prevents phagosome-lysosome fusion, and therefore phagosomes containing the wild type strain do not have LAMP-1 in macrophages from parent mice.

Parent or PrP^C-deficient mice infect with *B. abortus* to find if this defect in intracellular replication of *B. abortus* correlates with an inability to establish infection in the host. Many bacteria can be recovered from the spleen of B ALB/c and C57BL/6 mice infected with the wild type strain at 10 days after infection, but few bacteria are recovered from PrP^C-deficient mice, based on the number of CFU in each spleen. Fewer bacteria are recovered from the spleen of the three mice

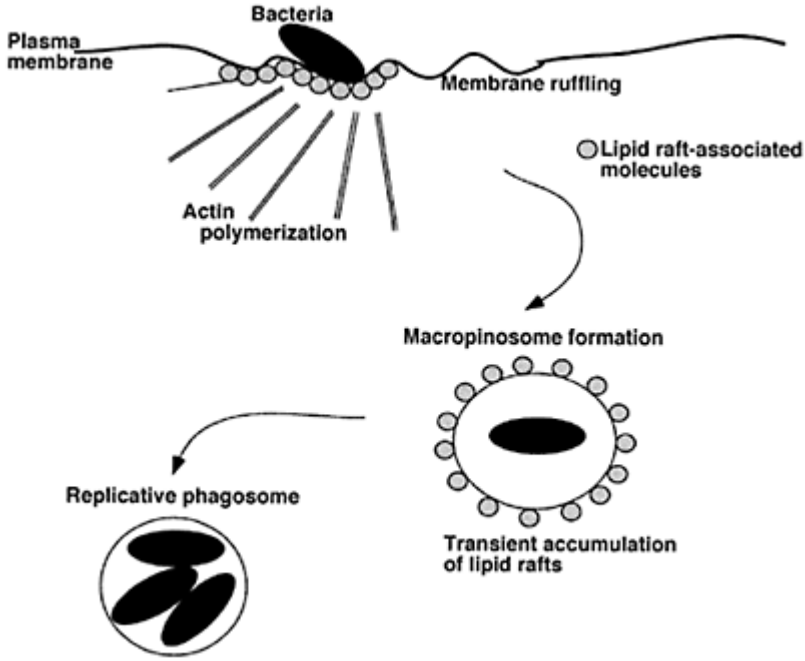

Figure 3. Model depicting interaction between *B. abortus* and lipid raft-associated molecules, and replicative phagosome formation.

strains infected with the *virB4* mutant (Watarai *et al.*, 2002a). These results suggest that replicative phagosome formation and proliferation in mice of *B. abortus* require an uptake pathway associated with PrP^C.

Several phenotypes ascribed to Ngsk PrP^C-deficient mice are most likely caused by up-regulation of PrP-like protein doppel rather than by ablation of PrP^C (Moore, *et al.*, 1999). To investigate the participation of doppel expression in *B. abortus* infection, Zrch PrP^C-deficient mice (Bueler, *et al.*, 1992) that has no up-regulation of doppel can be used in an infection assay. Phenotypes of Zrch PrP^C-deficient mice are almost the same as Ngsk PrP^C-deficient mice in *B. abortus* infection. And PrP^C transgenic Ngsk PrP^C-deficient mice are successfully rescued from the inhibition of bacterial intracellular growth. Therefore, doppel expression does not participate in *B. abortus* infection.

The function of the *B. abortus virB* locus is essential for intracellular survival, both in cultured cells and in the mouse model (O'Callaghan *et al.*, 1999; Foulongne *et al.*, 2000; Hang *et al.*, 2000; Sieira *et al.*, 2000; Sum *et al.*, 2002). Niemann-Pick type C1 gene (NPC1) regulates the internalisation and intracellular replication of *B. abortus* and also contributes to bacterial proliferation in mice (Watarai *et al.*, 2002b). Macrophages from NPC1-deficient mice do not support internalisation and intracellular replication of *B. abortus*. Internalisation is not inhibited in macrophages from PrP^{C}-deficient mice (Watarai *et al.*, 2003). In NPC1-deficient mice macrophages, lipid raft-associated molecules, such as cholesterol, GM1 ganglioside and GPI-anchored proteins, accumulate only in intracellular vesicles (Watarai *et al.*, 2002c). In contrast, these molecules are in both plasma membrane and intracellular vesicles of macrophages from PrP^{C}-deficient mice, and in macrophages from parent mice (unpublished results). Therefore, lipid raft-associated molecules on the plasma membrane are essential for the internalisation of *B. abortus*, and PrP^{C} promotes the bacterial swimming internalisation (Figure 3).

6. Roles of Host Membrane Lipids in Other Pathogens

Lipid rafts participate in infection by several intracellular pathogens. For example, cholesterol is essential for the uptake of *Mycobacterium bovis* by macrophages (Gatfield and Pieters, 2000). Cholesterol accumulates at the site of *M. bovis* entry and depleting plasma membrane cholesterol specifically inhibits *M. bovis* uptake; *M. bovis* has a high binding capacity for cholesterol (Gatfield and Pieters, 2000). Macropinosomes containing *L. pneumophila* include lipid raft-associated molecules (Watarai *et al.*, 2001b). GPI-anchored proteins are in *Toxoplasma gondii* and *Plasmodium falciparum* vacuoles (Mordue *et al.*, 1999, Lauer *et al.*, 2000). The intracellular parasite *Leishmania donovani* can actively inhibit the acquisition of flotillin-1-enriched lipid rafts by phagosomes and the maturation of these organelles (Dermine *et al.*, 2001). Lipid platforms have been implicated in the budding of human immunodeficiency virus (HIV) and influenza virus (Scheiffele *et al.*, 1999; Nguyen and Hildreth 2000). Ebola and Marburg viral proteins form compartments within lipid rafts during viral assembly and budding (Bavari *et al.*, 2002). PrP is attached to membranes by a GPI-anchor associated with lipid rafts. Conversion of raft-associated PrP^{C} to the protease-resistant state requires insertion of PrP^{Sc} into a contiguous membrane (Baron *et al.*, 2002). Thus lipid rafts, including PrP^{C}, may have an important role as a gateway for the intracellular trafficking of pathogens (Duncan *et al.*, 2002).

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Chapter 14
Invasion, Intracellular Trafficking and Replication of *Brucella* Organisms in Professional and Non-Professional Phagocytes

Edgardo Moreno* and Jean-Pierre Gorvel

From: *Brucella:* Molecular and Cellular Biology. Edited by: Ignacio López-Goñi and Ignacio Moriyón

Abstract

Brucella organisms are intracellular parasites of mammals, including humans. Initially bacteria seem to bind lipid rafts and to different membrane receptors of macrophages. In these cells, the cyclic-AMP/protein kinase-A pathway is activated followed by phosphorylation of transcription factors. In epithelial cells, the bacterium activates small GTPases of the Rho subfamily and attains a modest recruitment of actin cytoskeletal structures. In macrophages, most of the ingested *Brucella* are routed to phagolysosomes and only a few bacteria arrive at endoplasmic reticulum, the compartment that constitutes the *Brucella* replicating niche. In epithelial cells, on the contrary, most of the bacteria are directed to the endoplasmic reticulum and not to lysosomes. For internalisation, *Brucella* requires the competence of the BvrS/BvrR regulatory system, while the VirB type IV secretion apparatus is needed for intracellular trafficking. The expression of stationary phase genes seems to be required during the replicating stage. During this period *Brucella* are able to prevent apoptosis. Replicating bacteria release large quantities of lipopolysaccharide (LPS) within the host cells. The LPS recycles to the cell membrane forming stable complexes with MHC-II proteins in lipid megarafts. These surface lipid-LPS-protein macrodomains hamper the presentation of peptides to T-cells. The *Brucella* LPS also triggers regulatory T cells recognising MHC-II-LPS macrodomains on the surface of antigen presenting cells.

1. Introduction

Brucella organisms have been found to survive and replicate within membrane-bound compartments of professional and non-professional phagocytes. Despite the tropism of these pathogens for reproductive organs, the bacteria also localise within a variety of cell types at different stages of the infection (Enright, 1990). *In vivo*, *Brucella* has been described within bovine, caprine and murine trophoblasts, in caprine lymphocytes, M cells, chicken embryo fibroblasts, as well as a number of professional phagocytes lining different tissues (Holland and Pickett, 1956; Richardson and Holt, 1964; Anderson and Cheville, 1986; Anderson *et al.*, 1986; Ackermann *et al.*, 1988; Detilleux *et al.*, 1988; Cheville *et al.*, 1992; 1996). *In vitro*,

* For correspondence email emoreno@medvet.una.ac.cr

Brucella is able to replicate within hamster kidney cells (Hatten and Sulkin, 1966a; 1966b), primary cultures of bovine adult and foetal cells (Richardson and Holt, 1964), and a number of epithelial cells and macrophages (Detilleux *et al.*, 1990a; 1990b; Baldwin and Winter, 1994; Caron *et al.*, 1994; Liautard *et al.*, 1996; Pizarro-Cerdá *et al.*, 1998a; Sola-Landa *et al.*, 1998; Celli *et al.*, 2003). Among non-professional phagocytes, epithelial HeLa and fibroblastic Vero cell lines have been the most extensively used for studying *Brucella* replication and intracellular trafficking. Among professional phagocytes, murine J774, murine peritoneal macrophages, murine bone marrow macrophages, human monocytes, bovine mammary gland macrophages and human neutrophils have been the most widely used cells.

Intestinal M cells, neutrophils, non-activated macrophages from newly infected hosts, activated macrophages from immune animals and non-professional phagocytes, all serve a different purpose during the course of *Brucella* infection (reviewed by Moreno and Moriyón, 2002). While the translocation of ingested *Brucella* organisms occurs through M cells, the first leucocytes confronting *Brucella* are neutrophils, (Ackermann *et al.*, 1988). These phagocytic cells do not serve as a substrate for *Brucella* replication due to their short life span; however, a significant proportion of the ingested bacteria are capable of withstanding destruction inside these leukocytes (Kreutzer *et al.*, 1979; Young *et al.*, 1985). In turn, this event may favour the spread of the parasite from neutrophils to other tissues (Ackermann *et al.*, 1988; Enright, 1990). The second line of defence are macrophages; cells that destroy an important proportion of ingested *Brucella* but which also serve as a substrate for *Brucella* replication as well as vehicles for spread to other tissues (Celli *et al.*, 2003). In the pregnant animal, *Brucella* invades the erytrophagocytic trophoblasts, which are the preferred replicating host cells and the site from which the bacteria spread to the foetus (Anderson and Cheville, 1986; Anderson *et al.*, 1986; Tobias *et al.*, 1993). Generally, vaccinated immune animals are capable of controlling the infection via stimulation of the macrophagic system through the concourse of Th and Tc cells. Depending upon the animal species, the humoral response may serve as an important aid for phagocytosis and for directing the intracellular route of the ingested bacteria to destructive compartments.

Important findings revealing the interaction between *Brucella* and host cells have been documented in recent years. At the molecular level, the information is still incomplete.

Analysis and identification of molecules in the host cells, receptors at the cell surface and bacterial components involved in the invasion process and trafficking are necessary to understand the events leading to the establishment of the infection. In addition, isolation and characterization of the different *Brucella*-containing compartments is necessary for the identification of those molecular determinants relevant for the bacterial intracellular life and for the cross-talk between these pathogens and their host cells. This review concentrates in those experimental works in which macrophages and epithelial cells infected with *Brucella abortus, Brucella melitensis* or *Brucella suis* have been used as models to understand the intracellular life of *Brucella* organisms

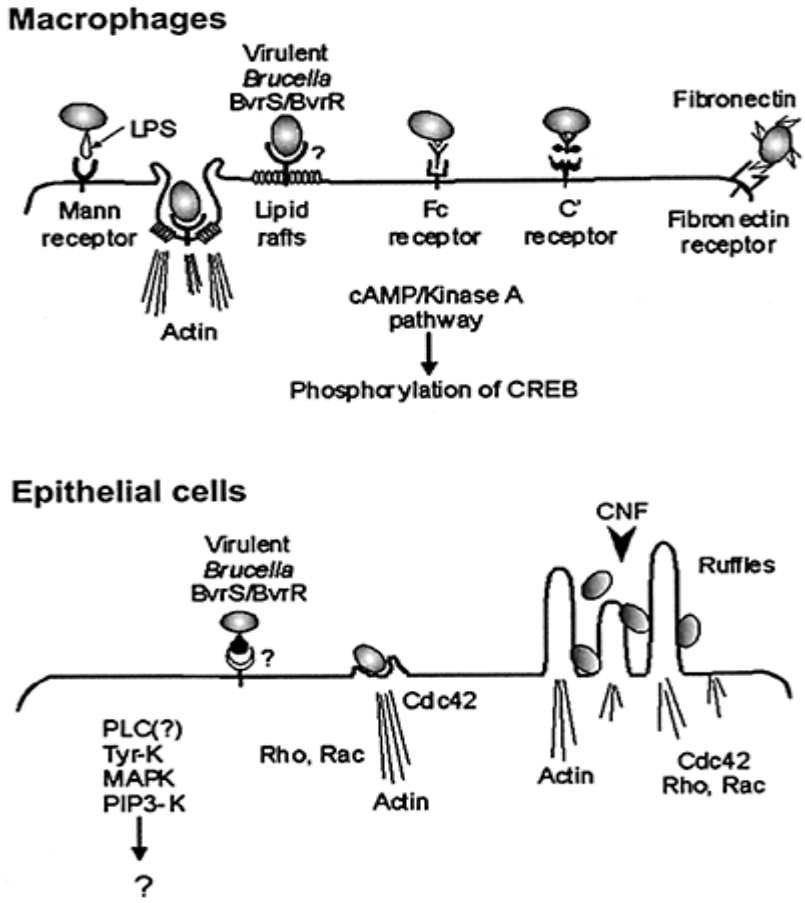

Figure 1. Schematic model of *B. abortus* invasion in macrophages and epithelial HeLa cells. In macrophages *Brucella* organisms bind to discrete sites of the cell membrane via known

(FcR, C3bR, MannoR, and fibronectin) and unknown receptor molecules and thereafter ingested via lipid rafts. The bacterium is commonly ingested by a zipper-like phagocytosis with moderate recruitment of actin filaments and activation of the cyclic AMP/kinase pathway and phosphorylation of transcriptor factor CREB. In HeLa cells, *Brucella* organisms bind to discrete sites of the cell membrane via unknown receptor molecules (?), and penetrate by phagocytosis via moderate recruitment of actin filaments, activation of small GTPases (Cdc42, Rac, and Rho), mainly Cdc42, and signals mediated by second messengers (Tyr-K, MAP-K, and PI3-K). *Brucella* organisms attach in larger numbers and are internalised more efficiently after intoxication of HeLa cells with the cytotoxic necrotizing factor (CNF), which deamidates the small GTPases Rho, Rac, and Cdc42, and induces ruffles and stress fibre formation. Functional BvrS/BvrR two component regulatory system is required for invasion and survival in both cell types.

2. Binding and Penetration to Cells

The mode by which *Brucella* invade professional and non-professional phagocytes considerably differs (Figure 1). The uptake of *Brucella* organisms varies depending upon the type of cell as well as the bacterial strain used. For example, experiments have revealed marked differences between trophoblasts, intestinal M cells, epithelial cell lines and professional phagocytes (reviewed by Moreno and Moriyón, 2002). Moreover, some cells such as binucleated trophoblasts, lymphocytes and neutrophils do not sustain *Brucella* replication, either because they do not become infected or because they are short term living cells devoted to immediate killing mechanisms (Kreutzer *et al.*, 1979;

Ackermann *et al.,* 1988; Samartino and Enright 1992). In addition, a collection of *Brucella* mutants generated under laboratory conditions have demonstrated defects in binding and invasion to cells (Sola-Landa *et al.*, 1998).

The initial number of virulent *Brucella* organisms per infected epithelial cell and the percentage of infected cells is low, (with one or two bacteria per cell) (Detilleux *et al.,* 1990a; 1990b; Pizarro-Cerdá *et al.,* 1998a). Even if the bacterial inoculum is augmented in several logs, the rate of infection per cell remains low, suggesting that not all cells are permissive (Sola-Landa *et al.,* 1998) or that not all bacteria are capable of binding to epithelial cells. Alternatively, both phenomena may act in concert contributing in this manner to the low binding of *Brucella* organisms to non-phagocytic cells. Macrophages in contrast, bind and internalise *Brucella* in higher numbers due to their phagocytic nature (Celli *et al.,* 2003). Independently of the cell type, once the brucellae bind, the penetration efficiency is close to 100%. Intestinal M cells and other epithelial cells preferentially ingest *Brucella* by a classical zipper-like mechanisms (Ackermann *et al.,* 1988); in these cells however, the initial membrane rearrangements are modest and do not involve an extensive recruitment of actin filaments. In epithelial cells, *Brucella* preferentially attaches to cellular extensions that are compatible with adhesion plaques and between cell-to-cell contacts (Guzmán-Verri *et al.,* 2001). Uptake of killed or alive *Brucella* by epithelial cells is suppressed by inhibitors of both energy metabolism inhibitors of receptor-mediated endocytosis and by repressors of endosomal acidification (Detilleux *et al.,* 1991). These drugs are capable of inhibiting penetration when added at the same time as the bacterial inoculum, but not when added after the inoculation period, suggesting that the infection process occurs via receptor molecules and requires energy input from the host cell. Inhibition of actin polymerisation by drugs (Detilleux *et al.,* 1991; Guzmán-Verri *et al.,* 2001), or by glucosylation of Rho small GTPases, key modulators of this cytoskeletal structure, hamper *Brucella* internalisation but not binding to cells (Guzmán-Verri *et al.,* 2001). Similarly, epithelial cells constitutively transfected with dominant negative Rho, Rac and Cdc42 proteins are considerably less infected than control cells. On the contrary, the dominant positive counterparts of these small GTPases expressed in epithelial cells stimulate penetration of *Brucella*. Virulent *Brucella*, but not defective Bvr mutants, selectively activates Cdc42 reaching a maximum of accumulated GTP-loaded Cdc42, 30 minutes after bacterial contact with cells. Moreover, *B. abortus* is internalised more efficiently after intoxication of cells with the cytotoxic necrotizing factor (CNF), which activates Rho, Rac, and Cdc42, and induces membrane ruffles and stress fibre formation (Guzmán-Verri *et al.,* 2001). This phenomenon emphasises the role of these small GTPases in the internalisation events of *Brucella*. Infection can also be inhibited by chemicals and toxins that increase the levels of cyclic-AMP, but it is stimulated by toxins and chemicals that increase the levels of cyclic-GMP, suggesting an inverse relationship between these two second-messengers during *Brucella* infection. Similarly, inhibition of the PI-3 kinase considerably reduces the internalisation of *Brucella* in epithelial cells, indicating the involvement of phosphorylations mediated by this enzyme (Guzmán-Verri *et al.,* 2001). Since the level of cyclic GMP usually increases when the inositol phospholipid pathway is activated, it is likely that binding of *Brucella* to cells also stimulates the generation of IP3 via phospholipase C activation. Other cellular kinases, such as tyrosine kinases and MAP kinases, seem to be required for physiological internalisation to non-professional phagocytes cells (Guzmán-Verri *et al.,*

2001). Microtubule depolymerising agents such as nocodazole, partially reduce the internalisation but not the replication of *Brucella* in epithelial cells (Guzmán-Verri *et al.*, 2001). The general strategy used by *Brucella* to invade epithelial cells (characterised by the use of chemical and biological tools) differs from those employed by other intracellular pathogens, such as *Salmonella* and *Shigella*. On the other hand and despite the obvious differences between *Brucella* and *Listeria*, both pathogens seem to display similar strategies (Finlay and Cossart, 1997).

In macrophages, lipid rafts seem to provide a port for *Brucella* entry under non-opsonic conditions. Indeed, it has been observed that phagocytosis occurs by exclusion of membrane negatively charged groups (Gay *et al.*, 1981) on sites enriched in cholesterol, glycosylphosphatidylinositol and GM1 gangliosides as well as actin recruitment (Gay *et al.*, 1981; Kuzumawati *et al.*, 2000; Naroeni and Porte, 2002; Watarai *et al.*, 2002a; 2002b; see also Chapter 13). Under opsonic conditions, macrophages ingest *Brucella* via Fc, complement or fibronectin receptors depending upon the coating molecule. Opsonization seems to negatively affect the rate of survival and multiplication of *Brucella* within phagocytes as has been observed in other bacteria. That is, Fc or complement receptor-mediated phagocytosis favour the host cells rather than the bacteria (Harmon *et al.*, 1988; 1989; Caron *et al.*, 1994; Gross *et al.*, 1998). This phenomenon is more clearly observed in activated than in non-activated professional phagocytes (Young *et al.*, 1985; Harmon *et al.*, 1988; Gross *et al.*, 1998; Arenas *et al.*, 2000; Eze *et al.*, 2000; Gross *et al.*, 2000a). The fact that non-opsonized *Brucella* organisms bind, penetrate and reproduce *in vivo* and *in vitro* within macrophages from naïve animals (Campbell *et al.*, 1994; Sola-Landa *et al.*, 1998; Gross *et al.*, 2000a; Kuzumawati *et al.*, 2000) indicates the existence of a receptor-ligand mechanism that is independent from the Fc and complement receptors. Penetration of *Brucella* into bovine mononuclear phagocytes is inhibited by bacterial cell envelopes, antibodies against the a chain of the MAC-1 integrin (CD11b), *O*-polysaccharide and mannan, suggesting at some point the participation of integrins and scavenger lectin-like receptors. In macrophages, the cyclic-AMP/protein kinase A pathway is activated upon *Brucella* infection (Gross *et al.*, 2003). This activation results in a prolonged phosphorylation of the transcription factor CREB. It seems that the activation of the cyclic-AMP/protein kinase-A pathway is essential for the survival and intracellular replication of *Brucella* in macrophages.

At present, very little is known about the *Brucella* molecular determinants involved in the attachment and penetration of *Brucella* to cells. It has been stated that non-virulent two regulatory component *Brucella* BvrS/BvrR mutants are less efficient at penetrating cells, despite the fact that they bind in larger numbers to cells than wild type bacteria (Guzmán-Verri *et al.*, 2001; see also Chapter 10). Moreover, these mutants fail to stimulate any of the cellular small GTPases previously indicated (Guzmán-Verri *et al.*, 2001). The defects found in the BvrS/ BvrR mutants pinpoint alterations in the structure of the outer membrane, including the absence of outer membrane proteins such as Omp3a (Omp25) and Omp3b (Omp22) as well as defects in LPS biosynthesis (Guzmán-Verri *et al.*, 2002; López-Goñi *et al.*, 2002). The BvrS/BvrR mutants not only present augmented sensitivity to lysosomal cationic peptides, but also are readily destroyed after phagocitosis. As consequence of this alteration, these mutants display reduced virulence in mice (Sola-Landa *et al.*, 1998).

It has been known for long time that the absence of O-chain and concomitantly native hapten (NH) polysaccharides alters the binding and survival of *Brucella* in both professional and non-professional phagocytes (Braun *et al.*, 1958; Harmon *et al.*, 1988; Detilleux *et al.*, 1990a; Sola-Landa *et al.*, 1998; Freer *et al.*, 1999). The augmented binding to cells seems to be the combination of newly exposed molecules and changes in the hydrophobicity on the surface of rough bacteria (Weber *et al.*, 197; Schurig *et al.*, 1981; Martínez-de-Tejada and Moriyón, 1993; Freer *et al.*, 1999), all properties that are hidden by the O-chain in smooth cells. In some cases, rough *Brucella* (live or dead) seem to be toxic for cells monolayers, probably due to the strong hydrophobicity and detergent-like properties displayed by the outer membrane (Braun *et al.*, 1958; Detilleux *et al.*, 1990a; Sola-Landa *et al.*, 1998; Freer *et al.*, 1999). Rough *Brucella* lacking O polysaccharides and NH are more readily destroyed within phagolysosomes that the smooth counterparts (Kreutzer *et al.*, 1979; Sola-Landa *et al.*, 1998), a property that is also reflected in the reduced virulence of these rough bacteria (Moriyón *et al.*, 2003). The reason why these rough bacteria are less virulent is related to their higher sensitivity of bactericidal cationic peptides and lysosomal substances (Martínez-de-Tejada *et al.*, 1995; Freer *et al.*, 1996).

It has been proposed that the *Brucella* VirB type IV secretion system is involved in the entry process to macrophages and epithelial cells (Watarai *et al.*, 2002a; 2002b; 2003; see also Chapter 13). According to this scheme, *Brucella* entry through lipid rafts is specifically mediated by the binding of VirB-dependent surface-exposed Hsp60 to the cellular prion protein PrP^{C}. This interaction would allow virulent bacteria to totally escape the endocytic pathway and arrive to in its replicating niche in macrophages (Watarai *et al.*, 2003). Although the same authors claim that the VirB type IV secretion system participates in the invasion of the bacteria to epithelial cells, the observed mechanism of entry differed to that envisioned in macrophages. Despite these claims, several works have demonstrated that there are not significant differences in the mode and kinetics of entry between wild type *Brucella* and VirB mutants in macrophages or epithelial cells, precluding differential trafficking decisions at entry mediated by this system (Commerci *et al.*, 2001; Boschiroli *et al.*, 2002; Celli *et al.*, 2003). Moreover, several lines of evidence against a role for the VirB secretion machinery in this process have been recently summarised (Celli and Gorvel, 2004) and resumed as follows. It is known that a large proportion of the ingested virulent *Brucella* are killed by macrophages within the first hours after entry (Rittig *et al.*, 2001; Celli et. al., 2003; Gross *et al.*, 2003), implying that they cannot escape the endocytic pathway and, therefore, the potential for intracellular survival is not fully established upon entry (Celli *et al.*, 2003). It is also known that the *Brucella* VirB apparatus is activated intracellularly (Boschiroli *et al.*, 2002) following *Brucella*-containing vacuole acidification (Porte *et al.*, 1999). Moreover, Hsp60 surface detection is surprising, since outer membrane proteins are only accessible to antibodies in rough strains but not in smooth *Brucella*, due to the O-chain of the LPS and NH polysaccharide covering the bacterial surface (Bowden *et al.*, 1995). Finally, the endogenous PrP^{C} is internalised through clathrin-coated pit-mediated endocytosis (Sunyach, 2003), making its use to avoid to the endocytic pathway, unlikely.

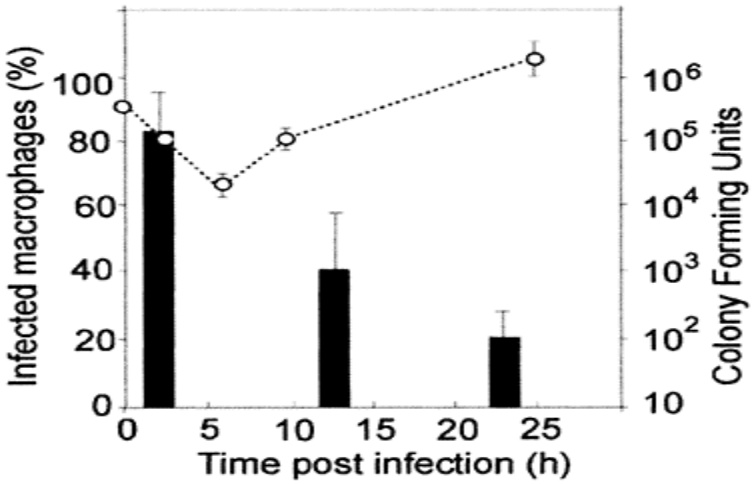

Figure 2. Survival and replication of *B. abortus* in murine macrophages RAW 264.7. The black bars (left scale) indicate the number of *Brucella* infected macrophages in time, while the doted line (right scale) indicates the number of colony forming units counted in time. Notice that the number of macrophages containing live bacteria diminishes over time, while the number of bacteria decreases at early times but increases at later times. This phenomenon is the result of a combination of two events: killing by macrophages at early times and replication of bacteria once they have reached the endoplasmic reticulum.

3. Intracellular Trafficking

Before describing the intracellular trafficking of *Brucella,* we must persevere in clarifying a few aspects that frequently constitute a source of confusion and misinterpretation. In the first place we must emphasise that rough *Brucella* binds differently to cells and are more readily killed than the smooth counterparts; a

phenomenon that has been evident for several decades (Braun *et al.*, 1958) and which differs between phagocytic and non-phagocytic cells (Detilleux *et*

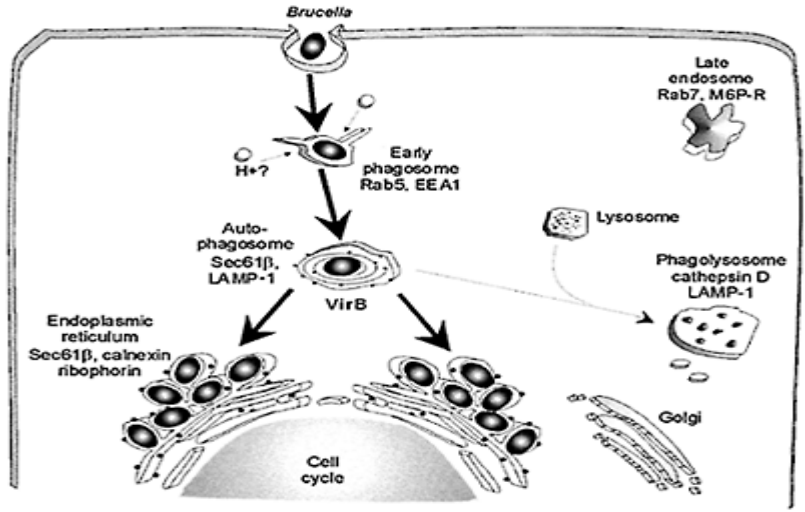

Figure 3. Schematic model of *B. abortus* intracellular trafficking in epithelial HeLa cells. The ingested bacterium is initially routed to early phagocytic compartments (marked by Rab5 and EEA1) which may be acidified (H+?) by acquisition of specific proton pumps (short dotted arrows). In HeLa cells most of the ingested virulent *Brucella* are routed (thick arrows) to the endoplasmic reticulum (marked by calnexin, ribophorin and Sec61β) through the autophagocytic route (marked by LAMP1 and Sec61β), whereas only a few bacteria are directed to phagolysosomes and destroyed (thin dotted arrows). Functional bacterial VirB type IV secretion system is required for fusions with the endoplasmic reticulum and to hamper

interactions with lysosomes. *Brucella* organisms do not transit through late endosomes or Golgi compartments during intracellular trafficking. Once in the endoplasmic reticulum, *Brucella* organisms replicate extensively without hampering DNA synthesis, karyokinesis or cytokinesis.

al., 1990a; 1990b). In turn, this creates different intracellular vacuolar patterns observed under the microscope, which are difficult to interpret. The main source of confusion, however, has been the differences in the intracellular biogenesis of *Brucella* organisms observed between epithelial cells and macrophages. Indeed, the intracellular trafficking of *Brucella* in epithelial cells is relatively homogeneous, with only a few bacteria invading the cell. Then, the internalised bacterium follows a trafficking that generally proceeds to its replicating niche without confronting cellular killing mechanisms (Pizarro-Cerdá *et al.*, 1998a; 1998b). In this model the experimenter has the advantage to follow the intracellular fate of a bacterium without major interferences. In contrast, a large proportion of the ingested *Brucella* is destroyed within the first 8 hours within the phagolysosmes of macrophages and only a small number of the internalised bacteria succeeds in reaching the replicating niche (Celli *et al.*, 2003). As pointed out before, this phenomenon is

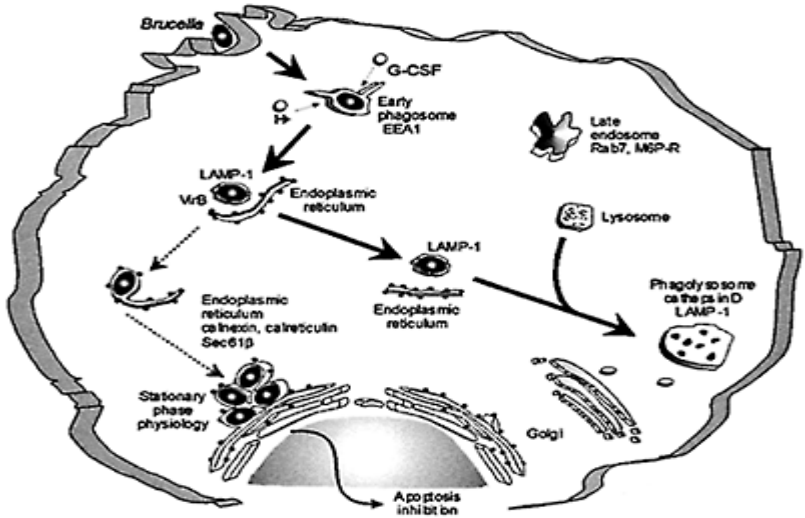

Figure 4. Schematic model of *B. abortus* intracellular trafficking in macrophages. After phagocytosis

Brucella organisms are localised within early phagocytic compartments marked by early endosomal antigen-1 (EAA1) which seems to acidified (H^+) by acquisition of specific proton pumps (short dotted arrow). Granulocyte colony-stimulating factor (G-CSF) is involved in promoting fusions between endocytic vacuoles and *Brucella*-containing vacuoles (short dotted arrow). Initially, the *Brucella*-containing vacuoles acquire LAMP-1 and are found surrounded by, or in close contact with the endoplasmic reticulum. In these phagocytic cells, most of the ingested bacteria do not maintain sustained interactions with the endoplasmic reticulum over time; in consequence they are routed to fuse with lysosomes (thick arrows) and finally destroyed within phagolysosomes (marked by cathepsin D and LAMP1). A few *Brucella*-containing vacuoles, however, are able to maintain sustained interactions with the endoplasmic reticulum via the virB type IV secretion system (thin dotted arrows), fuse and replicate within the cisternae of this compartment (marked by calnexin, calreticulin and Sec61β). *Brucella* organisms do not transit through late endosomes or Golgi compartments during intracellular trafficking. Inhibition of apoptosis is promoted in both, infected and non-infected macrophages. Adapted from Celli *et al.*, 2003.

more evident in activated macrophages and when bacteria are opsonized before phagocytosis (Eze *et al.,* 2000). Moreover, an important proportion of infected macrophages are able to completely clear the infection after 24–48 hours (Figure 2). As expected, this phenomenon causes a highly heterogeneous intracellular pattern of the *Brucella*-containing compartments. Therefore, when not taken into consideration this heterogeneity may be turned into a source of error at the time of interpreting the intracellular trafficking as well as describing the bacterial factors involved. Indeed, *Brucella* already found within digestive vacuoles must confront stressing conditions that differ from those organisms that have avoided lysosomal fusion. As consequence, a diverse pattern of bacterial responses concomitant to the heterogeneous pattern of vacuoles containing bacteria, is generated. In order to resolve this it is necessary to use an adequate set of vacuolar markers at different times of infection and a combination of different microscopic conditions (Celli *et al.,* 2003). It is also indispensable to make a clear distinction between live and dead *Brucella* in order to discern between vacuoles containing bacteria that are directed to lysosomes and those that are trafficking to their replicative compartment (Celli *et al.,* 2003).

Immediately after phagocytosis, virulent *Brucella,* interact with a compartment related to the early endosomal network in both macrophages and epithelial cells (Figures 3 and 4). This is confirmed by the presence of certain markers, such as the transferrin receptor, the small GTP-binding protein rab5, or the early endosomal antigen 1 (EEA1) in the *Brucella*-containing vacuole (Pizarro-Cerdá *et al.,* 1998; Chaves-Olarte 2002; Celli *et al.,* 2003). The association of *Brucella* with the early endocytic network is transient, since 10 min after internalisation, the number of *Brucella*-containing compartments labelled either with rab5 or EEA1 decreases significantly, and no labelling is detected with these markers after 30 min post-inoculation (Pizarro-Cerdá, 1998a, 1998b; Chaves-Olarte, et al 2002). The integrity of the early endosomal system is necessary FOR the subsequent normal trafficking of *Brucella* in host cells. For instance, in cells lines expressing and activated form of rab5 (bound to GTP), an important fraction of the internalised brucellae are unable to escape from the early containing compartments, supporting limited bacterial replication within giant vesicles labelled with rab5 (Chaves-Olarte, *et al.* 2002). However, 48 h after infection, the bacterial proliferation is attenuated in these transfected cells in comparison to the wild type counterparts, indicating that this altered compartment is not suitable for intracellular bacterial replication. In macrophages, a significant number of *Brucella*-containing vacuoles are surrounded by endoplasmic reticulum structures immediately after phagocytosis. Thereafter, the number of *Brucella*-containing vacuoles surrounded by these structures, decreases. This phenomenon is reminiscent of the internalisation of latex beads and of some microbial pathogens by these phagocytic cells (Gargon *et al.,* 2002; Celli *et al.,* 2003).

During the first hours (1–6 h), no clear signs of intracellular bacterial replication are demonstrated. Although *Brucella* organisms do not travel through the late endosome network, vacuole acidification seems to be required since substances that inhibit endosomal acidification are capable of reducing the bacterial number at early but not at later times after infection (Detilleux *et al.,* 1991; Porte *et al.,* 1999). Acidification of this *Brucella*-containing compartment may be necessary for the activation of virulence genes (Antoine *et al.,* 1990; Buchmeier and Heffron, 1990), as it seems the case for the expression of the VirB type IV secretion system (Boschiroli *et al.,* 2002). Following these

initial steps, the *Brucella*-containing compartment is gradually transformed by acquiring (one h after internalisation), the lysosomal-associated membrane protein (LAMP)-1, but not the luminal lysosomal hydrolase cathepsin D. This finding supports the proposition that virulent *Brucella* inhibits the fusion of its phagosome with lysosomal compartments (Frenchick *et al.,* 1985). However, beyond this point and before *Brucella* organisms reach their replicating niche, a striking difference in the biogenesis of this intracellular bacterium is observed between epithelial cells and macrophages (Pizarro-Cerdá *et al.,* 1998a; 1998b; Celli *et al.,* 2003). Indeed, while in epithelial cells *Brucella* are subsequently localised within multimembranous compartments compatible with autophagosomes (Figure 3), in macrophages the *Brucella*-containing vacuoles never acquire characteristics of autophagosomes but rather maintain sustained interactions with the endoplasmic reticulum (Figure 4). In spite of this difference, *Brucella*-containing vacuoles finally fuse with cisterns of the endoplasmic reticulum and the bacteria establish their replicating niche within this compartment, independently if the host cell is a macrophage or an epithelial cell.

In addition to ribosomes bound to the *Brucella*-containing vacuole (Anderson and Cheville, 1986a, 1986b; Detilleux *et al.,* 1990a; 1990b; Pizarro-Cerdá *et al.,* 1998a; 1998b; Celli *et al.,* 2003), the nature of the *Brucella* replicating niche is also revealed by the presence of endoplasmic reticulum markers such as the ribosome translocator protein sec61β, the luminal protein disulfide isomerase and the chaperone proteins calreticulin and calnexin (Pizarro-Cerdá *et al.,* 1998a; 1998b; Chaves-Olarte *et al.,* 2003; Celli *et al.* 2003). Additional evidence confirms the endoplasmic reticulum as the final *Brucella* replication niche in these host cells. First, treatment of infected cells with brefeldin A, which normally induces the reorganisation of the Golgi complex around the endoplasmic reticulum, induces the colocalisation of Golgi markers around the *Brucella*-containing compartments (Pizarro-Cerdá *et al.,* 1998b; Celli *et al.,* 20003). Second, treatment of infected cells with proaerolysin, a toxin from *Aeromonas hydrophyla* that induces vacuolisation of the endoplasmic reticulum, also generates vacuolisation of the *Brucella*-containing compartments (Pizarro-Cerdá *et al.,* 1998a; Celli *et al.,* 2003). Treatment of target cells with proaerolysin before *Brucella* inoculation impairs the bacterial replication and induces the degradation of virulent strain, suggesting that the integrity of the endoplasmic reticulum is required. Third, in heavily infected cells (48 h post infection) there is a dramatic reorganisation of the endoplasmic reticulum which is restricted to the bacterial replication area (Celli *et al.,* 2003). This phenomenon is commonly accompanied by compression of the nucleus periphery by the replicating bacteria without invasion of the nucleoplasm (Anderson and Cheville, 1986a; Anderson *et al.,* 1986; Detilleux *et al.,* 1990a, 1990b). Altogether, these data convincingly demonstrate that *Brucella* organisms replicate within the endoplasmic reticulum of macrophages and epithelial cells, including throphoblasts, the preferred host cells in the pregnant animal.

Trafficking through autophagosomes is not an exclusive property of *Brucella* organisms, since other bacteria such as *Legionella* and *Porphyromonas* have also been found to travel through and even replicate within these organelles (Dorn *et al.,* 2002). Presently there is not a clear explanation for bacteria trafficking through autophagosomes. One of the initial hypotheses is that degradation products found in this compartment may serve as a source of nutrients for the intracellular bacteria (Dorn *et al.,* 2002). In the case of *Brucella,* this proposition seems unlikely, since this intracellular

bacterium does not replicate within autophagosomes of epithelial cells; rather it is only transiently found within this compartment (Pizarro-Cerdá *et al.,* 1998a). Moreover, in macrophages *Brucella* do not travel through autophagosomes, but the bacteria containing vacuoles progressively associate with endoplasmic reticulum structures acquiring specific markers of this compartment through limited fusion events (Celli *et al.,* 2003). In any case, and in spite of the differences observed between macrophages and epithelial cells, it seems that fusion of the *Brucella*-containing vacuole with its final niche, is achieved by selective acquisition of endoplasmic reticulum components and elimination of endosomal proteins such as LAMP and that this process is mediated by the bacterium. Indeed, in both macrophages and epithelial cells, this fusion process is mediated and regulated by the VirB apparatus (Comerci *et al.,* 2001; Delrue *et al.,* 2002; Celli *et al.,* 2003), which seems to be activated by acidic conditions within the vacuolar environment (Boschiroli *et al.,* 2002). It is important to underline, however, that during the *Brucella* journey to this suitable replicative compartment, the bacterium does not divide. Furthermore, in addition to the VirB system, the control of the intracellular trafficking seems to require the adequate expression of stationary-phase genes. Indeed, it has been shown that stationary phase physiology is needed for intracellular long viability within macrophages (Roop *et al.,* 2003). Some of these genes, such as the *hfq,* stress proteins and iron capturing molecules seem to be necessary during intracellular life (see Chapter 12).

In epithelial cells the trafficking and final fate of the internalised *Brucella* are independent events which can be dissected from the mode of entry (Chaves-Olarte *et al.,* 2003). In macrophages, however, this is not to the case since the mode of internalisation (e.g. opsonization by antibodies or complement) influences the efficiency of the infection in these cells, as stated before. Although the reasons behind this difference are not known, it seems that the phenomenon is related to the cell properties rather than to the bacterium characteristics. Indeed, certain activation processes such as the inducible nitric oxidase synthase or NADPH oxidase mechanisms of macrophages seem to be independent of the virB system (Sun *et al.,* 2002). As stated before in macrophages the usage of Fc, complement or scavenger receptors seems to activate macrophages and in consequence making the killing mechanisms more efficient. This is also supported by the fact that knockout macrophages deficient in vacuolar fusion events controlled by certain cytokines such as granulocyte colony stimulation factor, allowed the extensive replication of attenuated strains of *Brucella* (Pizarro-Cerdá *et al.,* 1999). Similarly, macrophages activated through the cannabinoid receptor CB1, acquire the capacity to control *Brucella* infection indicating that the CB1 receptor-triggering engages the microbicidal activity of phagocytes (Gross *et al.,* 2000a).

4. The Replicating Niche

Besides *Brucella,* other bacteria such as *Legionella pneumophila* and parasites such as *Toxoplasma gondii* and simian virus 40 multiply in endoplasmic reticulum derived vacuoles, revealing a path of convergent evolution in non-related organisms (Swanson and Isberg, 1995; Sinai *et al.,* 1997a; Stang *et al.,* 1997). The benefits involved in the association of these pathogens with the host-cell endoplasmic reticulum have not been characterised yet. In addition to the obvious advantage of being in an intravacuolar

environment in which the bacterium avoids lysosomal fusion, association with the host endoplasmic reticulum must be a means for the *Brucella* to obtain the adequate nutrient supply for reproduction. Therefore, the inside milieu of the invaded endoplasmic reticulum must provide for all the components necessary for an extensive bacterial replication which occurs at later times (from 24–72 h) of the infection. It is also feasible that *Brucella* could extract the required substances for replication under microaerobic conditions, if that would be the case inside the replicating niche. Indeed, contrary to the general believe, the growth requirements of *Brucella* organisms are not excessive and the bacteria are able to growth under minimal nutrient conditions (Gerhardt, 1958) and under microaerobic environments. These properties have been reinforced by the complete genomic analysis of various *Brucella* strains which have demonstrated the presence of genes coding for these metabolic alternatives, some of which may be more important during intracellular replication (DelVecchio *et al.*, 2002; Paulsen *et al.*, 2002).

The overall higher hydrophobicity of *Brucella* cell envelopes, the close association among the macromolecules of the outer-membrane and the properties of the porins are factors implicated in the selective penetration of nutrients inside the bacterial cell (reviewed by Moreno and Moriyón, 2001). For instance, it has been suggested that some porins not expressed *in vitro* may be specifically expressed during intracellular parasitism (Ficht *et al.*, 1989; Marquis and Ficht, 1993) and that the permeability of the *Brucella* outer membrane to hydrophobic substances may allow the uptake of hormones and siderophores. Indeed, it has been found that *Brucella* infected trophoblasts produce cortisol, a steroidal hormone not normally generated by the placenta (Enright and Samartino, 1994) and that iron-capturing bacterial siderophores possess the adequate structure for crossing the outer membrane layer (López-Goñi *et al.*, 1992). The absence of a barrier to hydrophobic substances is linked to the structure of the *Brucella* LPS core and lipid A (Martínez-de-Tejada and Moriyón, 1993; Freer *et al.*, 1996; Velasco *et al.*, 2000). The advantages of a hydrophobic envelope for intracellular α-2 Proteobacteria are illustrated by the finding that *Rhizobium* LPS becomes highly hydrophobic during bacteroid development (Kannenberg and Carlson, 2001). The net result of this structural change in the LPS is that intracellular bacteroids have a more hydrophobic outer membrane than the free living rhizobiae. This adaptive condition could promote the exchange of nutrients and favour intracellular life of the bacteroids. Obvious comparisons between the intracellular lifestyle of *Brucella* and *Rhizobium* emerge, since these two bacteria are phylogenetically close relatives (Moreno *et al.*, 1990).

Once *Brucella* organisms arrive at the endoplasmic reticulum, they start replicating until they achieve a limiting stationary bacterial density. It seems that in this compartment the intravesicular pH rises (Köhler *et al.*, 2002a; 2002b; see also Chapter 15), giving opportunity for a set of bacterial mechanism to be turn off while others are turned on. Indeed, quorum sensing hydrophobic molecules such as acyl-homoserine lactone released by *Brucella*, down regulate the expression of virB genes *in vitro* (Taminiau *et al.*, 2002), suggesting that this small hydrophobic molecules could play a role in the control of the type IV secretion system, necessary for the intracellular trafficking, but unnecessary during intracellular growth inside the endoplasmic reticulum. At this point it seems logical that the expression of stationary-phase genes reinitiate adjusting the bacterial physiology to the needs of the intracellular colony (Köholer *et al.*, 2002a; 2002b; Roop *et al.*, 2003), inhabiting practically every single cistern of the

endoplasmic reticulum. This hypothesis is supported by the expression of similar patterns of *Brucella* cytoplasmic proteins during stress conditions and intracellular growth (Rafie-Kolpin *et al.,* 1996) as well as by the diversity of bacterial genes that seem to be employed during the replicating phase (Köhler *et al.,* 2002a; 2002b; Kim *et al.,* 2003). However, we should be cautious in defining the intracellular conditions prevailing within the replicative compartment of macrophages, based solely in the expression of *Brucella* genes. The broad expression of bacterial genes observed in macrophages may be the consequence of the diversity in bacterial responses which are located within different compartments, rather that the reflection of the conditions prevailing in the replicative niche. In this sense, it is relevant to pay attention to the heterogeneous patterns of intravacuolar *Brucella* observed in professional phagocytes.

One of the striking properties induced by intracellular replicating *Brucella* organisms is the ability to inhibit programmed cell death (Gross *et al.,* 2000b). Similarly, heavily infected *Brucella* trophoblasts, epithelial cells and macrophages do not display obvious signs of toxicity (although the final outcome of infected cells might proceed by necrosis) (Anderson *et al.,* 1986; Anderson and Cheville, 1986; Detilleux *et al.,* 1990b; Jiang and Baldwin, 1993; Tobias *et al.,* 1993). The absence of cytotoxicity has been also demonstrated by the sustained ability of heavily infected cells to proceed with DNA synthesis, microtubule spin formation, chromosome migration, karyokinesis and cytokinesis (Chaves-Olarte *et al.,* 2002). As a consequence, dividing cells filled with *Brucella* are frequently observed *in vivo* and *in vitro* (Detielleux *et al.,* 1990a; 1990b; 1991). In CNF-treated HeLa cells, cytokinesis is inhibited without affecting nuclear division. When these cells are infected with *Brucella,* karyokinesis proceeds without signs of degeneration, despite the large number of intracellular *Brucella* within the endoplasmic reticulum (Chaves-Olarte *et al.,* 2002). Replicating intracellular *Brucella* prevents apoptosis of human monocytes and macrophages (Gross *et al.,* 2000b; Wang *et al.,* 2001). Since both invaded and non-invaded cells, including lymphocytes (cultured in proximity to the infected cells) are guarded against apoptosis, it has been suggested that this protective mechanism is mediated through soluble substances released by the infected cells during bacterial infection. It seems that the apoptosis inhibition mediated by the infected cells requires the over expression of the *A1* gene, a member of the *bcl*-2 family involved in the survival of blood-forming cells (Gross *et al.,* 2000b). Moreover, the anti-apoptotic activity is evident at later times of infection (beyond 24 h), suggesting that this effect is mediated by the replicating bacteria within the endoplasmic reticulum and not during the intracellular trafficking towards this compartment. Indeed, apoptosis mediated by nitric oxide (NO) produced by iNOS protein of activated macrophages (Wang *et al.,* 2001), is inhibited by *Brucella* infected cells at later (beyond 24 h) but not at earlier times. Since NO produced by activated macrophages also accelerates the killing of *Brucella* at early but not at later times, it is reasonable to believe that replicating bacteria could express genes that counteract the effect of NO (Wang *et al.,* 2001). Alternatively, *Brucella* may activate genes to utilise NO as a nitrogen source within the endoplasmic reticulum. This last asseveration is supported by the recent complete genome analysis of *Brucella* which has revealed the presence of genes coding for nitric and nitrous oxide reductase (DelVecchio *et al.,* 2002; Paulsen *et al.,* 2002).

The obvious explanation for prolonging the life of cells and not producing acute cytotoxic effects is that the bacterium would be better suited for parasitism if it could

sustain replication for longer times in the host cell. In addition of being an advantage, this phenomenon could be related, at least partly, with the ability of *Brucella* organisms to generate chronic infections (Tolomeo *et al.,* 2003). We have observed in cell cultures of epithelial cells and macrophages that while some cells are heavily infected with *Brucella*, others maintain a limited number of bacteria for prolonged periods of time, even after 5 weeks of culture under conditions in which not infected cells died after 4 days (E.Moreno, unpublished). The fact that some bacteria promote programmed cell death whereas others are prone to prevent it and even prolong cell life, is suggestive and works in favour of this hypothesis. Indeed, within the group inducing apoptosis there are bacteria that produce acute infections, such as *Shigella* and *Salmonella* (Navarre and Zchylinsky, 2000). Interestingly, in the second group we have Proteobacteria of the a subdivision, such as *Rickettsia* and *Brucella*, as well as other intracellular bacteria such as *Chlamydia*, all capable to prevent apoptosis (Clifton *et al.,* 1998; van Zandbergen *et al.,* 2004).

5. *Brucella* Molecules Released During Intracellular Life: The LPS Case

Although the main intracellular trafficking pathways and the site of replication of *Brucella* have been established in professional and non-professional phagocytes, practically nothing is known about the molecular determinants involved in these processes. As indicated before, it has been recognised that only living but not killed or attenuated *Brucella* are able to avoid fusion with lysosomes. Moreover, this phenomenon is more conspicuous in non-professional phagocytes than in macrophages. In addition to energy input, it has been proposed that the VirB type-IV secretion apparatus may be the main structure responsible for secreting molecules that hamper phagosome-lysosome fusion (Comerci *et al.,* 2001; Delrue *et al.,* 2001; Boschiroli *et al.,* 2002; Celli *et al.,* 2003). Indeed, the function of this secretion system seems to be activated by acidification during early times of intracellular trafficking and seems to be turned down during the replication phase within the endoplasmic reticulum (D.Comerci, personal communication). In addition, VirB mutants do not sustain competent fusions with the endoplasmic reticulum, and some non-polar mutants, although do not fuse with lysosomes, are incapable of reaching their replication niche. Altogether these experiments strongly support the idea that this secretion system releases substances that control bacterial trafficking during the early stages of intracellular parasitism.

Until now, no other *bona fide* secretion system has been described in the *Brucella* genus. In spite of this, it has been shown that mutations in “putative” flagellar genes produce attenuated bacteria incapable to achieve extensive replication in host cells counterparts (Léonard *et al.,* 2003). Although flagellar-like structures have been occasionally reported under the electron microscope (J.Girón, personal communication), it is known that *Brucella* organisms are essentially non-motile. Therefore, it may well be that the flagellar proteins constitute a second secretion apparatus devoted to release substances for the control of different functions during the *Brucella* intracellular life (Moreno and Moriyón, 2001).

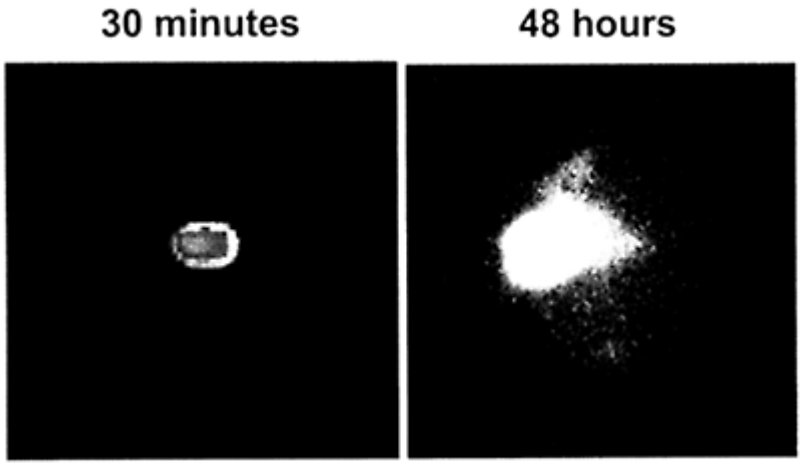

Figure 5. Detection of LPS by immunofluoresence in epithelial HeLa cells. At 30 minutes post-infection no LPS is detected outside the perimeter of the *Brucella*-containing compartment. However, after 48 hours, during the bacterial replicating period, large quantities of released LPS can be detected in the periphery of the compartment.

Indeed, from the genetic point of view it seems possible that the flagellar proteins are able to construct a basic structure with the characteristics of a secretion apparatus (DelVecchio *et al.,* 2002; Paulsen *et al.,* 2002). If this experimental data is consolidated (Leonard *et al.,* 2003), the next step would be to determine if this flagellar system could be involved in attachment and invasion, intracellular trafficking, replication or even in regulating programmed cells death.

A striking feature recently described (Lapaque *et al.,* 2004), is that replicating *Brucella* release large quantities of LPS inside cells (Figure 5). This phenomenon is relevant from several perspectives. In the first place, it has been known for more than 20 years that *Brucella* LPS displays very little endotoxicity and that it is a poor inducer of cytotoxic mediators (Moreno *et al.,* 1981; Rasool *et al.,* 1992). This property has been confirmed (Goldstein *et al.,* 1992; López-Urrutia *et al.,* 2000) and complemented by a second characteristic, showing that *Brucella* LPS is also the molecule responsible for conferring resistance to bactericidal lysosomal substances and cationic peptides (Martinez-de-Tejada *et al.,* 1995; Freer *et al.,* 1999). Third, *Brucella* LPS is initially directed to late endosomes then to lysosomes where it resists degradation. Thereafter the LPS molecules proceed to a compartment in which they intersect MHC-II proteins

forming large complexes that migrate to the cell surface (Forestier *et al.*, 1999; 2000). *Brucella* LPS molecules form stable complexes with MHC-II but not with MHC-I proteins. In contrast to antigenic peptides intersecting MHC-II containing compartments, the intracellular trafficking of *Brucella* LPS is a slow process that takes several hours (Forestier et. al., 1999; 2000; Lapaque *et al.*, 2004). Once in the cell membrane, the *Brucella* LPS forms large macrodomains identified as lipid mega rafts in which MHC-II as well as other proteins and lipid molecules such CD-14, cholesterol, gangliosides and GPI are captured (Figure 6). Remarkably, *Brucella* LPS complexes remain in macrophages up to 3 months without been degraded (Forestier *et al.*, 2000).

One striking finding has been the observation that *Brucella* LPS is capable to impair the MHC-II presentation pathway, but not MHC-I presentation of foreign

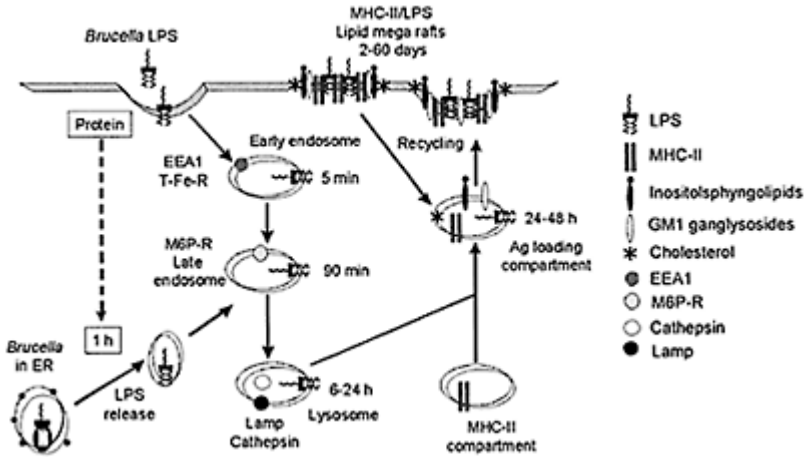

Figure 6. Intracellular trafficking of *Brucella* LPS inside macrophages. LPS released by replicating *Brucella* or ingested from the outside milieu is incorporated into vacuoles that fuse with lysosomes and then to compartments loaded with MHC-II and lipid rafts. Then the LPS-MHC-II lipid rafts complexes are clustered in mega rafts and recycled to the cell membrane. This phenomenon proceeds with a very slow kinetics as compared to proteins intersecting MHC-II containing compartments. *Brucella* LPS complexes remain in

macrophages for long time (up to 3 months) without signs of degradation.

peptide antigens (Forestier *et al.*, 2000; Lapaque *et al.*, 2004). This impairment is not the result of a reduced MHC-II surface expression, deficient uptake or poor catabolism of the native antigen. Moreover, this phenomenon is not due to a reduced number of B7 membranous co-stimulatory molecules, a defective alpha/beta dimmer formation or a direct suppressive action of LPS on T cells, independent of macrophages. More likely, *Brucella* LPS molecules are capable of sequestering surface MHC-II molecules in mega rafts, hampering these proteins from reaching functional lipid rafts and consequently impairing presentation of peptides to specific CD4+ T cells. The *in vitro* inhibition of the immune response correlates with that observed *in vivo* upon infection with *Brucella*. It is worth noting that chronic brucellosis may be accompanied by a general immunosuppression that can be revealed by using an IL-2 detection system (Zhang 1992; Zhang *et al.*, 1993). An additional consequence of LPS binding to MHC-II is that epitopes of this bacterial glycolipid seem to be presented within this context by antigen presenting cells to CD4+ CD25+ lymphocytes, with a phenotype compatible with regulatory T cells (Forquet, Jolly, Lapaque, Moriyón, Gorvel and Moreno, unpublished results). *Brucella* LPS recognition depends on the O-polysaccharide moiety and on MHC-II but not MHC-I molecules as demonstrated by the use of inhibitory antibodies as well as MHC-II and MHC-I deficient mice. The low frequency of LPS-reactive T cells as compared to mitogen reactive T cells is consistent with a specific LPS T cell recognition.

All these properties, which are related to the particular structure of the *Brucella* LPS, such as long chain hydroxylated fatty acids (up to 30 C long), and distinctive acylation patterns on a diaminoglucose backbone (Moriyón, 2003), may be envisioned as evolutionary advantages for the adaptation to intracellular life (Freer *et al.*, 1996; Moreno, 1992; Rasool *et al.*, 1992; Velasco *et al.*, 2000). Consequently, delaying proinflammatory responses and avoiding efficient macrophage killing mechanisms during intracellular life constitute obvious advantages for slow growing bacteria that otherwise are destroyed more readily by activated cells. At the same time, the LPS conferring bacterial resistance to killing microbicidal molecules such as defensins and lysozyme, represent an additional benefit for standing the harsh conditions that exist during intracellular trafficking. Then, the invading *Brucella* may simple delay the killing processes, giving time to the bacterium to arrive at the endoplasmic reticulum, which constitutes a safer compartment for replication and release of LPS. Finally, inhibiting presentation of peptide antigens to T cells and generation of regulatory T lymphocytes against *Brucella* LPS may constitute further advantages, since these phenomena may delay the immune response and favour the establishment of chronic infections. As a final remark it is worth noting that some of the structural and low endotoxic properties of *Brucella* LPS are also shared by other intracellular animal pathogens such as *Porphyromonas, Rickettsia, Legionella, Coxiella, Chlamydia* and *Bartonella* and even by some plant pathogens and endosymbionts such as *Agrobacterium* and *Rhizobium* (Schramek *et al.*, 1977; Hollingdale *et al.*, 1980; Helander *et al.*, 1982; Amano *et al.*, 1987, 1993; Tanamoto et al., 1997; Neumeister et al., 1998; Brade *et al.*, 1988; Vandenplas *et al.*, 2002). Therefore, it may not come to surprise that the LPSs of these bacteria eventually could display similar behaviour as the *Brucella* LPS.

6. Acknowledgments

We are thankful to Esteban Chaves-Olarte for the revision of the manuscript. This work was partially supported by grants from INSERM (4N004B); institutional grants from INSERM (biotechnologies), CNRS (PICS 558), Research contract ICA4-CT-1999–10001 from the European Community, RTD project NOVELTARGETVACCINES, and by the Ministerio de Ciencia y Tecnología/ Consejo Nacional de Ciencia y Tecnología, Costa Rica.

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Chapter 15
The Intramacrophagic Environment of *Brucella* spp. and Their Replicative Niche

Véronique Jubier-Maurin, Séverine Loisel, Jean-Pierre Liautard, and Stephan Köhler*

From: *Brucella:* Molecular and Cellular Biology. Edited by: Ignacio López-Goñi and Ignacio Moriyón

Abstract

The intracellular bacteria *Brucella* spp. replicate within their host cell, the macrophage. This relationship is slowly being elucidated, especially the characterization of the compartment containing the pathogen, and the adaptive response of the latter to this environment. Entry of brucellae via a LPS O chain-lipid raft interaction is critical and leads to inhibition of early phagosome-ly sosome fusion. Rough strains, devoid of O antigen, do not use lipid rafts for entry and cannot avoid fusion. Major stress proteins allow the pathogen to resist at this stage of infection. In a second step, activation of specific genes such as *virB* in an acidic compartment deprived of amino acids results in the establishment of the replicative niche termed “brucellosome”. Escape into this final compartment allows the onset of multiplication and necessitates expression of genes we defined as the intramacrophagic “virulome”. Its analysis allows an indirect characterization of the brucellosome, resulting in the conclusion that the environment encountered by *Brucella* inside the macrophage is deprived in amino acids, poor in nutrients, and characterised by low oxygen tension. Brucellae live in a specific, isolated niche, and infection does not seem to affect the main functions of the cell. These observations contributed to their nickname, “stealthy bacteria”.

1. Introduction

1.1. *Brucella* is an Intracellular, Facultatively Extracellular Bacterium

Evolution of the knowledge on the interaction between *Brucella* spp. and their host leads to the definition of these bacteria as intracellular, facultative extracellular (Moreno and Moriyon, 2002). This view of the life of *Brucella* implies that it subverts the cellular host to obtain a safe environment resulting in efficient replication. The type of cell used by *Brucella* to multiply in is not indifferent. *In vitro, Brucella* can replicate in epithelial cells such as HeLa cells (Tatum *et al.,* 1992) and Vero cells (Detilleux *et al.,* 1990), or in macrophage cell lines (Caron *et al.,* 1994c) or monocyte-derived macrophages (Frenchick *et al.,* 1985; Ottones *et al.,* 2000). *In vivo,* the multiplication of *Brucella* in the trophoblastic cells of

* For correspondence email kohler@univ-montp2.fr

the placenta of domestic animals is a well-documented phenomenon (Meador and Deyoe, 1989). However, after challenge, invading *Brucella* are distributed to regional lymph nodes by lymphatic drainage, probably within phagocytic cells (Enright, 1990). Failure to destroy *Brucella* within the draining lymph nodes results in persistence of infection, and in eventual escape via the blood using monocytes or macrophages as vehicle. This results in bacteraemia, quite uncommon in domestic animals (Enright, 1990) but a general feature in human infection, resulting in the well-known undulant fever and a positive blood culture (Young, 1989). Spleen invasion characterises the secondary localisation. This means that *Brucella* multiplies in the macrophages, a cell type involved in the physical elimination of invading pathogens.

1.2. Macrophages are Specific Targets of *Brucella* for its Multiplication

Macrophages are dangerous cells for the bacteria, and only a specific inhibition of bactericidal activities and the avoidance of cell defence mechanisms can allow intracellular multiplication. On the other hand, they are particularly important for the survival and spreading of *Brucella* spp. during infection (Liautard *et al.,* 1996). Complex mechanisms have been developed by *Brucella* to cope with the weapons of the macrophage and to modify its own intracellular trafficking, allowing the creation of the replicative niche it needs. Inhibition of the activation of the macrophage is the first action of the bacteria. Indeed, *Brucella* inhibits activation of the human macrophage by blocking TNF-α production (Caron *et al.,* 1994d; Jubier-Maurin *et al.,* 2001a), and increasing cellular cAMP (Gross *et al.,* 2003). The second action is to keep the macrophage alive as long as possible, this is obtained by inhibiting apoptosis (Gross *et al.,* 2000). However, these modifications of the macrophage are necessary but not sufficient to allow

colonisation by the bacteria. The pathogen must find a safe way to penetrate the macrophage and then prepare and build a niche where it can multiply safely.

1.3. A Bacterial Point of View

Analysis of the fate of *Brucella* inside the macrophage can be performed either from the point of view of the bacterium or from the one of the cell. The different compartments where the bacteria live can be characterised either by their cellular markers, or by the modification of the bacterial metabolism, reflecting the perception of the environment by the parasite. This second approach to analyse the environment of *Brucella* is more functional and reveals a genuine strategy developed by the parasite to avoid the destruction by macrophages. We therefore propose to define the environment encountered by brucellae inside the macrophage by analysing mainly the response of the bacteria to the challenging modifications of its surrounding during intracellular life.

2. Entry of *Brucella* into the Macrophage

Many receptors present on the macrophage surface can be used by *Brucella* spp. to penetrate the cell. It is quite clear that the type of receptor used will determine the fate of the bacteria inside the cell (Gorvel and Moreno, 2002). The receptor(s)

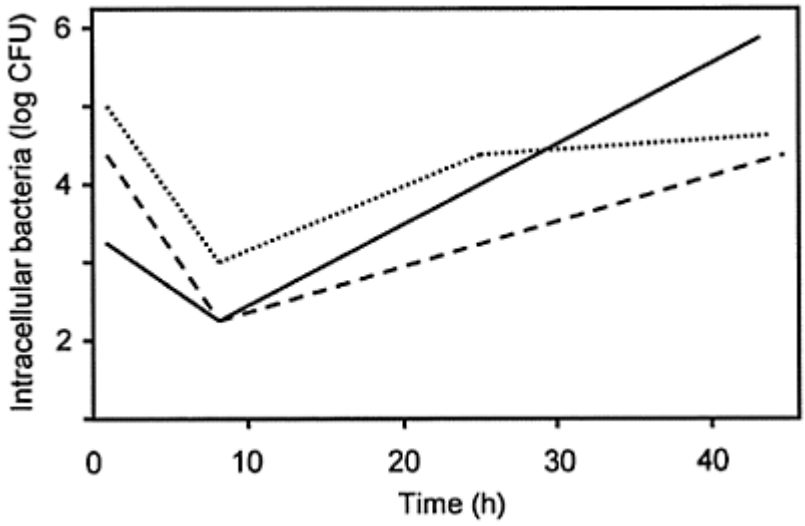

Figure 1. Schematic presentation of the intramacrophagic survival profiles of smooth and rough *B. suis* strains in a macrophage model of infection. Solid line: smooth, non-opsonized

bacteria; dashed line: smooth, antibody-opsonized bacteria; spotted line: rough, non-opsonized bacteria.

used by *Brucella* has not yet been identified without ambiguity, although lectines, fibronectin and recently prion protein have been suggested (Campbell *et al.*, 1994; Watarai *et al.*, 2003). Furthermore, despite the fact that the different species of *Brucella* are genetically very close (Verger *et al.*, 1985), striking differences in their behaviour have been published for different species. We present and compare below the results obtained with *B. abortus* and *B. suis*.

2.1. *B. suis* Utilises its O-antigen to Target the Lipid Rafts of the Macrophage

In order to understand the importance that has to be attributed to the portal of entry for the evolution of macrophage infection, we propose to compare the fate of *Brucella* depending on the cell surface properties of the bacteria: antibody-opsonized *B. suis*, rough mutants of *B. suis*, and wild type smooth *B. suis*. The intramacrophagic multiplication is schematically represented in Figure 1. Penetration by the Fc-receptors is about 10 times more efficient as compared to uptake of non-opsonized bacteria. However, decrease of the viable *B. suis* at 7–10 hours post infection is higher, and the rate of replication thereafter is obviously reduced. Different explanations have been put forward to explain this phenomenon, such as activation of the oxidative burst by opsonized bacteria (Caron and Hall, 1998), or general activation of macrophages by Fc-receptors (Liautard *et al.*, 1996). Anyway, it is evident that the penetration route is totally different from the one involved in the uptake of non-opsonized wild type *B. suis*. These observations make clear that the door of entry is of great importance for the bacteria. Rough brucellae adhere very strongly to the cell surface (Fernandez-Prada *et al.*, 2003; M.P.Jimenez de Bagues and J.Dornand, unpublished), resulting in a very high rate of phagocytosis by macrophages, but a very poor intracellular multiplication (Figure 1) (Porte *et al.*, 2003). Analysis of the entry mechanism has clearly shown that rough mutants do not enter by the same receptors as smooth brucellae. Indeed, wild type smooth *B. suis* need intact lipid rafts to penetrate into the macrophages (Naroeni and Porte, 2002); on the other hand, rough mutants are totally indifferent to disorganisation of cholesterol-rich lipid rafts for invasion of macrophages (Porte *et al.*, 2003).

2.2. *B. abortus* Needs Functional VirB and Targets Prion Protein on Mouse Macrophages

The results described above were obtained with *B. suis*, and differ considerably from those obtained with *B. abortus* by the group of Watarai (see Chapter 13). Indeed, the VirB type IV secretion system seems to be involved in the lipid raft-dependent internalisation of *B. abortus* by mouse macrophages, which then decides of the subsequent fate of the bacteria (Watarai *et al.*, 2002). On the contrary, it should be noted that Delrue *et al.* (2001) could not find a correlation between VirB and phagocytosis of *B. abortus*. An interesting finding may explain the discrepancy of the results obtained with

the two species: *virB* appears to be induced only intracellularly in *B. suis* (Boschiroli *et al.*, 2002b), and is therefore not present on the surface of the bacteria during phagocytosis, while in *B. abortus* it is expressed in the late log phase of growth in broth culture (Sieira *et al.*, 2000; Rouot *et al.*, 2003) and is present on the cell surface during phagocytosis experiments.

More recently, Watarai *et al.* (2003; see also Chapter 13) have shown that the Hsp60 homologue resides on the surface of *B. abortus* possessing a functional VirB system. They show that presence of this stress protein correlates with swimming internalisation that uses lipid rafts. Furthermore, this phenomenon is dependent on the presence of the Prion protein that is inserted into the macrophage surface by a GPI-anchor. Interaction between Hsp60 and Prion protein has been demonstrated (Edenhofer *et al.*, 1996) and quantified (Guerin *et al.*, 2001), and has been published lately for *B. abortus* Hsp60 (Watarai *et al.*, 2003). In macrophages isolated from mice where the Prion protein had been knocked out, *B. abortus* are not able to multiply. In our hands, we have been neither able to find an interaction between recombinant hamster Prion protein and *B. suis* nor to notice a modification of the phagocytosis rate or intracellular replication rate using THP-1 cells and antibodies against human Prion protein (Köhler, Gross & Liautard, unpublished). The question arises if these different routes of entry may result in the creation of different compartments inside the macrophage.

3. The Early *Brucella*-Containing Phagosome Avoids Lysosome Fusion: The Loop Line

What happens to *Brucella* after penetration into macrophages? Their fate depends on the structure of the LPS in the case of *B. suis*, and on the presence of an active VirB machinery for *B. abortus*. A strong inhibition of short-term survival (1 h) of the smooth *B. suis* strain is observed when cells are pre-treated with cholesterol-binding or -depleting molecules or with a ganglioside GM1-binding molecule (Naroeni and Porte, 2002). Therefore, we propose that bacteria must associate with lipid rafts to escape killing, and to survive inside the macrophage. On the contrary, short-term survival of the rough mutants of *B. suis* was not affected following treatment of the cells with these drugs, and thus did not involve lipid rafts (Porte *et al.*, 2003).

Why are rough *B. suis* mutants so sensitive to macrophage killing? The answer was obtained by analysing the fusion of the compartment containing *Brucella* with lysosomes. Phagosomes containing rough mutants are prone to rapid fusion with lysosomes that unload deleterious components killing most of *B. suis* (Porte *et al.*, 2003). In contrast, smooth wild type *B. suis* stay in a membrane-bound structure that does not fuse with lysosomes. This phenomenon is entirely due to the O-chain of the LPS because killed *B. suis* are able to avoid lysosome fusion during the first hours (Porte *et al.*, 2003). LPS O-antigen may govern entry via the lipid rafts and determine the genesis of phagosomes. We can suppose that the LPS O-chain may be involved in an interaction between *B. suis* and the lipid rafts of the cell membrane, either directly or, more likely, via an unknown receptor. After vacuole formation, the interactions between *B. suis* and the lipid rafts in the phagosomal membrane remain strong enough to impair phagosome-lysosome fusion. Therefore, these results support the hypothesis that lipid rafts may be implicated in the

entry of some microorganisms into host cells, resulting in an endocytic pathway which avoids rapid fusion with lysosomes. Although the mechanisms of entry appear to be different for *B. abortus*, involving VirB, Hsp60, and Prion protein, the final targets are obviously rich in lipid rafts. This leads to the same type of compartment as the one described above for *B. suis*.

Taken together, these observations suggest that bacterial entry by way of lipid rafts is essential for the maturation of the nascent vacuole. As described below, it is known that, following uptake, brucellae-containing phagosomes rapidly acidify (Porte *et al.*, 1999; Arenas *et al.*, 2000). It has been shown that subunits of the proton pump V-ATPase concentrate inside the raft structure (Gagnon *et al.*, 2002). Thus, it is possible that the association with lipid rafts is responsible for the rapid decrease of the intravacuolar pH.

4. Acidification of the Early *Brucella*-Containing Phagosome is Essential for Intracellular Replication

The acidification of pathogen-containing vacuoles to a low, harmful pH, often associated to phagosome-lysosome fusion, is part of the instruments that phagocytic cells have developed to kill pathogens or at least inhibit their growth. Some facultative intracellular bacteria, such as *Coxiella burnetii* (Maurin *et al.*, 1992), *Francisella tularensis* (Fortier *et al.*, 1995), and *Salmonella typhimurium* (Rathman *et al.*, 1996), however, have adapted to acidic phagosomes or phagolysosomes. In general, such an adaptation is accompanied by the synthesis of a specific set of proteins by the pathogen.

In murine macrophages infected by *B. suis*, intraphagosomal pH was determined by measuring the pH-dependent fluorescence intensity of brucellae labelled by a fluorescent dye. Vacuoles containing live *B. suis* acidify within one hour after phagocytosis to a pH of about 4.0 to 4.5 (Porte *et al.*, 1999), and are therefore as acidic as the vacuoles with killed *B. suis*. The major mechanism of acidification of *Brucella*-containing vacuoles appears to be proton pumping via vacuolar proton-ATPases sensitive to bafilomycin. Interestingly, the low phagosomal pH is crucial to the survival and replication of the pathogen: neutralisation of the vacuolar pH by various reagents considerably reduces short-term intracellular survival of *B. suis*. Investigations over a longer period of infection (48 h) made clear that early neutralisation results in complete elimination of intramacrophagic brucellae, whereas intracellular bacteria develop normally when the compartments are neutralised several hours after the onset of infection (Porte *et al.*, 1999). Obviously, intravacuolar pH becomes less acidic or even neutral at 7–12 h post infection when bacterial replication starts, as brucellae cannot grow at a pH lower than 5.5–6 (Kulakov *et al.*, 1997). The acidification of the vacuole containing *B. suis* can be therefore considered as an environmental signal that triggers an adaptive bacterial response, allowing the pathogen to reach a safe haven. The system in charge of sensing the environmental pH is still unknown. In several enterobacteria, a two component system transmits the signal to the bacterium (Bearson *et al.*, 1997). The sequencing of the *B. melitensis* and *B. suis* genomes has revealed the existence of at least 20 putative signal-transduction systems (DelVecchio *et al.*, 2002; Paulsen *et al.*, 2002). However, none of them has yet been attributed specifically to pH sensing.

It has been reported in early work on brucellae that this pathogen remains in a vacuole throughout its intracellular life (Harmon *et al.*, 1988), in contrast to other facultative intracellular bacteria such as *Listeria monocytogenes* and *Shigella flexneri* which have developed the strategy to rapidly escape into the cytoplasm by membrane lysis using cytolysins such as listeriolysin. The question, whether *B. suis* could survive or even replicate in the absence of an intact phagosomal membrane, was of interest to the general understanding of *Brucella* spp. virulence mechanisms, especially knowing that the early acidification of *B. suis*-containing phagosomes is crucial for the intracellular replication of the bacteria in macrophages. To this end, listeriolysin was expressed in *B. suis*, and its secretion into the culture supernatant was obtained. In human macrophage-like cells, these bacteria are unable to replicate, and survive at low level (Köhler *et al.*, 2001). Electron microscopy revealed that the membrane structure of the vacuoles containing listeriolysin-secreting brucellae is altered and partially degraded, allowing leaking of the vacuolar content (Köhler *et al.*, 2001). This lack of multiplication can be explained by early neutralisation due to listeriolysin-induced membrane lysis, and collapse of the pH-gradient between the phagosomal compartment and the cytosol is a direct consequence. It is interesting to note that the expression of an essential virulence factor of the Gram positive pathogen *L. monocytogenes* in the Gram negative pathogen *B. suis* completely abolishes the capacity of intramacrophagic replication of the latter, hence demonstrating that a specific intracellular strategy for a given pathogen cannot be altered. *B. suis* is not adapted to replication in a cytoplasmic environment.

5. The Importance of Major Stress Proteins in Resistance to the Harsh Conditions in the *Brucella*-Containing Vacuole

5.1. The Important Role of Hsp70 (DnaK)

Among the identified virulence genes induced under acid pH conditions are major stress proteins and the type IV secretion system VirB described hereafter. DnaK is a major heat shock protein and molecular chaperone of the hsp 70 family, and the corresponding gene in *Brucella* spp. has first been cloned from *B. ovis* (Cellier *et al.*, 1992). Protection of *B. suis* and other intracellular pathogens against antimicrobial macrophage factors may require the induction of chaperones such as DnaK for the synthesis and activation of virulence factors, as exposure to low pH, oxygen radicals, etc. results in rapid degradation of extracellular proteins. This hypothesis has been supported by the finding that complementation of a DnaK-deficient *E. coli* strain with the *dnaK/dnaJ* operon of *B. ovis* reduces initial intracellular killing in a human monocytic cell line (Caron *et al.*, 1994a).

In *Brucella* spp., *dnaK* is induced under heat shock and acid pH stress *in vitro* (Köhler *et al.*, 1996; Rafie-Kolpin *et al.*, 1996; Teixeira-Gomes *et al.*, 2000). Intramacrophagic bacteria also induce the expression of *dnaK*, undetectable in complex medium at 37°C (Köhler *et al.*, 1996; Rafie-Kolpin *et al.*, 1996). A *dnaK* null mutant of the pathogen is characterised by its increased sensitivity to oxidative stress (Köhler *et al.*, 2002a), acid pH, and by the lack of intracellular replication (Köhler *et al.*, 1996). Recent work describes the chromosomal replacement of the native heat shock promoter of *dnaK* of *B.*

suis by the β-lactamase promoter, resulting in constitutive, low-level expression of *dnaK*. Such a mutant is characterised by its high sensitivity to oxidative stress and by rapid elimination in murine macrophagic cells and in the murine model of infection (Köhler *et al.*, 2002a). Non-inducible expression of *dnaK* is therefore not sufficient to allow survival in a macrophage model of infection and in a host organism. These findings correlate well with the hypothesis that DnaK indeed plays an important role in the protection of brucellae from the effects of low pH and other stresses in the early phase of infection.

5.2. Other Stress Proteins Involved in *Brucella* Virulence

The protease Lon has been described as another stress response protein from *B. abortus* that is required for wild type virulence during the early phase of infection in the mouse model, and for survival in isolated murine macrophages (Robertson *et al.*, 2000). It is, however, not essential for the establishment and maintenance of chronic infection. Besides its potential role as a generalised stress response protease in early colonisation, it is possible that Lon has a function in the production or degradation of regulatory proteins involved in the expression of virulence factors (Robertson *et al.*, 2000).

Adaptation to the harsh intramacrophagic environment may also necessitate the regulation of the stationary phase stress resistance. A major bacterial protein involved in this phenomenon is Host Factor I (HF-I), which has been described in well-studied bacteria such as *E. coli* to regulate the stationary phase response via the alternative sigma factor RpoS. In *B. abortus*, the gene *hfq* encoding Host Factor I contributes *in vitro* to resistance to H_2O_2, to acidic conditions, and to stationary phase growth (Robertson and Roop, 1999), conditions that brucellae encounter or may encounter in the vacuole of the macrophage. Indeed, HF-I is also crucial for the intramacrophagic survival of *B. abortus* in murine macrophages and in the murine host (Robertson and Roop, 1999). The exact function of HF-I in *Brucella* is not yet clear, as the RpoS homologue has not been identified. It is likely though, that brucellae respond through this factor to the nutritionally poor intramacrophagic environment.

In addition, brucellae possess a homologue of the high-temperature-requirement A (HtrA) protein, which is an ATP-independent serine protease induced under certain stress conditions such as elevated temperature and exposure to reactive oxygen intermediates (Elzer *et al.*, 1994; Roop *et al.*, 1994). The specific knockout of the gene in *B. abortus* results in a strain that is more sensitive to oxidative killing *in vitro*, and less resistant in the initial phase of infection to killing by macrophages than the virulent strain. However, the multiplication rate, starting at 24 hours post infection, is comparable to the one observed with the wild type (Phillips and Roop, 2001). Therefore, HtrA may be important for the adaptation to the intracellular environment before establishment of the final replicative niche, and it may be responsible for the resistance of the pathogen to reactive oxygen intermediates possibly encountered under certain experimental conditions (Phillips and Roop, 2001).

Among other stress response proteins that have been investigated in brucellae are ClpA and ClpB, members of the Clp ATPases family. ClpA is known to form a complex with the protease ClpP, and presents substrates for degradation by this protease. Although a *clpA* mutant of *B. suis* is not attenuated in the macrophage, a *clpA/B* double mutant shows increased sensitivity to oxidative stress (Ekaza *et al.*, 2000; Ekaza *et al.*, 2001).

ClpB, in contrast, behaves as a typical stress protein, and is involved in resistance to thermal, acid, and ethanol stress *in vitro,* but does not directly contribute to *Brucella* survival in macrophages (Ekaza *et al.,* 2001).

5.3. The Role of Reactive Oxygen Intermediates (ROI) in Macrophage Control of *Brucella* Infections

Many of the above described stress proteins are involved in resistance to oxidative stress. In this context, it is therefore of interest to ask if reactive oxygen intermediates (ROI) play a role during *Brucella* infection. In bovine mammary gland macrophages, opsonized *B. abortus* produce an oxidative event, whereas non-opsonized do not (Harmon and Adams, 1987; Harmon *et al.,* 1988). This is consistent with results from our laboratory describing the absence of an oxidative burst during the interaction of human macrophage-like cells with non-opsonized *B. suis* (Caron *et al.,* 1994b), and with other results confirming the production of ROIs during the infection of murine macrophages with opsonized *B. abortus* (Jiang *et al.,* 1993). In conclusion, ROIs may be produced under certain conditions during *Brucella* infection, and bacterial stress proteins could play an important role in resistance to these specific antimicrobial macrophage factors.

6. The Type IV Secretion System VirB of *B. suis* is Induced in the Early Phase of Macrophage Infection

Type IV secretion systems have been described in several bacterial pathogens of plants and animals and are involved in the transport of pathogenicity-related protein effector molecules from bacteria to eukaryotic cells. *Brucella* spp. possess such a type IV secretion system, first described in *B. suis* (O'Callaghan *et al.,* 1999). Independent mutants obtained in different *virB* genes are highly attenuated in *in vitro* infection models with human macrophages (O'Callaghan *et al.,* 1999; Foulongne *et al.,* 2000; Köhler *et al.,* 2002b), and in the mouse virulence model (Hong *et al.,* 2000; Sieira *et al.,* 2000). DNA sequence analysis of the *virB* regions of three *Brucella* species shows that they are composed of 12 genes *(virB1-virB12),* of which the encoded proteins VirB1-VirB11 are highly homologous to VirB of *A. tumefaciens* and *B. pertussis,* and that they form operons (Boschiroli *et al.,* 2002b).

We will focus here on the intramacrophagic induction of the *virB* operon, and on the identification of the corresponding environmental signals. Using *virB* promoter fusions to *gfp,* it was possible to study *virB* induction using either flow cytometry or fluorescence microscopy (Ouahrani-Bettache *et al.,* 1999; Boschiroli *et al.,* 2002b). The fusion can be induced within 3 h of infection in macrophages, indicating that there exist specific signals in the *Brucella*-containing vacuole. A similar induction is obtained in minimal medium acidified to pH 4.5, but not in complex medium (Boschiroli *et al.,* 2002b). In contrast, inhibition of phagocytosis by cytochalasin D prevents *virB* induction, making clear that these virulence genes are not expressed prior to *B. suis* entry. In the species *B. abortus* and *B. melitensis,* however, *virB* is expressed in rich medium and at neutral pH (Rouot *et al.,* 2003), and induction occurs in the late log phase (Sieira *et al.,* 2000). These discrepancies may reflect differences in the functions of the VirB system during infection

of the macrophage host cell. From these *in vitro* results obtained for *B. suis,* it can be hypothesised that an intramacrophagic environment poor in nutrients and of acidic pH is the intracellular signal resulting in *virB* induction. Additional experimental evidence supports this hypothesis: the neutralisation of the intracellular compartments of infected macrophages abolishes *virB* induction in *B. suis*. Furthermore, the absence of amino acids appears to be a crucial signal for the induction of *virB.* The sensing mechanism may involve the gene product of *rsh,* (p)ppGpp, which is involved in counteraction of starvation to which bacteria are exposed. Most of the attenuated mutants obtained in *B. suis* cannot multiply in the macrophage, but are not or only very slowly eliminated. Only few mutants, among which are essentially *virB* null mutants, and, surprisingly, a *rsh* null mutant obtained by non-polar allelic exchange, are rapidly eliminated (S.Köhler, unpublished). Very recent results from our laboratory also revealed that *virB* from *B. suis* is not expressed *in vitro* under normally inducing conditions (Boschiroli *et al.,* 2002b) in a *rsh* mutant (Köhler and Boigegrain, unpublished).

To day, the exact role of the VirB system in virulence of *Brucella* spp. is still unknown. The effector molecules most likely exported by the apparatus have not yet been identified. In HeLa cells, *B. abortus* and *B. melitensis* VirB have been described to be essential for the modulation of the endosomal pathway and for the ability of the pathogen to reach its final replication compartment (Comerci *et al.,* 2001; Delrue *et al.,* 2001; see also Chapter 14). Although the analysis of the intracellular traffic of brucellae in macrophages has given less conclusive results, a *virB10* mutant of *B. suis* colocalises with cation-independent mannose-6-phosphate receptor-positive vesicles. This marker of late endosomes and lysosomes is never seen to be associated with wild type *B. suis* (Boschiroli *et al.,* 2002a). Detailed analysis of the virulence genes involved in intramacrophagic multiplication reveals that mainly *virB* mutants are actually eliminated (Köhler *et al.,* 2002b), suggesting the participation of these genes in the active process of establishing the replicative niche that we call the “brucellosome”.

Key events reflecting the different processes described above are recapitulated in Figure 2: the early induction of stress proteins to counter harsh intraphagosomal conditions following uptake of brucellae; the early expression of *virB*, and, most likely, of amino acid biosynthesis genes, coupled to low pH are also essential for the pathogen to reach the brucellosome. On the other hand, evolution of these factors within the final niche is often unknown due to the lack of experimental data, and indicated curves are hypothetical.

7. Lysosomes Do Not Fuse With the Brucellosome: Escape Action

It has been shown several decades ago (Oberti *et al.,* 1981) and confirmed later (Naroeni *et al.*, 2001) that *Brucella* spp. remain isolated from the lysosome compartment during their replicative intracellular life. Although the description of this phenomenon is widely accepted, the mechanisms involved are not completely understood. Three possible mechanisms can be put forward to explain this phenomenon: (i) brucellae inject a molecule that blocks the cellular mechanism of fusion; (ii) brucellae enter a cellular compartment that never fuses with lysosomes; (iii) brucellae modify the membrane of their vacuole to avoid fusion (see also Chapter 14). These three strategies have been used by various pathogenic bacteria: *Mycobacterium tuberculosis* modifies the membrane

(Ferrari *et al.,* 1999), *Salmonella enterica* blocks the cellular mechanism (Uchiya *et al.,* 1999) and *Legionella pneumophila* locks itself up in a reticulum-like structure (Kagan and Roy, 2002). We have tested the first hypothesis by analysing the fusion between

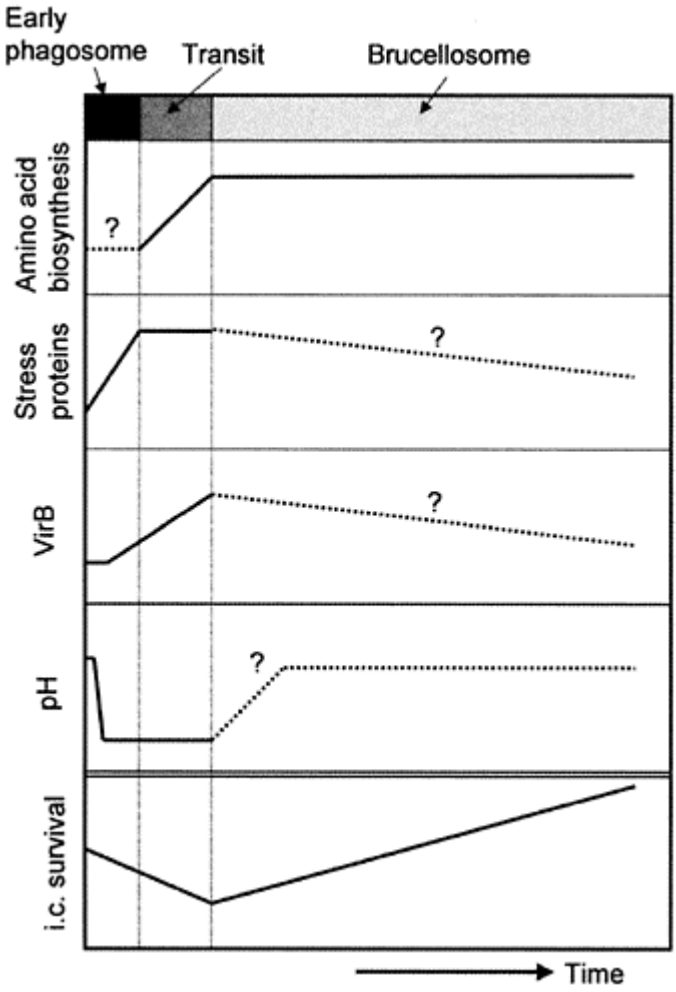

Figure 2. Alignment of the evolution in time of some key factors involved in intramacrophagic multiplication of *B. suis*. The analysed periods of time cover the short period immediately after phagocytosis (1–2 h post infection), the transit period of 3–7 h allowing set-up of the replicative niche

(brucellosome), and the period of bacterial multiplication in the final replicative niche. Dotted lines indicate that direct experimental data are not yet available. Intracellular survival of brucellae over the same periods of time is shown at the bottom of the diagram.

lysosome and latex beads-containing phagosomes during infection. The results unambiguously demonstrated that the fusion machinery of the macrophages is not altered by the intracellularly developing *B. suis* (Naroeni *et al.,* 2001). To confirm this approach, we have set up an *in vitro* fusion system that was strictly dependent on the addition of a cytoplasmic fraction i.e. the cellular molecules involved in organelle trafficking (Naroeni *et al.,* 2001). The compartment containing live *B. suis,* i.e. the brucellosome, cannot fuse with lysosomes, even if the cytoplasmic fraction is prepared from uninfected cells (Naroeni *et al.,* 2001). This allows us to conclude that *B. suis* does not subvert the cellular machinery but modifies the cellular membrane of the phagosome to build its replicative niche. It should be stressed that this is an active process, as killed *B. suis* reside at 24 h post infection in a compartment that fuses with lysosomes (Porte *et al.,* 2003). With killed smooth, wild type *B. suis* this fusion appears only several hours after phagocytosis, suggesting that the early phagosome slowly matures into a phagolysosome when the bacteria cannot induce their virulence machinery. This view is confirmed by the observation that *virB* mutants behave as killed brucellae (Porte *et al.,* 2003), confirming the central role of this type IV excretion system for the building of the brucellosome. However, no experimental data are available today determining whether the VirB machinery excretes protein(s) that are inserted into the membrane of the vacuole to block the interaction with the ly sosomes, or whether it contributes to the invasion of the cellular endoplasmic reticulum (as proposed by Comerci *et al.,* 2001).

8. Adaptation of *Brucella* spp. to the Environment of the Brucellosome

Once the pathogen has avoided the bactericidal mechanisms of the host cell and reached its replicative niche, it has to cope with the conditions it encounters in this final compartment. Adaptation to these conditions will allow brucellae to multiply inside the macrophage.

In the search of the virulence factors of *Brucella* spp., various strategies have been applied: (i) the identification of genes specifically induced intracellularly using the approach of “Differential Fluorescence Induction” (DFI); (ii) signature-tagged mutagenesis (STM) in various models of infection; and (iii) a large-scale Tn5 mutagenesis approach in human macrophage-like cells. In addition, specific genes potentially involved in virulence have been studied individually by cloning and insertional inactivation.

8.1. Genes From *Brucella* spp. Specifically Induced Inside the Macrophage

The DFI strategy for the screening of intramacrophagic gene expression was originally developed for *S. typhimurium* (Valdivia and Falkow, 1997). Based on the broad host range vector pBBR1MCS (Kovach *et al.,* 1994), we have designed promoter fusion vectors for *Brucella* and other Gram negative bacteria, using *gfp* as reporter gene (Köhler *et al.,* 1999; Ouahrani-Bettache *et al.,* 1999). Screening of 5,000 random fusions of *B. suis* DNA in pBBR1-KGFP yielded a certain number of constitutively expressed and not further characterised genes, and 12 clones containing promoter sequences specifically induced in the murine macrophage model J774 (Köhler *et al.,* 1999). The corresponding genes encode mainly amino acid and sugar uptake systems, a nickel transport system, and a glutaredoxin-like protein that could help to maintain a reducing environment, countering oxidative stress. These results give a first indication that brucellae encounter an environment rather poor in nutrients. A similar approach has been performed later by Eskra *et al.* (2001) for the identification of intracellularly induced *B. abortus* genes. From the 13 genes isolated that were associated with 24 h infection of the mouse macrophage cell line RAW 264.7, only three yielded sequence similarities with sequences available in GenBank: They encode a sulfonate transport protein, an iron binding protein, and a protein involved in rhizopine catabolism. Again, the induction of certain transport systems in the late phase of infection indicates that the pathogen lacks these compounds. The major disadvantage of this approach in the search of virulence factors is the fact that one cannot automatically deduce that intracellularly induced genes are essential for the virulence of brucellae. Only labour-intensive, individual inactivation of every identified gene can give an answer. In fact, very few of the genes published that we have isolated by this approach (Köhler *et al.,* 1999) contribute to intramacrophagic multiplication of *B. suis* (S.Köhler, unpublished).

8.2. Genes Essential for Intramacrophagic Multiplication of *Brucella* spp.

8.2.1. Identification by Signature-Tagged Mutagenesis

Various mutagenesis protocols have been developed that allow direct isolation of attenuated mutants in the infection model of choice. Signature-tagged mutagenesis (STM) is such a technique (Hensel *et al.,* 1995). It is based on the comparison of an input and an output pool of individually tagged transposon mutants. Mutants that are eliminated during infection can hence be easily identified and the inactivated gene may be characterised. In *Brucella* spp., STM has been applied to *B. suis*, *B. abortus*, and *B. melitensis* (Foulongne *et al.,* 2000; Hong *et al.,* 2000; Lestrate *et al.,* 2000). Different infection models have been used for these three approaches: the human macrophage cell line THP-1 for *B. suis*, and the murine model for *B. abortus* and *B. melitensis*. For the latter, isolated attenuated mutants have also been analysed in J774 murine macrophages. The important role of VirB was confirmed for *B. suis* in the screen with THP-1 cells. In addition, genes involved in regulation and encoding enzymes participating in biosynthetic or metabolic pathways were identified (Foulongne *et al.,* 2000), among which *aroC*.

AroC belongs to the enzymes essential for the synthesis of aromatic compounds which are obviously available only in small amounts to brucellae inside macrophages, as the inactivation of this gene strongly reduces intracellular multiplication (Foulongne *et al.*, 2001). Some, but not all STM mutants of *B. melitensis* isolated in the mouse model are also attenuated in J774 macrophages, giving additional indication that in the mouse, macrophages play a central role in *Brucella* infection. The affected genes encode transport systems, proteins participating in amino acid and DNA metabolism, peptidoglycan synthesis, transcriptional regulators and proteins of unknown function (Lestrate *et al.*, 2000). Unfortunately, only relatively small numbers of mutants were screened in these assays (between 200 and 1,100 mutants). It is therefore difficult to assess the pathogen's strategy of intramacrophagic multiplication on the sole basis of STM results.

8.2.2. Identification by Large-Scale Tn5 Mutagenesis

An alternative approach is classical Tn5 mutagenesis and screening, where the obtained mutants are studied individually with respect to their capacity of replication inside the host cell. The use of cell culture models allows a high throughput of mutants, well-defined interaction with the host that may be limited to a single cell type, and rapid results. We developed and improved this assay for *B. suis* mutants in THP-1 macrophages (Köhler *et al.*, 2002b). The use of a constitutively (GFP) fluorescent acceptor strain simplified the screening procedure consisting of a first selection using fluorescence microscopy, and allowed the analysis of more than 10,000 mutants in a reasonable lapse of time. Attenuated Tn5 mutants affected in 59 different genes could be isolated, and a putative function was ascribed to 53 of them according to homologies obtained by sequence comparisons with the available databases. Mutation of the genes necessary for multiplication inside the replicative niche was expected to result in survival without replication, whereas the lack of escape should have led to elimination. Only a few attenuated mutants, mainly *virB* mutants, were actually eliminated, meaning that the majority of the inactivated genes are necessary for replication in the final compartment. The identified genes were classified as follows in nine groups (Köhler *et al.*, 2002b). (1) Global adaptation to the intracellular environment: *rsh* is essential for intracellular growth of *B. suis* and encodes (p)ppGpp, which is a transcriptional activator of numerous metabolic pathways under starvation conditions; genes encoding stress-induced proteins such as Host Factor I, the protease HtrA, and the protease Lon were also identified, suggesting that stress response is crucial for the adaptation to the intraphagosomal life style. (2) Amino acid and (3) nucleotide synthesis: most of the pathways encoding enzymes necessary for amino acid biosynthesis were targeted, indicating that these are not available in the compartment containing the pathogen; the synthesis of purines and pyrimidines is also crucial. (4) Sugar metabolism: the pentose phosphate pathway, direct use of pentoses, and pyruvate carboxylase are important for intramacrophagic multiplication of brucellae; the latter catalyses an anaplerotic reaction restocking the tricarboxylic acid cycle, confirming that the metabolites of the TCA cycle are derived for the biosynthesis of amino acids and bases. (5) Oxidoreduction: the importance of the cytochrome *bd* oxidase, which has been described to be important under microaerophilic conditions, is confirmed. (6) Nitrogen metabolism: enzymes involved in NH_4^+ utilisation

are important, indicating that *Brucella* spp. use NH_4^+ for amino acid and nucleotide biosynthesis. (7) Regulation: only one of the two component systems, BvrR-BvrS, was identified, confirming its previously described participation in virulence (Sola-Landa *et al.*, 1998). (8) Disulphide bond formation: the proteins DsbAB may be involved in the assembly of yet-to-be-identified virulence factors. (9) Lipopolysaccharide biosynthesis: smooth LPS containing intact O-antigen is crucial for virulence of *B. suis* in macrophages, and confirms results described above (Naroeni and Porte, 2002; Porte *et al.*, 2003). Intriguingly, "classical" virulence factors such as toxins or other exported proteins are absent, except the known VirB secretion system which was targeted in about 25% of the mutants. The majority of the mutations affected house-keeping genes, confirming the results previously obtained by STM or DFI. Figure 3 summarises the functional groups of factors which are essential for survival and replication of brucellae in the vacuole of the macrophage.

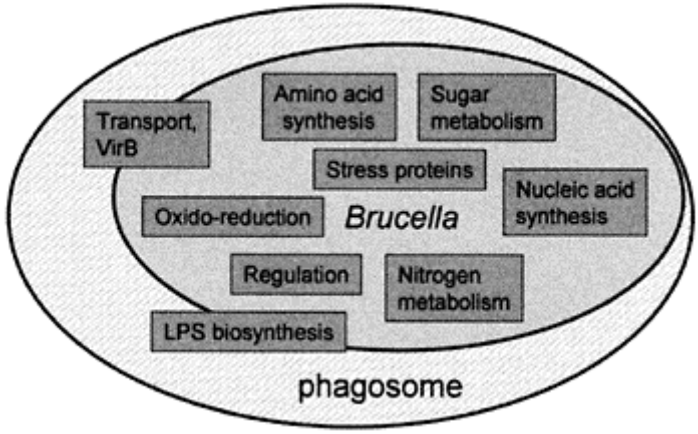

Figure 3. General overview of groups of *Brucella* factors essential for intracellular replication in the macrophage model of infection, classified according to their functions.

8.3. Evidence for Low Oxygen Tension Within the Brucellosome

We have previously studied the environmental conditions that activated the *nikA* gene, one of the genes of *B. suis* specifically induced in murine macrophages, as revealed by DFI (see above). The *nikABCD* genes are very similar to those of *E. coli* in their sequences and their genetic organisation, both operons encode specific transport systems for nickel. Our results show that activity of the *nikA* promoter-*gfp* fusion is also enhanced under *in vitro* microaerobic conditions (Jubier-Maurin *et al.*, 2001b). This finding parallels the anaerobic activation of the *E. coli nik* operon under the control of the general anaerobic transcriptional factor Fnr. This protein is functional when oxygen is absent and

it looses its activity upon direct interaction with oxygen. Furthermore, several sequences homologous to the binding site for Fnr (Fnr box) were identified upstream of the *B. suis* putative *nikA* promoter but they are not located at the proper spacing (−41 from the transcription initiation site) described for promoters positively controlled by Fnr. To examine the possible regulation of the *B. suis nikA* gene expression by a transcriptional factor binding upstream of the promoter, we eliminated this region in the *nikA-gfp* fusion. *In vitro*, fluorescence analysis of this *nikA* deletion-gfp fusion shows very high expression of the promoter under the experimental conditions not activating the original construct (V.Jubier-Maurin, unpublished). This observation indicates that binding of a protein to this region may repress the native *nikA* promoter. Therefore, a putative regulator protein may function under aerobiosis as a repressor of the *nikA* gene. If it is a "Fnr-like" factor, this would imply that the situation encountered in *B. suis* is opposite to that reported for the *E. coli nik* operon, positively controlled by Fnr under anaerobiosis.

Another study identified a *cydB* mutant of *B. abortus* lacking the cytochrome bd oxidase of high affinity for oxygen, highly attenuated in the mouse model of infection (Endley *et al.*, 2001). This finding was corroborated by our description of the virulome of *B. suis* that comprises the *cydD* gene, part of the operon encoding the cytochrome bd oxidase (Köhler *et al.*, 2002b). The *B. abortus cydB* mutant exhibits high sensitivity in response to oxidative stress and respiratory inhibitors. This phenotype led the authors to interpret the decreased viability of the mutant at stationary phase as an inability to prevent the build-up of oxidative radicals due to the increased oxygen level in the absence of the cytochrome bd oxidase. However, the production of reactive oxygen intermediates has been reported only for the use of opsonized *Brucella* during macrophage infection (see above). Taken together, these results present additional evidence that the intramacrophagic environment of *Brucella* is characterised by a low oxygen concentration. We suggest that oxygen-dependent gene regulation could take place in the replicative niche of *Brucella*.

Analysis of *B. suis* and *B. melitensis* genome sequences detected a gene predicted to encode a putative transcriptional regulator that possesses particularities of oxygen sensors. This gene was annotated as *fixK* in *B. melitensis* because of the similarity of its product to homologues in some *Rhizobiaceae* species, due to a fair conservation of the putative DNA binding domain. Despite a lower identity to Fnr, the *B. suis*-predicted protein possesses in the NH_2-terminal region and in the central part the cysteines characteristic for this oxygen-responsive protein class. In *E. coli*, these amino acids are ligands to the iron-sulphur cluster whose oxidative state drives the transcriptional activity of Fnr. They are absent from FixK of *Sinorhizobium meliloti*. In this species, oxygen sensing is carried out by the FixLJ two component system which controls the *fixK* activity. In turn, the FixK transcription regulator activates numerous genes or operons, including the *fixNOQP* operon which is responsible for the synthesis of a high affinity cbb3-type terminal oxidase for respiration under microaerobiosis. Sequence analysis gave no clear indication of the mode of regulation potentially used by *Brucella*. There is no evidence for the presence of a FixL homologue in the *B. suis* genome. A gene was detected whose product has only low homology within the histidine kinase domain of FixL. However, the adjacent gene may encode a protein showing similarity to the overall amino acid sequence of the FixJ protein (Figure 4). This indicates that both genes may

belong to an operon, as do *fixLJ* of *S. meliloti*. In a recent phylogenetic study (Körner *et al.* 2003), the *B. suis* Fnr-like factor was found to cluster with the members of the FnrN class, which comprises for example FnrP from *Paracoccus denitrificans*, FixK1 from *Bradyrhizobium japonicum* and FnrN from *Rhizobium etli* and *R. leguminosarum* bv. *viciae*. They have in common the conserved cysteine motif of Fnr. We propose to call the *B. suis* protein of the FixK/Fnr family "FnrN".

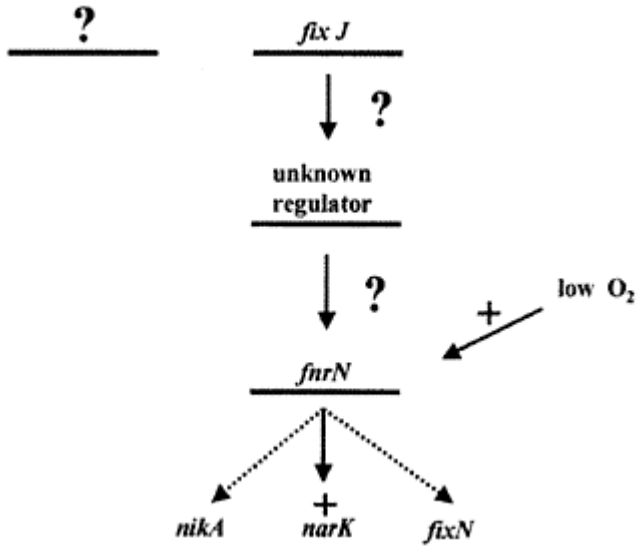

Figure 4. Role of *fnrN* in oxygen-dependent gene regulation in *B. suis*. In accordance with the close phylogenetic relationship of *fnrN* to *B. japonicum* and *R. etli* regulators (see the text for further explanations), the existence of an unknown regulator can be postulated. Dotted lines indicate that results are preliminary. Positive regulation is indicated.

The *fnrN* gene appears to be a good candidate to study how regulation by low oxygen tension can influence expression of genes implicated in the adaptation of *B. suis* to the environmental conditions of the phagosome. Expression of this gene in *B. suis* was analysed by the use of a transcriptional fusion with a promoterless *gfp* gene contained in a plasmid construct. Measurement of fluorescence intensity revealed a high level of expression of the *fnrN* promoter in *B. suis* cultures under aerobic conditions, and an increased activity under microaerobic conditions (S.Loisel and V.Jubier-Maurin,

unpublished). However, at 48 h post-infection, in bacteria prepared from infected cells, the level of expression is significantly reduced. Therefore, in the phagosomal compartment, the activity of the *fnrN* promoter is not under the simple control of a regulatory mechanism dependent on the microaerobic state (Figure 4). This situation is in contrast to that encountered in most of the *Rhizobiaceae* species studied to date. Moreover, expression rates of the *fnrN* promoter are identical in the wild type and in the *fnrN* mutant strain. Thus, unlike all the *fixK* or fnrN regulators involved in *Rhizobium* (for review, see Körner *et al.* 2003), *fnrN* expression is not regulated by its own product.

According to sequence analysis, *B. suis* possesses a *nar* operon predicted to encode a respiratory nitrate reductase homologous to that of *E. coli*. However, genomic organisation differs, and *narK*, whose product is involved in nitrate transport, is the first gene of the *B. suis* operon instead of being an independent gene upstream of the *narGHJI* genes. This particular arrangement was previously described for the denitrifying soil bacterium, *Paracoccus pantotrophus* (Wood *et al.*, 2001). Another feature common to *narK from B. suis* and *P. pantotrophus* is the presence of two *narK*-like sequences fused into a single ORF. *Brucella* possesses all the genes needed for a complete denitrification pathway transforming nitrates into nitrogen. By contrast, no homology with genes coding for an assimilatory nitrite reductase producing ammonia has been detected in its genome. Accordingly, we found that *B. suis* is unable to use nitrate as the sole nitrogen source for its metabolic activity. Therefore, *B. suis* has no aerobic assimilatory nitrogen pathway. However, alternative electron acceptors such as nitrate may also be used by brucellae under anaerobic conditions for energy production. Expression of *narK*, a potential FnrN target promoter as underlined by the finding of a perfect “Fnr-box” in the putative promoter region, was examined in the wild type and in the *fnrN B. suis* strains. Unexpected findings revealed that *narK* is expressed by *B. suis in vitro* under normal oxygen tension. The expression level is reduced in the *fnrN* mutant thus indicating that the *fnrN* product is implied in the *nar* promoter induction (Figure 4). In the macrophage, the *narK* promoter is repressed but not inactivated. This negative effect was abolished in the strain devoid of FnrN showing identical *narK* promoter activity in free bacteria and in bacteria obtained from infected cells. This could indicate that the intracellular repression of the *nar* promoter was a consequence of the strong reduction of *fnrN* activity under the same conditions (S.Loisel and V.Jubier-Maurin, unpublished). These results reveal that the *B. suis nar* promoter is active under aerobic conditions, as opposed to results obtained for *P. pantrophus*, whose homologous promoter is inactive under aerobiosis and active under anaerobiosis (Wood *et al.*, 2001), as it is also the case for other denitrifying bacteria. In the same manner, the synthesis of the *E. coli* nitrate reductase from the *nar* operon is strictly dependent on anaerobiosis.

8.4. The Virulence Genes of *Brucella* spp. Indirectly Characterise the Niche in the Macrophage

We have ascribed the term intramacrophagic “virulome” to the group of genes that are involved in virulence (i. e. intracellular survival) of brucellae. The analysis of the virulome of *B. suis* reveals not only which bacterial genes are participating in the construction of and the adaptation to the final replicative niche of this pathogen, but it reveals also an extended picture of the niche itself based on

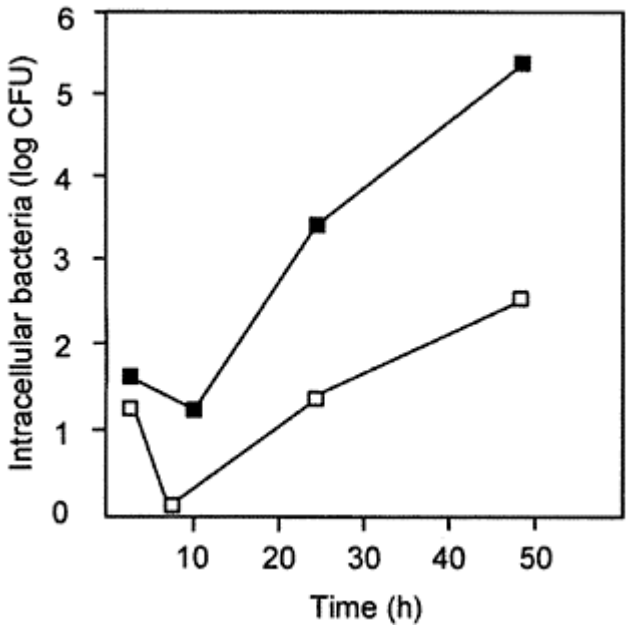

Figure 5. The *eryB* mutant (*eryB*::Tn5) of *B. suis* is attenuated in the human macrophage-like THP-1 cells. Closed squares: *B. suis* 1330 (wild type); open squares: *B. suis eryB.*

indirect evidence (Köhler *et al.,* 2002b). The nature of the *Brucella*-containing vacuole was poorly defined before, and these recent data together with previously published results allow to describe the strategy the bacterium uses to subvert the functions of the phagocyte. In its final niche, *Brucella* is exposed to an environment poor in nutrients, explaining the necessity of genes encoding amino acid and nucleotide biosynthesis. The essential character of cytochrome bd oxidase for intramacrophagic replication (Endley *et al.,* 2001; Köhler *et al.,* 2002b) indicates that this compartment possesses a lowered oxygen tension. This pathogen-specific niche for multiplication within the cells is different from all naturally existing cellular organelles, and we therefore proposed to call this specific compartment the "brucellosome" (Köhler *et al.,* 2003).

Before the publication of the importance of *purD, purF,* and other nucleotide biosynthesis genes for intramacrophagic multiplication of *B. suis* (Köhler *et al.,* 2002b), work focused on *purE* of *B. melitensis* led to similar conclusions (Drazek *et al.,* 1995). In human monocyte-derived macrophages, the *purE* deletion mutant fails to replicate, whereas the complemented strain grows as the wild type, yielding additional evidence that the compartment containing *Brucella* spp. is poor in purines and pyrimidines.

Among the genes identified for the first time in a mutagenesis screen are *eryB* and *eryC,* encoding enzymes involved in erythritol degradation (Köhler *et al.,* 2002b). The

high concentrations of erythritol in the trophoblasts of the placenta, and the capacity of brucellae to use this molecule as a carbon source, have been given as an explanation for the abortions observed in gravid animals suffering from brucellosis (see also Chapter 11). Here, the *ery* genes are shown to be important in a macrophage model of infection, their inactivation reduces survival of *B. suis* by more than 2 logs over 48 h of infection (Figure 5). As growth of *ery* mutants is inhibited in the presence of erythritol *in vitro* (Sangari and Aguero, 1994), two possible explanations of the phenomenon exist: erythritol is present in the macrophage and leads to partial growth inhibition of the corresponding mutants, or the encoded enzymes catabolize additional, yet unknown substrates essential for brucellae. Our preliminary results are in favour of the first hypothesis, as a spontaneously erythritol-resistant *eryC* mutant recovers its entire capacity of intramacrophagic replication (S.Köhler, unpublished). This yields indirect evidence (1) that macrophages may contain erythritol, and (2) that this erythritol reaches the brucellosome, hence limiting the replication of the mutant. In the literature, no information is available with respect to the possible presence of erythritol in macrophages.

9. *Brucella* spp. Inhibit Apoptosis of the Host Cell

It can be deduced from the high number of genes necessary to multiply inside mammalian cells that brucellae are characterised by a long co-evolution with their host. Although it can be considered that *Brucella* spp. are related to other intracellular bacteria such as *Sinorhizobium*, it is difficult to know if the intracellular form of life is primitive or of derived character. A very important fact in favour of a genuine intracellular life and a true adaptation to intramacrophagic multiplication is the inhibition of apoptosis of infected cells. Indeed, it was clearly demonstrated that *Brucella* does not induce apoptosis. Looking at an infected macrophage is very instructive (O'Callaghan and MacMillan, 2001). The macrophage can be totally filled with bacteria (1,000 bacteria or more), without that any alteration of the structure of the cell nucleus can be detected, suggesting that the cell metabolism is still functioning. Study of the macrophage during infection has shown that it is protected from apoptosis: *Brucella* infection inhibits spontaneously occurring apoptosis in human monocytes, and it renders macrophage-like cells resistant to IFN-γ-induced apoptosis (Gross *et al.*, 2000). These results show that brucellae modify the cellular signalling of the macrophage. Recent results on the increase of cAMP in the infected macrophages (Gross *et al.*, 2003) may explain these observations: increased levels of cAMP induce a deactivation of the macrophage. This consequence is of advantage for the bacteria, because it diminishes the unfavourable conditions that the macrophage can generate for the pathogen.

Altogether, it is obvious that *Brucella* spp. need to prolong the life of the host cell in order to multiply as much as possible, confirming the hypothesis that they are genuine intracellular bacteria. The molecular mechanism involved in this phenomenon is still unknown. Two hypotheses can be put forward. (1) During penetration of the macrophage, *Brucella* spp. use a receptor that induces a "life" signal in the macrophage. This hypothesis is in line with the very rapid rise in cAMP, however it is more difficult to understand why the inhibition lasts for the whole period of infection. (2) On the other

hand, it could be guessed that brucellae induce a signal once inside the cell. This signal can be a molecule injected into the cytoplasm, or a consequence of bacterial multiplication. This latter hypothesis would rule out the possibility that *Brucella* spp. reside inside the endoplasmic reticulum, as filling of the reticulum would result in a stress that induces apoptosis (Ferri and Kroemer, 2001). The hypothesis of the injection of a molecule into the cytoplasm (a protein for example) has not been demonstrated yet.

10. Intracellular Traffic of *Brucella* spp. in Macrophages. Where do the Membranes Come From?

As described at the beginning of this chapter, macrophages are the most important host cells of brucellae in the infected organism. The pathogen develops inside the cells until the whole cytoplasm is filled with bacteria-containing vacuoles, leading finally to destruction of the host cell and release of the bacteria. Electron microscopy observations reveal in an impressive way the accumulation of brucellae in the macrophage. Besides the major questions concerning strategies of entry, avoidance of bactericidal mechanisms, and adaptation to the replicative niche, the question about intracellular trafficking and the origin of the membranes surrounding the bacteria arises (see Chapter 14). It is still widely accepted that the plasma membrane is the main source of membrane used for phagosome formation, although more recently the idea has come up that endomembranes are recruited to the cell surface and involved in this process (Holevinsky and Nelson, 1998). In addition, proteomic approaches analysing phagosome composition led to the identification of about 600 proteins associated with latex-beads-containing compartments and indicate that the plasma membrane is probably not the major source of membrane for phagosome formation (Garin *et al.*, 2001). In fact, many markers of the endoplasmic reticulum (ER) are present, resulting in the hypothesis that ER may be involved in the biogenesis of phagosomes (Garin *et al.*, 2001). The same authors were then able to show that ER is recruited to the cell surface, where it fuses with the plasma membrane underneath phagocytic cups, hence supplying membrane for the formation of phagosomes (Gagnon *et al.*, 2002). This ER-mediated phagocytosis appears to be a general mechanism of entry into macrophages.

For *Brucella* spp., it has been proposed that in trophoblasts and in non-professional phagocytes such as HeLa cells, the ER is the replicative niche to which the pathogen induces its transfer (Anderson and Cheville, 1986; Pizarro-Cerda *et al.*, 1998). But in the light of what has been described above, is this replicative niche a natural organelle, or is it a compartment that exhibits markers which do not really define its function (i.e. ER-derived)? Indeed, the shortage of amino acids and nucleic acid bases deduced from the analysis of the virulome in the macrophage (Köhler *et al.*, 2002b) suggests an isolated compartment that does not contain peptides and proteins, strengthening the hypothesis that the brucellosome is rather ER-derived. A plausible working model proposes that the default pathway of entry into macrophages for *Brucella* spp. is ER-mediated phagocytosis, and that the pathogen inhibits the maturation of the ER-derived phagosome at a certain stage to avoid fusion with lysosomes (Desjardins, 2003). However, in contrast to what has been described for HeLa cells, in murine J774 macrophages only a very low percentage of *B. abortus* is found in compartments containing markers for

autophagosomes and for endoplasmic reticulum (Arenas *et al.*, 2000). Very recent work by Celli *et al.* sheds light on the intracellular traffic of *Brucella*-containing vacuoles in bone marrow-derived macrophages (Celli *et al.*, 2003). The authors show that the surviving bacteria replicate in vacuole segregated from the endocytic pathway. The maturation of this vacuole involves interaction and fusion with the ER, creating an ER-derived replicative organelle. A *virB* mutant used in this system is unable to sustain interaction and to fuse with the ER, and is eventually killed via fusion with lysosomes. Vir B hence is essential for the maturation events necessary for the creation of the final replicative niche in macrophages. Based on these results, the intense recruitment of ER-membranes to the vacuoles of dividing brucellae is certainly crucial for the replication phase of this pathogen in the macrophage.

11. Conclusions and Future Prospects

In this chapter, we have tried to give a general survey of the knowledge accumulated on the interaction between *Brucella* and its major host cell, the macrophage, from the mechanisms of entry to its final replicative niche. Most of the facts presented here were unknown as little as six years ago, showing how rapidly science has progressed in the field. The study of *Brucella* in its eukaryotic host cell is of such great interest, because it is a “stealthy” bacterium that subverts the macrophage in a subtle way, without causing direct damage to the host. The route of entry is apparently the key strategy that enables the pathogen to considerably delay in time the event of phago-lysosome fusion, thereby allowing expression and action of VirB, resulting in building of the replicative niche. In addition, analysis of the interaction, as seen by the pathogen, allows several interesting indirect conclusions regarding the characterization of the macrophage environment, without making use of cell biology approaches.

Several major questions for future research are of interest: (1) are the lipid rafts recruited to the site of entry of *Brucella*, and if yes, how?; (2) what are the sensors/regulators in *Brucella* involved in sensing the environment in the early phagosome as well as in the brucellosome?; (3) based on the assumption that the role of VirB as a virulence factor is linked to the secretion of an effector molecule: what is the nature and the biological role of the latter?; (4) what are the C- and N-sources available to *Brucella* in its vacuole?; (5) how does the recruitment of pre-existing cellular membranes, in particular from the ER, to the *Brucella*-containing vacuole take place?; (6) last not least, a field of research that is only at the beginning of its development concerns the aspects of host specificity: what are the molecular explanations for the observation that certain species of brucellae are pathogenic in one host, but not in another? Physiopathological studies of *Brucella* infections will certainly also contribute to a better understanding of these phenomena.

We are of course aware of the fact that the macrophage is only one element of the natural line of defence encountered by brucellae during infection of an organism. However, it allows the pathogen to shelter from numerous other systems set up by the host that may nevertheless become fatal to *Brucella*, explaining spontaneous recovery from brucellosis in some cases. Continuation of the study of the activation of macrophages, and their interaction with other cell types of the immune system such as T-

helper cells during infection is therefore of great interest and essential for a more general understanding of host-pathogen interaction.

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Chapter 16
Host Cellular Immune Responses Against *Brucella* spp. Evaluated Using the Mouse Model

Cynthia L.Baldwin* and Radhika Goenka

From: *Brucella:* Molecular and Cellular Biology. Edited by: Ignacio López-Goñi and Ignacio Moriyón

Abstract

Brucella spp. are able to survive phagocytosis by several adaptations to intracellular life including the ability to prevent phagolysosomal fusion in professional phagocytes such as macrophages and dendritic cells and by activation of a set of genes in response to the acidic environment. In this way they are functionally related to other intracellular prokaryotes such as the acid-fast bacteria *Mycobacterium tuberculosis* and intracellular eukaryotes such as the protozoa *Leishmania major* and *Toxoplasma gondii* that also live within membrane-bound vacuoles in phagocytic cells albeit using different mechanisms to survive in such an environment. Because these organisms are replicating inside of host cells it is logical that an effective immune response must include a cell-mediated immune response. Immunity is based most importantly on production of interferon-γ (IFN-γ) since in its absence mice die of brucellosis. Production of IFN-γ is controlled by IL-12 *in vivo* and its effective functioning for activation of macrophages depends upon TNF-α. It is likely that IFN-γ is made by both CD4 and CD8 T cells in response to infections with the attenuated *B. abortus* strain 19 but only by CD4 T cells in response to the virulent strain 2308. Both reactive oxygen intermediates and nitric oxide contribute to control within macrophages and IFN-γ serves to increase anti-*Brucella* activities. The involvement of CD8 T cells in control of infections with attenuated but not virulent strains of *B. abortus* and the implications with regard to cross-presentation of antigens is discussed.

1. Introduction to Cellular Immunity

Brucella spp. are able to survive phagocytosis by several adaptations to intracellular life including the ability to prevent phagolysosomal fusion in professional phagocytes such as macrophages and dendritic cells and by activation of a set of genes in response to the acidic environment (Cheers and Pagram, 1979; Sowa *et al.*, 1992; see also Chapters 14 and 15). In this way they are functionally related to other intracellular prokaryotes such as the acid-fast bacteria *Mycobacterium tuberculosis* and intracellular eukaryotes such as the protozoa *Leishmania major* and *Toxoplasma gondii* (Sher and Sachs, 2002) that also live within membrane-bound vacuoles in phagocytic cells, albeit using different mechanisms to survive

* For correspondence email cbaldwin@vasci.umass.edu

in such an environment. Because these organisms are replicating inside of host cells it is logical that an effective immune response must include a cell-mediated immune response.

In general, the cell-mediated adaptive immune response comprises (i) type 1 or Th1 cytokines including interferon-γ (IFN-γ) which is produced by T cell receptor (TCR) αβ-bearing CD4 and CD8 T cells, (ii) production of IgG_2 antibodies by B cells, and (iii) cytotoxic TCR αβ CD8 T cells. The production of the important effector cytokine IFN-γ results in activation of cells of the innate immune system, i.e. professional phagocytes such as macrophages, for more efficient killing and inhibition of replication of intracellular microbial pathogens. IgG_2 antibodies are particularly effective opsonins meaning they promote phagocytosis of microbes by the ability of the antibody Fc region to bind the Fc receptor of phagocytes. Cytotoxic T cells kill the infected host cells either by perforin-mediated cytolytic activity or Fas-fas ligand interaction but they may also be responsible for killing microbes within the target cells by granulysin (Stenger *et al.*, 1998a). While collectively these responses traditionally have been referred to as cell-mediated or cellular immunity it may be more appropriate to refer to them as type 1 immunity as an abstraction from the original Th1 CD4 T cells that produce IFN-γ. The cellular or type 1 immune pathway is a coordinated complex of responses however it should be noted that an individual response may not involve all components of type 1 immunity. That is, all components may not be activated in response to infection or vaccination nor may they be necessary for effective immunity to a particular infection.

Cells of the innate and bridging immune systems also contribute to cellular or type 1 immunity. They do this either (i) as the end-stage effector cell (the IFN-γ activated macrophage), (ii) as producers of type 1 cytokines that may direct the adaptive immune response (i.e., responses by αβ T cells and B cells that have immunological memory) down a type 1 pathway, and/or (iii) initially holding the infection at bay until the adaptive immune response has been established. Examples include IFN-γ production by the primitive T cells known as natural killer (NK) cells as well as by bridging immune system cells including γδ T cells and NKT cells. We refer to these as bridging cells since they have characteristics of both the adaptive immune system cells (clonally diverse T cell receptors) and the innate immune system (do not conclusively have immunological memory). In addition to producing IFN-γ, γδ T cells may also be cytolytic (Ottones *et al.*, 2002) as can NK cells. Macrophages and dendritic cells also direct the immune response

since they can produce the cytokine IL-12 (Mosser, 2003) which is known to stimulate IFN-γ production by NK cells as well as by classical αβ T cells and γδ T cells. Macrophages have been recently categorised as M1 or M2 cells according to their ability to direct the immune response to the Th1 (type 1/cellular) or Th2 (type 2/humoral) pathways, respectively.

This chapter will concentrate on reviewing the activation of components of the cellular immune system by *Brucella* principally drawn from reports employing the mouse model but with reference to those involving humans or livestock species where appropriate. Since there are several species and strains of *Brucella* examined as well as hosts, the relative importance of the roles of antibodies, IFN-γ, cytotoxic T cells, etc. in protection may vary accordingly and thus care will be taken to be precise with reference to those. By way of examples, for virus infections (viruses being obligate intracellular pathogens) antibodies are often the first level of control while cytotoxic T cells act to clear the infection. However the contribution of antibodies and T cells varies with the virulence of the strain of the virus. A similar situation exists with regard to protective immunity to *Brucella*. Vaccine strains (*B. abortus* strain 19, *B. suis* strain 2 and *B. melitensis* Rev 1) derived for use in livestock and which represent the three major human pathogenic species of *Brucella* are attenuated in the mouse model as they are in the livestock species (Bosseray and Plommet, 1990). Thus modulation of the host responses to them may be different than it is to a virulent field strain. The nature of a protective response may differ as well. For example, while antibodies are effective in controlling challenge in mice with the attenuated *B. abortus* strain 19 they are less so against the virulent field strain *B. abortus* 2308 (Araya and Winter, 1990) regardless of whether the sera (and thus antibodies) was derived from strain 19 or strain 2308-infected animals. In contrast, adoptive transfer of T cells controlled both strains with a tendency for better control of strain 2308 (Araya and Winter, 1990). This correlates with the superior ability of strain 2308 to survive following phagocytosis by macrophages (Jones and Winter, 1992). In a more recent study discussed extensively below (Ko *et al.*, 2002), a mouse strain that had an interferon response gene deletion (IRF-1 −/− mice) survived infection with the rough mutant *B. abortus* RB51 but died when infected with the RB51 parent strain 2308 by 2 weeks after infection.

2. IFN-γ is Crucial for Survival of Brucellosis

Infection of normal mice with any of the virulent strains of *Brucella* that are particularly pathogenic in humans (*B. abortus*, *B. suis*, or *B. melitensis*) results in a chronic infection but one that is non-lethal (Bosseray *et al.*, 1982; Bosseray and Plommet, 1990). That is, mice can remain infected for more than six months with the number of bacteria generally slowly decreasing with time post-infection (for an example see Montaraz and Winter, 1986). However, disruption or deletion of the IFN-γ gene results in death of the inbred strains of mice BALB/C and C57BL/6 by 10.5 and 6 weeks, respectively, after infection with *B. abortus* strain 2308 (Murphy *et al.*, 2001a). Thus IFN-γ is absolutely crucial for survival of a *B. abortus* infection with the virulent field strain 2308. The BALB/c and C57BL/10 strains of mice used in those studies represent the prototypal susceptible and resistant phenotypes to infection with *B. abortus* 2308 (Montaraz and Winter, 1986) as

they are for several other intracellular microbial pathogens including *Leishmania major*. Thus we conclude from these results that a crucial component of immunity that results in maintenance of the chronic infective state and survival of the host is IFN-γ and will review this component of cellular immunity first.

The role of IFN-γ was first shown by *in vivo* studies with monoclonal antibodies that deplete or neutralise the activity of IFN-γ (Zhan and Cheers, 1993; Fernandes and Baldwin, 1995; Fernandes *et al.*, 1996). Those studies used C57BL/10, BALB/ c and CBA mice infected with either the virulent field strain *B. abortus* 2308 or the attenuated vaccine strain 19. The reciprocal studies were done as well as by administering recombinant IFN-γ to infected BALB/ c mice (Stevens *et al.*, 1992). While depletion of IFN-γ *in vivo* generally resulted in approximately a 10-fold

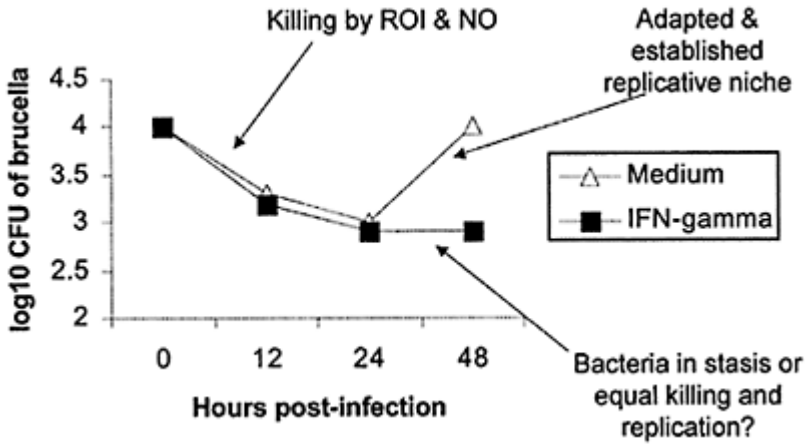

Figure 1. Schematic representation of the effect of IFN-γ on macrophage control of *Brucella*. Killing of intracellular *Brucella* occurs during the first 0 to 12 or 24 h after infection following which growth occurs in non-IFN-γ-activated macrophages but not in IFN-γ-activated macrophages.

increase in colony forming units (CFU) of *B. abortus*, supplementing mice with recombinant IFN-γ resulted in nearly a 10-fold reduction (Stevens *et al.*, 1992) by one week after infection. The spleen is the principal site of infection in mice and analysis of splenocytes following infection of CD1 mice confirmed the production of IFN-γ protein *in vivo* following *B. abortus* stain 2308 infection by extraction of the cytokine from the cells *ex vivo* (Fernandez-Lago *et al.*, 1996).

In vitro studies from our laboratory and others, done at approximately the same time, clearly showed that a direct effect of IFN-γ on macrophages was to increase intracellular

control of brucellae following phagocytosis by *in vitro* analyses (Jones and Winter, 1992; Baldwin *et al.*, 1993; Jiang and Baldwin, 1993a; 1993b; Jiang *et al.*, 1993). Moreover, in the mice that received recombinant IFN-γ the splenic and peritoneal macrophages were activated *in vivo* for increased brucellacidal activity by 48 h as shown by isolating those cells and evaluating them in *in vitro* bactericidal assays (Stevens *et al.*, 1992). While the activation by IFN-γ generally does not increase killing during the first hours after infection, since even unactivated macrophages kill the majority of phagocytosed brucellae (90–95%), it does inhibit subsequent replication intracellularly (Figure 1). Addition of IFN-γ even 2 h after infection has an effect however delaying it until 24 h post-infection does not even though the effects are most readily measured after 24 h (Jiang and Baldwin, 1993b). This suggests that the IFN-γ activation may prevent *Brucella* from adapting its intracellular environment for subsequent intracellular replication. However, it should be noted that even optimal IFN-γ treatment of the macrophages in terms of concentration and timing does not result in clearance of the bacteria from macrophages thus even in the worst case scenario the virulent *Brucella* are able to survive intracellularly. While they appear to not replicate, it is possible that the plateau in intracellular bacteria actually represents equal killing and replication (see Figure 1).

While all human pathogenic species of *Brucella* seem to be sensitive to IFN-γ control there is some difference in the susceptibility of strains to IFN-γ activated macrophage killing. IFN-γ activation has been shown to inhibit growth of *B. melitensis* strain 16M in mouse macrophages and the effect was especially pronounced when the bacteria were opsonized with antibody to the lipopolysaccharide (Eze *et al.*, 2000). While *B. suis* has not been evaluated in the murine system, it has been shown to be susceptible to control by IFN-γ activation of human monocytes (Ottones *et al.*, 2000). Jones and Winter (1992) showed that the attenuated *B. abortus* strain 19 is more readily killed than the more virulent strain 2308. By comparison, the highly attenuated rough strain *B. abortus* RB51 does not survive at all in macrophages being cleared by 24 h after infection even in the absence of IFN-γ activation (Baldwin, Jiang and Fernandes, unpublished). Because of the correlation between attenuation and intracellular survival of strains such as RB51 and strain 19, susceptibility to macrophage killing is often used as a criterion for measuring attenuation as a result of specific gene mutations in *Brucella* organisms.

Further studies by Splitter and co-workers have extended the *in vivo* observations regarding the critical role of IFN-γ in control of *B. abortus* infections. They showed that C57BL/6 mice die even more rapidly following infection with *B. abortus* 2308 when they have a deletion of the IFN regulatory factor-1 (IRF-1) gene (Ko *et al.*, 2002). IFN regulatory factors are a set of transcription factors that are induced in cells in response to IFN-γ as well as to type 1 interferons. Those IFN regulatory factors induced in response to IFN-γ are IRF-1, IRF-2 and IFN-consensus sequence binding protein (ICSBP). Using gene knock-out mice deficient in one of each of these factors it was shown that the IRF-1 deficient mice died within 2 weeks after infection in contrast to the IRF-2 mice that actually cleared the infection more efficiently. The ICSBP deficient mice did not die but had nearly 100-fold more bacteria per spleen at 4 weeks post-infection. The results from the study by Splitter and colleagues (Ko *et al.*, 2002) was able to shed additional light on the cause of lethal infection when IFN-γ was absent by comparing the cellular immune defects and immune regulatory components associated with IRF-1 and IRF-2 gene deficiencies.

The choice of C57BL/6 mice was particularly useful in these studies since Murphy *et al.* (2001a) showed that C57BL/6 mice are more susceptible to brucellosis in the absence of IFN-γ, dying in half the time of the infected IFN-γ-deficient BALB/c mice. In those studies the IFN-γ K/O C57BL/6 mice were shown to have a continual increase in splenic CFU until death while the IFN-γ K/O BALB/c mice held the number of brucellae in a *plateau* (Murphy *et al.,* 2001a) suggesting the B ALB/c mice use other alternative/back-up mechanisms in addition to IFN-γ for controlling brucellae replication (discussed further below). This may seem ironic since normally the BALB/c mice are more susceptible to infection (Montaraz and Winter, 1986).

Since both IRF-1 and 2 deficient mice have a deficiency of NK cells it could be concluded by Ko *et al.* (2002) that these cells may not play an important role in early control in mice. This is supported by an earlier study discussed below that specifically depleted **NK** cells in both C57BL/10 and BALB/c mice (Fernandes *et al.,* 1995). However, IRF-1 −/− mice have a deficiency in CD8 T cells and iNOS compared to normal functioning of those immune system components in IRF-2 −/− mice. They also have a deficiency in IL-12p40 induction whereas IRF-2 −/− mice have only a dysregulation of IL-12p40 (see Ko *et al.,* 2002 for review). Further analyses in that study used knock-out mice for these individual genes. The results indicated that nitric oxide played a role initially in controlling *B. abortus* 2308 infections in C57BL/6 mice but that it was not needed ultimately for control of the infection (Ko *et al.,* 2002). The role of nitric oxide will be discussed further below. A similar result was obtained with mice that were defective in production of reactive oxygen intermediates ($gp91^{phox}$ −/− mice), additional components of cellular immunity employed by macrophages to kill phagocytosed *B. abortus* (Jiang *et al.,* 1993) that also will be discussed below. In contrast the IL-12p40 −/− mice could not clear the infection with strain 2308 but nevertheless survived at least for the duration of the study (4 weeks). Bioactive IL-12p70 (which is a heterodimer of p35 and p40) drives IFN-γ production by T cells and NK cells and the IFN-γ in turn drives IL-12 production by macrophages suggesting this may be a necessary loop to ratchet up the amount of IFN-γ made during a brucellae infection. Since all of the mice with individual genes knocked out (iNOS, IL-12p40 and $gp91^{phox}$) survived infection it was concluded that the loss of these multiple functions early in the infection contributed together to the very early death of the IRF-1 −/− mice.

While the minimal conclusion is that nitric oxide, reactive oxygen intermediates and IL-12 all play a role in immune control of brucellosis in the mouse model and that their effective functioning is driven by IFN-γ there may be other deficiencies in the IRF-1 −/− mice that are crucial for control of brucellosis which have yet to be identified. Also there are other IFN-γ responding genes that may play a crucial contributing role. For example it has been shown that IFN-γ affects the p47 GTPases known as IRG-47, IGTP and IIGP and LRG-47. A defective in LRG-47 affects phagosomal functioning in that when murine macrophages are infected with *M. tuberculosis* the phagosomes cannot mature to prevent phagosomal acidification (MacMicking *et al.,* 2003). As a result the bacteria are killed since *M. tuberculosis* survives by maintaining phagosomal pH neutrality. Paradoxically however LRG-47 −/− mice also fail to acidify the phagosome as much as normally occurs (MacMicking *et al.,* 2003). This could affect the ability of *Brucella* to activate its acid response genes required for intracellular survival. While this is speculation at this point and such studies await investigation it will likely be necessary to go through the

shopping list of IFN-γ response genes as they are identified before the picture becomes totally clear. However, currently there is a great deal of interest in the endosomal compartment in which *Brucella* lives and evaluation of LRG-47 may make a useful contribution.

3. Role of IL-12 in Protective Immunity

Supporting results for the role of IFN-γ in protection against lethality due to brucellosis come from studies with IL-12. First it was shown that IL-12 is made in response to *Brucella* infections. Studies by Zhan and Cheers (1998) showed that intravenous infection of mice with *B. abortus* strain 19 resulted in detection of bioactive IL-12 briefly in the serum at 3 days after infection and the amount detected increased with the dose of infecting organisms. They also showed that homogenates of splenocytes contained IL-12 following infection. Other studies made similar evaluations with regard to infection with the virulent field strain 2308. Fernandez-Lago *et al.* (1999) showed using CD-1 mice that *Brucella* was a potent inducer of both IL-12p40 and the bioactive heterodimeric IL-12p70, although IL-12p70 was produced at much lower levels than IL-12p40. While there is still some work to be done in the area, it seems that the main inducer of IL-12 is the lipopolysaccharide/endotoxin (LPS) of *B. abortus* (Kariminia *et al.*, 2002; Huang *et al.*, 2003) which acts by stimulating macrophages and dendritic cells through their toll-like receptor 2 (TLR2).

Studies that have depleted IL-12 with antibodies *in vivo* or supplemented mice with recombinant IL-12 affected control of *Brucella* in a manner parallel to that which occurred when depleting or supplementing with IFN-γ (Zhan and Cheers, 1995; Zhan *et al.*, 1996; Sathiyaseelan *et al.*, submitted). That is, depletion of IL-12 by monoclonal antibody treatment increased the recovery of *B. abortus* strains 19 and 2308 from infected CBA or BALB/c mice. The effect of which could be measured for at least 6 weeks after infection (Zhan *et al.*, 1996). By way of comparison it is worth noting that there was no effect of TNF deletion by 6 weeks post-infection (the role of TNF-α will be discussed below in detail). Reciprocally, recombinant IL-12 administration reduced CFU of 2308 (Sathiyaseelan *et al.*, submitted) in the susceptible B ALB/c mice and the protective effect became more pronounced at 3 weeks. Together with the previously described IFN-γ evaluation, these results suggest that the endogenous production levels of both IFN-γ and IL-12 is insufficient in BALB/c mice to give optimal protection against the virulent strain 2308 since both recombinant IL-12 and IFN-γ increase control *in vivo*. This is the expected result based on the relative susceptibility of BALB/c mice to virulent infections (Montaraz and Winter, 1986). Others have shown that to get an optimal reduction in CFU in BALB/ c mice in the first week after infection with 2308 IL-18 must be combined with IL-12 (Pasquali *et al.*, 2002). Moreover, these authors have shown that IL-18 alone is actually more effective than IL-12 alone in controlling infection in the first week. IL-18 is generally though of as a cytokine that acts synergistically with IL-12 to stimulate IFN-γ production by T cells.

In the studies by Zhan and Cheers (1995) using strain 19 as well as that by Sathiyaseelan *et al.* (submitted) using strain 2308 the relationship of IL-12 to IFN-γ was shown. That is, when IL-12 was depleted and CBA mice infected with strain 19 there was

a 75% reduction in IFN-γ production as well as reduced splenomegaly (Zhan and Cheers, 1995; Zhan *et al.,* 1996). Splenomegaly occurs as a result of macrophage infiltration or hematopoiesis in the murine spleen in association with *Brucella* infections. Therefore these later results suggest that IFN-γ may be required to induce this response. In the study by Sathiyaseelan *et al.* (submitted) recombinant IL-12 required IFN-γ for its effectiveness in reducing 2308 CFU in BALB/c mice since administration of anti-IFN-γ antibodies reversed the effect of recombinant IL-12 at 3 weeks post-infection.

Zhan and Cheers showed in a subsequent study (1998) that *in vitro* naïve C57BL/10 splenocytes from TNF receptor −/− mice do not make measurable IL-12 in response to live *Brucella in vitro* while cells from wild type mice did suggesting a role for autocrine TNF stimulation for IL-12 production. Nevertheless when these TNF receptor −/− mice were infected with strain 19 there was IL-12 measured in their serum at 14 days, albeit at a reduced level. However the reduction in IL-12 did not affect the amount of IFN-γ made by splenocytes in a recall response to antigen. It is possible that the requirement for TNF for IL-12 production depends upon the strength of the macrophage/dendritic cell stimulus and that this is stronger *in vivo* than it was in *in vitro* assays with naïve splenocytes. It also suggests the wild type mice (C57BL/10) which are the more resistant phenotype may make excessive IL-12 with regard to the optimal needed for driving T cells for IFN-γ production as measured in the recall assay. However the caveat is that even though this "normal IFN-γ" occurred *in vitro* there were more CFU in both liver and spleen suggesting either that the *in vitro* recall assays may not be particularly quantitative for predicting resistance or that part of the role of IL-12 in protection is by IFN-γ-independent mechanisms and was indeed affected by the reduced IL-12 levels.

4. Which Cells Produce the Protective IFN-γ: NK Cells, γδ T Cells or αβ T Cells?

There are several cell types that can make IFN-γ: T lymphocytes expressing αβ TCR including CD4 and CD8 T cells, γδ TCR T cells, TCR-αβ expressing NKT cells as well as the more primitive TCR-negative NK cell. While the picture may not be entirely clear regarding the role of these lymphoid cell populations at various stages of *Brucella* infection, what is known is that CD4 T cells from 2308-infected mice are primed to produce IFN-γ early in infection in both BALB/c and C57BL/10 mice and do so in *in vitro* recall responses to antigen (Fernandes *et al.,* 1996). The CD4 T cells from the resistant C57BL/10 mice also produce more IFN-γ than those from the more susceptible BALB/c mice. This was shown using purified CD4 T cell populations. The production by CD8 αβ T cells was not evaluated in that study. Strain 19 infections induce IFN-γ production by both CD4 and CD8 T cells in CBA mice (Zhan *et al.,* 1996) and even killed *B. abortus* strain 19 can induce IFN-γ production by murine CD4 T cells (Svetic *et al.,* 1993). Following infection of cattle with *B. suis* their T cells have also been shown to produce IFN-γ in response to antigen stimulation *in vitro*. Using 2-color flow cytometry and intracytoplasmic staining for IFN-γ, the IFN-γ-producing cells were shown to be largely among the CD4 T cell population although a small contribution by CD8 T cells may have occurred (Weyants *et al.,* 1998). While we do not have any conclusive

evidence we presume the importance of IFN-γ to survival of the brucellosis extends to livestock and humans.

γδ T cells have only been examined in the human system. Ottones and co-workers (2002) have been shown that human γδ T cells are stimulated by brucellae and do produce IFN-γ. At least in *in vitro* assays the IFN-γ produced by γδ T cells is partially responsible for controlling the number of intracellular brucellae in human monocytes. However the mechanism by which γδ T cells control intracellular brucellae is not entirely attributable to IFN-γ since activated γδ T cells can lyse infected monocytes (Ottones *et al.,* 2002). γδ T cells have also been shown to increase in number in humans with brucellosis (Bertotto *et al.,* 1993) suggesting their ability to be stimulated by components of *Brucella* although this has not been directly shown except *in vitro*. It is possible that they are reacting only to cytokines produced by other immune system cells during an *in vivo* infection but this seems less likely.

Pathogenic intracellular bacteria that result in acute infection such as *L. monocytogenes* may rely more heavily on the control contributed by the innate immune system including NK cells than bacteria that cause chronic infection. In support of this, *L. monocytogenes* infection kills mice in the absence of NK cells (Dunn and North, 1991) but NK cells are apparently not needed for successful control of *B. abortus* 2308 in either the susceptible B ALB/c mice or the resistant C57BL/6 mice (Fernandes *et al.,* 1995). This was shown by depleting NK cells prior to infection with either anti-asialoGM1 antiserum or the monoclonal antibody anti-NK1.1 and evaluating the *Brucella* CFU in spleens and livers at 1 week after infection. This lack of a role for NK cells is despite the evidence that they are activated during *Brucella* infection in C57B/10 mice. A role in protection also was not evident even when the NK had been activated artificially by administration of poly A:U. In the later experiments, the NK cells were activated as assessed by *in vitro* cytolytic assays yet they did not enhance resistance to brucellosis *in vivo*. Thus we conclude that NK cell production of IFN-γ is not likely to be critical to outcome of a *Brucella* infection as was suggested by the studies of Ko *et al.* (2002) discussed above. As an aside we can also conclude cytolytic activity of NK cells does not contribute to control normally in the murine model. It has been observed that NK cell activity is suppressed in humans with brucellosis (Salmeron *et al.,* 1992). While this may indicate a contribution to chronicity of infection in humans (lack of NK cells response) it could also simply be a bystander response of infection and of no consequence as the reciprocal activation of NK cells was in the mouse model. As suggested it is likely that the difference in the role of NK cells for control of brucellosis and listeriosis may reflect the difference in chronicity of infections.

5. How Do Nude Mice Control Infection?

The results with nude mice that lack a thymus and thus thymus-derived αβ T cells including CD4 and CD8 subpopulations, NK cells and some populations of γδ T cells suggest that other components of the immune response can compensate by preventing death following infection with *Brucella,* at least temporarily (until 121 days post-infection which was the end of the study period; Cheville *et al.,* 1995). However these nude mice are unable to resolve the infection suggesting the effects of thymus-derived T

cells are required. While the normal euthymic mice cleared the granulomas in the liver early following infection the nude mice still had sustained extensive bacterial infection with *Brucella* found in luminal macrophages and intraepithelial monocytes. The nude mice also were unable to maintain granuloma formation and had what was described as diffuse lymphohistiocytic pericholangiitis (Cheville *et al.,* 1995). Granuloma formation is the perview of T cells including αβ T cells and γδ T cells (Mombaerts *et al.,* 1993).

It is of interest to note that the athymic nude mice actually had enhanced resistance in the first 10 days following infection. This suggests that other non-thymus dependent lymphoid subpopulations such as cells of the bridging immune system may produce IFN-γ for early control even though ultimately they are less efficient at clearing the infection. These seemingly disparate results (enhanced early control but ultimately an unresolved infection) may be explainable by the observation made with cytokine gene knock-out mice. That is, a discrepancy has been reported for antibody neutralisation of the cytokine interleukin-4 (IL-4) versus knock-out of the IL-4 gene with regard to the effects of the two treatments on control of leishmaniasis (Noben-Trauth *et al.,* 1999). It has been suggested that the knock-out mice develop compensatory mechanisms to attempt to fill in the gap. In this vein we postulate that in the nude mice the absence of αβ CD4 and CD8 T cells during development may have been compensated for by an actual increase in other IFN-γ producing T cells, i.e. NKT cells and γδ T cells which have thymus-independent subpopulations (Kikly and Dennert, 1992; Hashimoto *et al.,* 1995; Laky *et al.,* 1998). Since both NKT cells and γδ T cell subpopulations may be found in the spleen and liver (Eberl *et al.,* 1999), the main sites of *Brucella* infection, this is not implausible.

6. Control of *Brucellae* in Macrophages by ROI and Nitric Oxide

As discussed above the mechanism by which IFN-γ enhances control is likely to be principally related to increased control by phagocytes. Resistance to *Brucella* infection in murine macrophages was found to be largely mediated by the reactive oxygen intermediates (ROI) superoxide anion and H_2O_2 regardless of whether IFN-γ was present or not (Jiang *et al.,* 1993; Jiang and Baldwin 1993b). This was shown by using superoxide dismutase and catalase to catabolize those products. Neutralising nitric oxide with the L-arginine analogue N^GMMLA did not greatly increase the recovery of CFU (less than 0.3 log10) while catalase increased recovery most often by more than 10-fold at 12 and 24 h. It was ironic that less of a role was found for nitric oxide in the macrophages that had been pre-treated with IFN-γ than non-IFN-γ activated macrophages since IFN-γ activation increased nitric oxide production. A very similar limited contribution to killing by nitric oxide was seen with *B. melitensis* 16M in mouse macrophages (Eze *et al.,* 2000) and for evaluation of *B. suis* in the J774 mouse macrophage cell line under same conditions. That is, there was no role for nitric oxide for control of *B. melitensis* in IFN-γ-activated macrophages nor when un-opsonized *B. suis* was evaluated in IFN-γ-activated macrophages. However when the *B. suis* was opsonized with antibody, nitric oxide resulted in a 20–30-fold reduction in bacteria. This is the most dramatic effect reported for nitric oxide control of *Brucella*. In the study with *B. abortus* the bacteria were un-opsonized however in the *B. melitensis* study they were opsonized

with antibody so lack of opsonization cannot be held responsible for the inability to demonstrate a significant role for nitric oxide in all studies.

Others have suggested that *B. abortus* is not a good inducer of nitric oxide (Wang *et al.,* 2001) and thus this could account for the limited role it plays. In *in vitro* studies that evaluated its role, nitric oxide was shown to be produced in the infected macrophages cultures (Jiang *et al.,* 1993) however a direct comparison with *E. coli* indicated that *B. suis* even when opsonized with antibody induced considerably less iNOS protein following phagocytosis as shown by Westernblot than was produced following phagocytosis of *E. coli* (Gross *et al.,* 1998). Despite the lackluster role for nitric oxide *in vitro*, a role for nitric oxide synthase (iNOS), the enzyme responsible for production of nitric oxide, has been shown for control of brucellosis in two studies *in vivo* (Sun *et al.,* 2002; Ko *et al.,* 2002). Its contribution to control was at least as great as NADPH oxidase's contribution (NADPH oxidase is responsible for the oxidative burst and generation of ROIs) (Ko *et al.,* 2002) even though the ROIs appeared to be more important in *in vitro* analyses than nitric oxide (Jiang *et al.,* 1993). Since the iNOS studies were evaluations *in vivo*, antibody may have opsonized the *Brucella* and contributed to the effect measured for the role of iNOS/nitric oxide as it did for *B. suis*. It is also possible that iNOS/nitric oxide affected other parameters of the immune system in addition to intracellular survival and growth of brucellae in macrophages. Interestingly, the presence of nitric oxide, as predicted by a functional iNOS, actually inhibited control of brucellosis later in the infection (Ko *et al.,* 2002).

7. Role of TNF-α in Cellular Immunity to *Brucella*

Initial studies evaluated many exogenous cytokines for activating phagocytes for anti-*Brucella* activities and while IFN-γ was significantly more effective at enhancing intracellular control than any others (Jiang and Baldwin, 1993b) it required the presence of endogenous TNF-α for maximal control (Jiang *et al.,* 1993). TNF-α was also required for maximal killing of brucellae by macrophages in the absence of IFN-γ activation (Jiang *et al.,* 1993). That is, during the first 24 h after infection, phagocytosed brucellae are killed regardless of the presence of IFN-γ (see Figure 1) but it is dependent upon TNF-α. Subsequent to this initial killing period, the presence of IFN-γ largely prevents replication of the organisms rather than sustaining killing but again TNF-α is required for this effect. The dogma is that macrophage activation requires two signals: IFN-γ plus a second activator such as lipopolysaccharide (LPS) that ligates toll-like receptors (TLR) and induces TNF-α production. Although nitric oxide is not particularly effective in controlling intracellular brucellae, it has been noted that a functional TNF receptor is also necessary for nitric oxide production in response to *Brucella* (Zhan and Cheers, 1998) in accordance with the general literature regarding nitric oxide production.

Given the role of TNF-α it is of particular interest to note that *B. suis* actually inhibits TNF-α production when it infects human monocytes, thus being a stealth invader (Caron *et al.,* 1994). This inhibition has been attributed to a property of the *Brucella* outer membrane protein 24 (Jubier-Maurin *et al.,* 2001) and may be key to the parasitism of macrophages by *Brucella* since in its absence even IFN-γ would be less effective. This is illustrated by the studies of Golding and colleagues in conjunction with those by Zhan

and Cheers. Golding and coworkers (Huang *et al.,* 2003) have shown that *B. abortus* interacts with TLR2 of murine cells and results in stimulation of TNF-α and more recently of IL-12 p40. These two mediators of cellular immunity form a loop since Cheers and coworkers (Zhan and Cheers, 1998) showed that mice lacking the receptors for TNF (TNFR−/− mice) were 'severely deficient in IL-12 production' following infection with *B. abortus* strain. Since IL-12 is a product of macrophages and dendritic cells this suggests a controlling role for TNF-α not only in macrophage activation during a *Brucella* infection as it did the *in vitro* studies described above (Jiang *et al.,* 1993) but also for the production of IL-12 by them; the IL-12 would induce IFN-γ production by T cells which in turn activates macrophages.

In vivo evidence for this pivotal role for TNF-α was obtained by *in vivo* infection: as a result of lack of the TNF receptor the infection was exacerbated (Zhan *et al.,* 1996; Zhan and Cheers, 1998). The accompanying effects were that despite IFN-γ production in the TNFR−/− mice there were fewer macrophage in the spleen and thus a lessened splenomegaly and lack of nitric oxide production by the macrophages. Thus it would suggest that IFN-γ alone cannot control infection by attracting and activating macrophages for anti-*Brucella* activities in the absence of a TNF-α signal. However as mentioned above the role for TNF was no longer apparent at 6 weeks (Zhan *et al.,* 1996). Thus the role for TNF-α may only be in the early stages of infection.

We also have evaluated TNF-α for its role in control of *B. abortus* strain 2308 in BALB/c mice (Murphy *et al.,* 2001b). *In vivo* depletion of TNF-α with a monoclonal antibody resulted in a statistically significant increase in the mean CFU recovered from infected BALB/c mice at 3 weeks post-infection. However TNF-α depletion in IFN-γ gene-disrupted BALB/c mice resulted in a non-statistically significant increase in CFU (Murphy *et al.,* 2001b). Again this supports the role for the TNF-α—IL-12—IFN-γ loop for control of brucellosis. In the anti-TNF-α-treated IFN-γ gene-disrupted mice there were substantial changes in the spleen with approximately a ten-fold increase in macrophages, NK cells and neutrophils suggesting that in the absence of both TNF-α and IFN-γ that cells of the innate immune system were recruited or retained to try to control the infection. It is interesting that the observations with *B. suis* and human monocytes and *B. abortus* and murine macrophages are so different with regard to TNF-α production since the fact that *B. abortus* infections are exacerbated in the absence of TNF-α suggests it is indeed made during a *Brucella* infection.

8. Innate Resistance Mechanisms of Macrophages

The natural resistance-associated macrophage protein 1 gene known as *Nramp1* has been shown to control innate resistance to a number of intracellular microbial pathogens in mice including mycobacteria, salmonella and leishmania, and thus its early designation as the BCG/Ity/Lsh resistance gene. It is a divalent cation transporter for iron, zinc and manganese and found in the membranes of late endosomes and lysosomes. Its effects are independent of IFN-γ production. Early studies showed that monocytes from naturally resistant cattle tended to control intracellular *B. abortus* more efficiently than monocytes from more susceptible cattle (Price *et al.,* 1990; Qreshi *et al.,* 1996) and that this was correlated with *Nramp1* gene (Feng *et al.,* 1996). The difference in bovine resistant and

susceptible phenotypes is associated with a polymorphism in a microsatellite in the untranslated region of the *Nramp1* gene.

In an attempt to demonstrate its role more directly in brucellosis the bovine *Nramp1* gene homologues (resistant and susceptible types) were transferred into a murine macrophage cell line RAW264.7 (Barthel *et al.,* 2001). The resistant Nramp1 phenotype conveyed a statistically significant decrease in recovered CFU of *B. abortus*. By comparison in another *in vivo* study using Nramp1 resistant and susceptible strains of mice (Guilloteau *et al.,* 2003), the presence of the murine *Nramp1* resistance gene actually resulted in the recovery of more *B. melitensis* from infected mice than the presence of the *Nramp1* susceptible gene at 5 days post-infection. However, the difference was not statistically significant and no difference was found in the ability of the macrophages to control replication of *Brucella in vitro*. The cause of the increased susceptibility *in vivo* may have been related to the increase in the macrophage recruitment to the spleen. It has been seen in other studies that an increased splenomegaly, most often caused by an increase in splenic macrophages, does not necessarily correlate with increased control of infection (Mielke, 1991) although more often it does (Zhan and Cheers, 1998).

We have also compared the relative abilities of macrophages from BALB/c and C57BL/10 mice (both Nramp1 susceptible strains, but which are relatively resistant or susceptible to *B. abortus* 2308 infections) for their abilities to control intracellular brucellae with and without IFN-γ activation. Although both are Nramp1-susceptible, Ho and Cheers (1982) have shown that resistance in mice to brucellosis is a multigenic trait. Thus it seemed a reasonable speculation that differences could occur. However, extensive studies showed there was no difference in the ability of macrophages from BALB/c and C57BL/10 mice to control intracellular *Brucella* under any conditions (Sathiyaseelan *et al.,* 2000). Thus, it would seem based on the evidence to date that innate differences in macrophages control of *Brucella* do not contribute to control of disease.

9. Which is it: CD4 or CD8 T Cells That Are Key to Protection?

As reviewed above, IFN-γ is key to host survival of brucellosis. There is theoretical evidence to support a role for both CD4 and CD8 T cells in the control of brucellosis since both T cell subpopulations have the potential to produce IFN-γ. The evidence in favour of CD8 T cells as the major players for control has largely been obtained using the attenuated vaccine strain *B. abortus* strain 19 (Table 1). It has been obtained in several ways: (i) by depleting CD8 T cells with monoclonal antibodies to CD8 in CBA and BALB/c mice (Pavlov *et al.,* 1982; Mielke, 1991) (ii) using C57BL/10 β2-microglobulin gene knock-out mice (Oliveira and Splitter, 1995) which are deficient in class I MHC and thus do not induce response by αβ CD8 T cells and NKT cells that react with the MHC class I and MHC class I-like molecule CD1d, respectively; (iii) by adoptive transfer studies of CD8 subpopulations from infected BALB/c mice to naïve mice (Araya *et al.,* 1989); and (iv) histopathology studies by Mielke and colleagues which suggested that CD8 T cells surround infected macrophages and are key to reducing antigen load to allow re-emergence of a functional CD4 T cell protective response including production of IFN-γ and clearance of infection (Mielke 1991; Hort *et al.,* 2003). The main role for CD8

T cells in protection is an attractive hypothesis since they can potentially kill *B. abortus*-infected macrophages (Oliveira and Splitter, 1995) and they may also make granulysis which kills intracellular mycobacteria (Stenger *et al.*, 1998a).

However, despite the attractiveness of the hypothesis there is less evidence for the role of CD8 T cells in protecting against a virulent strain *B. abortus* strain 2308 using C57BL/6 mice. This mouse strain is virtually genetically identical to the C57BL/10 strain shown by Winter and colleagues to be relatively resistant to the infection (Montaraz and Winter, 1986). In these mice, deletion of the β2-

Table 1. Evidence for a role for CD4 or CD8 T cells *in vivo* for protection against *Brucella_abortus* virulent and attenuated strains in mice

Mouse strain	***B. abortus* strain**	**Method of analysis**	**Results**	**Reference**
CBA	19	Depletion of CD8 or CD4 T cells by monoclonal antibodies	CD8 were important for control	Pavlov *et al.*, 1982
BALB/c	19	Adoptive transfer of CD4 and CD8 T cells	Either CD4 or CD8 gave equal protection if adopted from mice that had been infected for at least 4 weeks	Araya *et al.*, 1989
BALB/c	19	Monoclonal antibody depletion of CD8 and CD4 T cells	Only CD8 T cells contribute to control at 18 days PI[1]	Mielke, 1991
C57BL/6	19	β2-microglobulin depleted mice (so no class I MHC expressed and thus no CD8 T cell response) and Class II MHC disruption (so no CD4 T cell response)	Only CD8 T cells contributed to control	Oliveira and Splitter, 1995
C57BL/6	2308	β2-microglobulin depleted mice—so no class I MHC expressed and thus no CD8 T cell response	CD8 T cells have a role at 1 week PI only—no role at 3 and 10 weeks	Murphy *et al.*, 2001
BALB/c	2308	Anti-CD8 monoclonal antibody	Slight (3-fold) but statistically significant increase in CFU at 3 weeks PI when CD8 T cells depleted	Murphy *et al.*, 2002
C57BL/6	2308	β2-microglobulin depleted mice (so no class I MHC expressed and thus no CD8 T cell response) and Class II MHC disruption (so no CD4 T cell	Equivalent effect of depleting either CD4 or CD8 T cells at 3 and 6 wk PI, but only CD4 have significant role in	Parent *et al.*, in preparation

	response)	protection at 10 weeks

1. PI=post-infection

microglobulin gene results in the loss of expression of major histocompatibility complex (MHC) class I molecules the thus the ability of CD8 T cells to interact with antigenic peptides for activation. So for our purposes they can be considered equivalent to a CD8 T cell knock-out. The stimulation of NKT cells that recognise CD1d presented molecules would also be absent since CD1d requires the presence of the accessory chain β2-microglobulin to be transported to the surface of host cells. These mice actually cleared the infection with strain 2308 more rapidly as did mice deficient in perforin (Murphy *et al.*, 200 1a). The only time where the β2-microglobulin or perforin deficient mice had less control of the infection was during the first week after infection. By 3 weeks post-infection they had identical CFU to the control mice. Since NK cells are not needed for clearance of *Brucella* it is unlikely that the role for perforin was attributable to mechanisms involving NK cell lysis of *Brucella*-infected cells. Therefore, perforin may be needed by CD8 T cells to lyse infected macrophages during the first week of infection with a virulent strain thereby releasing bacteria to phagocytes.

Another experiment was performed using the more susceptible BALB/c mice and infection with strain 2308. Despite the crucial requirement for IFN-γ to survive a *Brucella* infection in both strains of mice there is a significant difference in its role in BALB/c and C57BL/6 mouse strains (Murphy *et al.*, 2001a). First the number of CFU continually increased in the spleens of the IFN-γ-deficient C57BL/6 mice until they died at 6 weeks post-infection. As for normal mice, the spleen was the principal site of infection for murine brucellosis and at the time of death contained nearly 9 $\log_{10}$ CFU of brucellae. In contrast, in BALB/c mice the number of CFU at 3 weeks post-infection was equivalent in IFN-γ knock-out mice and normal mice and the CFU did not increase between then and the time of death (about 10–11 weeks post-infection). These data suggest the BALB/c mice have other mechanisms to control the number of CFU than IFN-γ. Thus we evaluated CD8 T cells for their role in control during this time (Murphy *et al.*, 2001b). We postulated a role for CD8 T cells since there was an influx of CD8 T cells in the spleens of the BALB/c IFN-γ knock-out mice at the time of their death (Murphy *et al.*, 2001a). *In vivo* depletion of CD8 T cells resulted in a statistically significant increase in the mean CFU recovered from infected BALB/c mice at 3 weeks post-infection but it was relatively small (3-fold increase in CFU only) compared to the 1,000-fold increase seen for strain 19 infection of C57BL/10 mice in the absence of CD8 T cell responses (Ko *et al.*, 2002).

Overall these studies suggest that the picture is quite different when a virulent strain of *B. abortus* 2308 is evaluated compared to an attenuated vaccine strain 19. That is, CD8 T cells may be very important for control of infections with attenuated strains but much less so for control of virulent field strains. However because the reciprocal experiment was not done with class II MHC-deficient mice in the Murphy study (2001) in order to analyse the role of CD4 T cells in control of 2308 infections, we began to wonder whether our suppositions were in error. Thus we repeated the initial experiment and evaluated the effect of the class II MHC deficient mice in parallel with class I deficient mice. Those results indicate that in the early stages of the infection (i) either cells of the innate/bridging immune system may be sufficient for control or (ii) that either CD4 or

CD8 αβ T cells may interchangeably do the job. These two possibilities that are as yet unresolved are based on the observation that depletion of either CD4 or CD8 responses did not affect the number of CFU recovered at 3 weeks post-infection. However, as we have contended, the CD4 T cells were ultimately needed to clear the infection in this strain of mice as shown at 10 weeks post-infection (Parent, Goenka and Baldwin, unpublished).

So what is the difference between control of infection with the attenuated strain 19 versus the field strain 2308? When one examines the number of CFU with time post-infection the profiles are quite different (Montaraz and Winter, 1986). For example in BALB/c mice strain 19 organisms reach very high numbers at 2 weeks after infection, being 30-fold higher than strain 2308 organisms at the same time. The strain 19 organisms then are cleared rapidly relative to clearance of strain 2308. The more rapid clearance of strain 19 may be reflecting clearance by CD8 T cells as discussed above. If CD8 T cells are important for clearance of strain 19 but not 2308 one viable hypothesis to explain this is that the strain 2308-infected host cells do not display appropriate levels of MHC class I or *Brucella* antigenic peptides on MHC class I molecules.

To test the first part of this hypothesis we examined the level of MHC class I and MHC class II expression on murine macrophages following infection with strain 2308. Decreased expression of MHC on macrophages has been substantiated for viruses which use more than one mechanism to decrease expression of MHC class I. Such mechanisms include inhibiting peptide transport across the endoplasmic reticulum (York *et al.,* 1994; Fruh *et al.,* 1995) and disruption of the Jak/Stat signalling pathway (Brander *et al.,* 2000). In addition similar results have been shown with another intracellular bacteria *M. tuberculosis* using human cells. *M. tuberculosis* down-regulates the expression of the antigen-presenting molecule CD1 on human cells (Stenger, 1998b). As discussed above this molecule displays pathogen molecules to NKT cells, a cell important in bridging immune responses.

To test this hypothesis *B. abortus* organisms expressing green fluorescent protein (GFP-*Brucella*) and three-colour flow cytometry was used to evaluate MHC expression on macrophages following *in vitro* or *in vivo* infection (Murphy *et al.*, 2002). In *in vitro* assays using the macrophage cell line J774, the expression of MHC class I and class II were the same or higher on macrophages with GFP-*Brucella* as on the uninfected macrophages. Because we could flow cytometrically gate on infected cells to distinguish them from uninfected, we could analyse both from the same cultures. Thus exposure to a different set of cytokines could not influence the MHC expression. In other words, any effect on level of MHC expression should be accountable by the fact they were infected or non-infected with brucellae. No difference in expression relative to infection level was found for *ex vivo* peritoneal macrophages from BALB/c and C57BL/10 mice, Finally macrophages from an *in vivo* infection were evaluated for the level of MHC class I and II. Here the infected cells tended to be as high as or higher than that on the GFP-negative cells. Thus level of MHC expression can not account for the lack of CD8 T cell engagement in protection against a virulent infection.

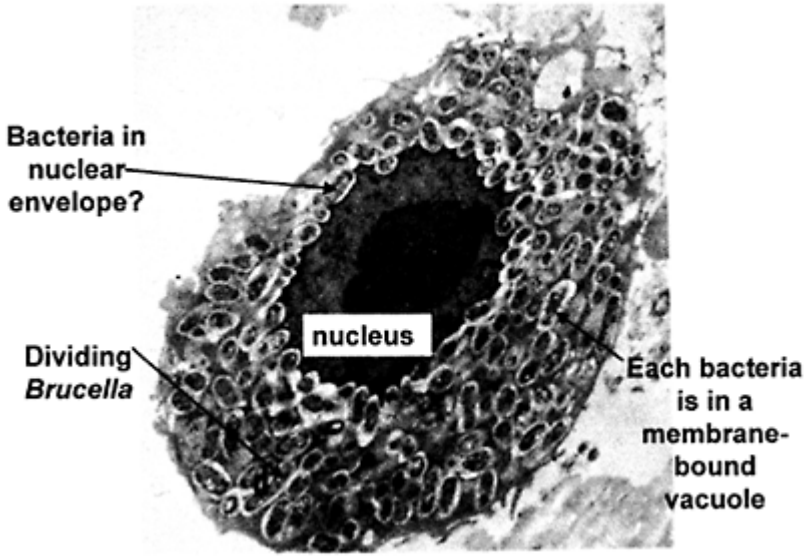

Figure 2. Electron micrograph section of a portion of a J774 mouse macrophage cell line following infection with *Brucella abortus* strain 2308 *in vitro* for 2 days. A portion of the nucleus is visible and the bacteria are visible in membrane-bound vacuoles throughout the cytoplasm as labelled and in what has been suggested to be the nuclear envelope. "d" is on a dividing bacteria.

10. The Implications of Cross-Presentation for *Brucella* Antigens

Since the lack of engagement of CD8 T cells in immunity to the virulent strain *B. abortus* 2308 could not be attributed to decreased MHC expression on infected host cells we will discuss the second part of our hypothesis: that CD8 T cells are not engaged in protective immunity in the later stages of the infection because of the inability of infected macrophages and dendritic cells to effectively display *Brucella* antigenic peptides on MHC class I molecules. While this is speculative, exciting work in the last few years on the nature of the *Brucella* replicative phagosome and cross-presentation of antigens on MHC class I molecules makes it timely. Intracellular trafficking events could potentially radically affect the presentation of *Brucella* antigens on MHC class I molecules for activation of CD8 T cells. That is, the type of endosomal compartment that strains 19 and

2308 are within in macrophages/dendritic cells could determine whether endogenous pathway presentation, or cross-presentation as it is called, of peptides with class I MHC molecules occurs.

B. abortus survives in multi-membrane bound compartments (Figure 2) that in some cases are lined with ribosomes that resembles modified endoplasmic reticulum structures (Pizarro-Cerda *et al.,* 1998; see also Chapter 14). Gorvel and colleagues showed that endoplasmic reticulum is involved early on during phagocytosis and that sequential sustained exchanges take place between "*Brucella* containing vacuoles" (BCV), and endoplasmic reticulum during the maturation of the *Brucella* containing phagosome (Celli *et al.,* 2003). In addition they showed that live BCV interact with early but not late compartments of the endocytic pathway. It was recently shown by Desjardins and coworkers (Gagnon

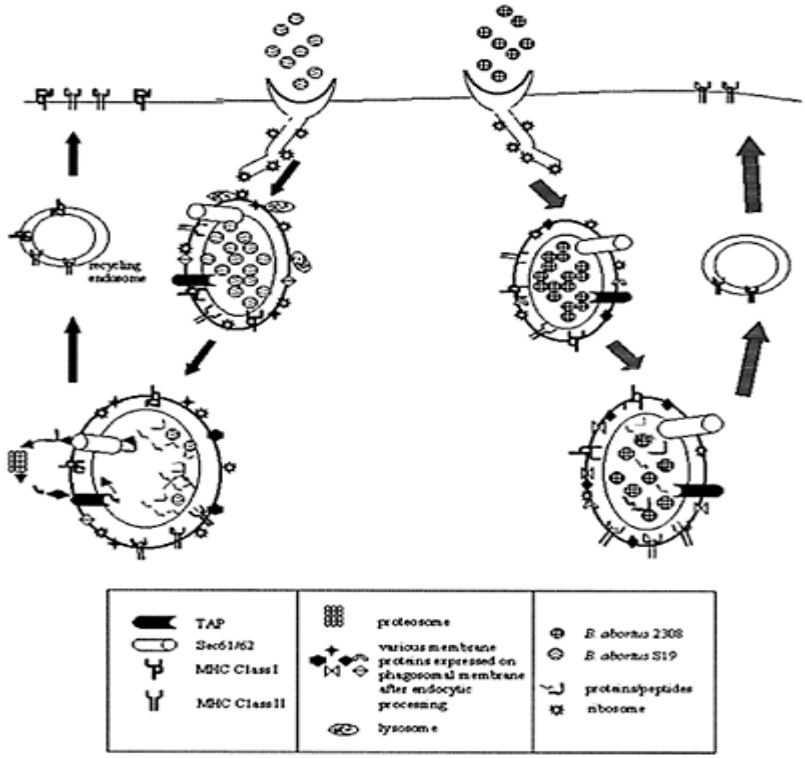

Figure 3. Theoretical schematic of phagocytosis of *Brucella abortus* strain 19 (left-hand side) and strain 2308 (right-hand side) to explain engagement of both CD4 and CD8 T

cells for protection of strain 19 but
only CD4 for protection of strain 2308.

et al., 2002) that fusion of the endoplasmic reticulum with the macrophage plasmalemma under the phagocytic cups is a source of membrane for phagosome formation in macrophages and referred to this as endoplasmic reticulum-mediated phagocytosis. Normally, these nascent phagosomes fuse with endocytic compartments and eventually with lysosomes and mature to become antimicrobial and highly degradative. They showed that the early recruitment of endoplasmic reticulum contributed to the phagosomal membrane endowing the phagosome with the MHC class I loading machinery for cross-presentation of antigens with MHC class I molecules. Amorigena and colleagues also showed that cross-presentation in dendritic cells occurs in a specialised, self-sufficient, endoplasmic reticulum-phagosome mixed compartment (Guermonprez *et al.,* 2003) while Cresswell and colleagues (Ackerman *et al.,* 2003) showed that internalised soluble antigens localised to an endoplasmic reticulum-like compartment by donation of endoplasmic reticulum membranes to the nascent phagosomes in dendritic cells. Both of the later groups also showed that phagosomes contain all the components necessary for class I MHC loading.

By entry into the professional antigen presenting cells with the involvement of the endoplasmic reticulum (Ackerman *et al.,* 2003; Gagnon *et al.,* 2002; Guermonprez *et al.,* 2003), both *B. abortus* strains 2308 and 19 would have access to the class I presentation machinery (Figure 3). However additional trafficking events may alter this prediction. With regard to this, Porte *et al.* (2003) showed that the O-side chain is involved in entry of *B. suis* into macrophages through lipid rafts (directly or through a receptor) and that this entry mechanisms subsequently directs phagosomes through an endocytic pathway which delays/impairs fusion with lysosomes while phagosomes formed around rough mutants of *B. suis* readily fused. Rough strains also do not use lipid rafts to enter the cell and it was suggested that this could be involved in directing subsequent events (Porte *et al.,* 2003). In addition, Gorvel *et al.* (Pizarro-Cerda *et al.,* 1998) demonstrated that *B. abortus* strains 2308 and 19 both use the autophagic machinery of the HeLa cells to establish themselves and that during the intracellular trafficking of both the attenuated strain 19 and virulent strain 2308 that phagosomal biogenesis involved fusion of the phagosome with the early endocytic compartments to form autophagosomes and that this was responsible for the subsequent avoidance of fusion with late endosomes and lysosomes (see also Chapters 14 and 15). However, even though both have O-side chains, attenuated strain 19 and virulent strain 2308 diverge in their trafficking profiles later on in the infection process. Strain 2308 gets delivered to the endoplasmic reticulum where massive replication takes place whereas strain 19 gets degraded following lysosomal fusion (Porte *et al.,* 2003) (Figure 3). Thus, while the LPS O-side chain may be an important determinant in the intracellular trafficking profile as described above, the fact that *B. abortus* strain 19 and 2308 end up in phagolysosomes and endoplasmic reticulum, respectively, indicates that there may be additional regulation events of this process. Strain 19 has reduced nucleoside inhibitors which may account for its inability to inhibit phagolysosome formation and hence accounts for its attenuation. Sowa *et al.* (1992) identified 14 putative virulence proteins that are present in strain 2308 and not in strain 19 while 25 proteins were present in strain 19 and not 2308 as determined by 2D gel

electrophoresis some of which may account for these differences in intracellular trafficking (Sowa *et al.,* 1992).

We postulate that this lysosomal fusion of the strain 19-containing phagosome may be required to facilitate cross-presentation on class I MHC and engagement of CD8 T cells (Figure 3). That is, the peptides generated in the phagolysosme may get released into the cytosol and through the cytosolic pathway of antigen presentation get associated with class I MHC molecules to activate CD8 T cells. By comparison we postulate that once strain 2308 has established itself it may replicate in the endoplasmic reticulum-derived organelles until the host cell dies and not release foreign proteins for association with class I MHC molecules. According to one of the new models (Gagnon *et al.,* 2003) for class I MHC presentation, pathogen specific foreign proteins that are either produced by the pathogen's metabolic activities (secreted) or as a consequence of the hydrolase degradation have to be retrotranslocated to the cytosol and degraded in the ubiquitin/proteasome complex to generate peptide fragments specific class I MHC molecules. Such proteasomes are called as immunoproteasomes because the specificity of the proteasome complex is altered to enhance the production of peptides that have been cleaved after hydrophobic and basic residues to facilitate binding to class I MHC molecules. IFN-γ is known to enhance the immunoproteasomes activity required for class I loading. Most of the peptides would be channelled back from the cytosol into the phagosomal lumen through the TAP to be loaded onto class I MHC molecules. While strain 2308 may initially be susceptible to the hydrolases generating peptides that can be presented with MHC class II and MHC class I molecules it may soon adapt and turn-on expression of adaptive/virulence genes that enable it to survive in the replicative niche without generating potential antigenic proteins that can be channelled out through sec61. This would explain why early in the infection both CD4 and CD8 T cells contribute to protection but at the later stages when clearance is occurring it seems to be restricted to CD4 T cells. In contrast, for strain 19 infections, CD8 T cells are particularly important at all times evaluated. The VirB Type IV secretion system has been recently shown to be involved in the intracellular trafficking profile, probably contributing to survival of 2308 survival by not allowing maturation of the nascent phagosome to a more degradative compartment (Celli *et al.,* 2003). As a result in our model class I presentation would be prevented and thus engagement of CD8 T cells.

11. Why Do BALB/c Mice Turn off the Protective IFN-γ Response During The Plateau Phase of Infection?

In the first studies on cellular immunity to *B. abortus* by Mackaness (1964), he found that the ability of macrophages from *Brucella*-infected mice to kill bacteria waned by day 35 when the infection was in the chronic or plateau phase. There is a hiatus of IFN-γ production by BALB/c splenoctyes during the plateau phase of infection with strain 2308 but not in C57BL/6 mice (Murphy *et al.,* 2001a). This has been corroborated by Mielke and coworkers using a mouse-adapted strain 19 (Hort *et al.,* 2003). We have postulated that the turn-off of IFN-γ may be necessary for survival of an overzealous inflammatory response.

One contribution to this we evaluated is blockade of functional bioactive IL-12 p70. While BALB/c splenocytes produced IL-12 during this time of IFN-γ hiatus, when they were evaluated *ex vivo* the supernatants from these cultures blocked the biological activity of recombinant IL-12p70 to induce IFN-γ production. This led us to suggest that the lack of IFN-γ production is due to a IL-12p40 homodimer blockade of the high affinity IL-12 receptor β2. Since prostaglandins induce IL-12p40 production (Kalinski *et al.*, 2001) and Stevens *et al.* (1992) showed that treatment of mice with indomethacin, an inhibitor of prostaglandins, increased control of brucellosis in BALB/c mice we also evaluated its effect on IL-12p40 production. While there was a substantial decrease in IL-12 p40 production in mice treated with indomethacin this did not increase the amount of IFN-γ (Baldwin and Parent, 2002). Thus IL-12 p40 blockade may not be a major contributing factor *in vivo*.

The IFN-γ hiatus is also not attributable to a switch to a Th2 response since IL-4 production by T cells from infected BALB/c mice was never measured although neutralising IL-4 did increase control of infection in one study (Fernandes *et al.*, 1995). However we have shown that IL-10 affects control *in vivo* and decreases IFN-γ production in *in vitro* studies (Fernandes and Baldwin, 1995). It has been shown that IL-10 affects IL-12 production (Aste-Amezaga *et al.*, 1998) and response to it since IL-10 is known to decrease IL-12Rβ2 expression (Wu *et al.*, 1997). Scott *et al.* (1997) also found that IL-10 inhibited the ability of *B. abortus* LPS to stimulate BALB/c splenocytes to produce IL-12 and IFN-γ. However another study undertaken by Cheers and colleagues (1998) using the resistant strain of mice C57BL/10 infected with the attenuated strain 19 found that neutralising either IL-4 or IL-10 did not prolong IL-12 production even in the presence of high bacterial numbers. Thus the role of these cytokines in control of IFN-γ and particularly in its shut-down during the plateau phase of infection in BALB/c mice is inconclusive.

Mielke and colleagues (Hort *et al.*, 2003) have suggested the hiatus is due to the disrupted architecture of the spleen during the plateau phase of the infection. As a result CD4 T cells and CD8 T cells are not appropriately juxtaposed with infected macrophages. They cite a profound depletion of both subpopulations in the periarteriolar lymphatic sheaths. They also could never demonstrate IL-4 production as assessed by the sensitive PCR method suggesting also that it is not a switch to a Th2 type response. Rather they suggest the shift towards clearance is mediated by CD8 T cells and this is supported by their prominent role in control of strain 19 infections. What shifts the balance in strain 2308 infections then since we have been unable to demonstrate a crucial role for these cells? This is an intriguing unanswered question.

12. Acknowledgements

We would like to acknowledge the contributions of former laboratory members to this work including Michelle, Parent, Xiaosui Jiang, Dancella Fernandes, Brian Leonard, Rita Benson, Janaki Sathiyaseelan and Erin Murphy, and thank our collaborators Drs. R.Martin Roop II and Phillip Elzer. Some of the work reviewed was funded by USDA NRI/CGP grants 9303615 and 9501995.

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Chapter 17
Development of New *Brucella* Vaccines by Molecular Methods

David L.Hoover*, Mikeljon P.Nikolich, Mina J.Izadjoo, Richard H.Borschel, and Apurba K.Bhattacharjee

From: *Brucella:* Molecular and Cellular Biology. Edited by: Ignacio López-Goñi and Ignacio Moriyón

Abstract

Brucellosis causes substantial morbidity in humans and exacts a considerable economic toll on both the health care and livestock industries. Recently, the long-recognized potential use of *Brucella* as a bioweapon coupled with growing concern over bioterrorism has led to its classification by the United States Centers for Disease Control as a Category B biological threat agent. Elimination of brucellosis in food animals is the preferred method to prevent naturally acquired disease in humans, but this approach would not protect against illicit use of the organism as a bioweapon. Development of a human vaccine would be valuable both as a biodefense strategy and as an interim solution for prevention of naturally acquired human disease in situations where economic or sociological factors prevent application of an effective animal disease control program. Indeed, the need to protect occupationally high-risk workers and other susceptible populations has led to a number of efforts at human vaccine development. These efforts have met with variable success, but none has resulted in a well-accepted product. The recent explosion in genetic information on *Brucella* as a result of complete sequencing of the *B. suis*, *B. melitensis* and *B. abortus* genomes may provide an opportunity to make safe and effective vaccines for humans and improve on those available for animals. Novel molecular based vaccine strategies, which may include DNA vaccination, new adjuvants and selection of immunogens based on genomic and proteomic screening methods may lead to development of subunit vaccines, with potential safety advantages over living organisms. In the near future, however, live attenuated vaccines will be the gold standard for efficacy.

In this chapter, we will focus on live, attenuated human vaccines. We will review previous human studies and examine aspects of interactions between *Brucella* and its host that are relevant for development of appropriate animal models for testing and for vaccine design. We will then discuss work in nonhuman

Note: The views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense (para 4–3, AR 360–5). Data discussed from unpublished work was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, NRC Publication, 1996 edition.
* For correspondence email david.hoover@na.amedd.army.mil.

primates and summarize results from our own program, which has established mucosal challenge models of infection to assess vaccine efficacy and uses molecular methods to develop live, attenuated vaccines. Finally, we will survey recent work that identifies other interesting targets for vaccine development.

1. Contrasts in Vaccine Strategies for Animals and Humans

Vaccination has been a key component of animal brucellosis control efforts, but attempts to develop vaccines for humans have met with limited success. These differences in success may reflect different goals for vaccination, which reflect differences in disease epidemiology, routes of infection, pathogenesis, and clinical manifestations that relate to the complex and varied interactions of different species of *Brucella* with different hosts. Vaccines for livestock are primarily aimed at interruption of transmission. Transmission may occur from mother to offspring by transplacental infection or through milk, or among mature animals venereally or by ingestion or inhalation of bacteria in infected placental tissue after parturition. The primary endpoint of vaccine efficacy in this setting is thus prevention of abortion or newborn infection. Preservation of seronegativity in vaccinated livestock is an important consideration, since it allows detection and elimination of animals that become infected despite vaccination. A less efficacious vaccine that preserves seronegativity may have greater utility than a more effective vaccine that compromises inexpensive identification of infected animals. In humans, in whom person-to-person transmission is of negligible epidemiological importance, the goal of vaccination is prevention or amelioration of disease. Preservation of seronegativity may be less important than safety and efficacy for a human vaccine. In humans, more expensive microbiologic tests and repetitive serologic testing can be used to establish a diagnosis and antimicrobial therapy may be based on a presumptive diagnosis if necessary. For both livestock and humans, vaccines must be safe and elicit a pathogenetically relevant immune response when administered by a feasible route. The safety threshold in humans, however, will necessarily be higher than that for animals.

2. Human Vaccination

Although several human vaccines have been tested to date, none is completely satisfactory. In an early experiment, immunization with killed, whole *B. melitensis* led to transient protection against illness in two volunteers challenged with 4.5 X 10^8 CFU of the homologous organisms, but one of the two volunteers developed brucellosis as a consequence of a laboratory accident 5 months after immunization (Elberg and Silverman, 1950). In another report (Howe *et al.*, 1948), 17 laboratory workers developed brucellosis with either *B. suis* or *B. melitensis* even though they had previously been vaccinated subcutaneously and had developed serum antibody. Interestingly, when disease occurred, workers developed erythema and edema at the site of previous vaccination or skin tests; reactions waxed and waned with exacerbations and relapses during recurrent disease. These studies suggested that local reactions may be a limiting factor for vaccines given cutaneously and that killed, whole cell vaccines may have limited efficacy. A more refined nonliving vaccine comprising a delipidated, phenol-insoluble fraction (PI) of strain 19 was tested in volunteers (Bentejac *et al.*, 1984; Hadjichristodoulou *et al.*, 1994) who had previously been screened and found nonreactive in intradermal skin tests. This vaccine was highly reactogenic, causing both local and systemic (fever, chills, malaise) reactions. It induced little or no delayed type skin hypersensitivity response to melitin, but about 18–25% developed a response to intradermally administered diluted vaccine. Blood mononuclear cells obtained from immunized subjects had weak lymphoproliferative responses to PI antigen unless donors had been occupationally exposed to *Brucella* for extended periods of time after vaccination (Bascoul *et al.*, 1976). The PI vaccine induced anti-*Brucella* antibodies, presumably directed against the OPS. Antibody titers peaked at 2 months after immunization and gradually declined over the next 18 months. There are no controlled trials indicating efficacy. The vaccine is no longer commercially available.

Live, attenuated vaccines for humans are theoretically attractive because, as indicated by experience in livestock, they are likely to elicit the most solid immunity against infection. A major hurdle to overcome, however, is finding a strain that is both suitably attenuated and sufficiently immunogenic. At least 3 live, attenuated vaccines have been tested or fielded in humans. A streptomycin-dependent variant of *B. abortus* 19 induced agglutinating antibodies in volunteers. Serum passively transferred from these vaccinees to mice reduced spleen colonization in animals challenged with strain 2308 (Sulitzeanu, 1955). A derivative of strain 19, called 19-BA, was developed and used extensively in the former Soviet Union. This vaccine had both local and systemic side effects, but was credited with reducing the 5-year incidence of brucellosis from 12.3% in nonvaccinated persons to 0.5% in vaccinated individuals (Elberg, 1973). When Spink *et al.* (1962) tested it in U.S. volunteers, however, they found it to be insufficiently attenuated.

The Soviets studied use of the BA vaccine as an aerosolized dry powder. Aerogenic vaccination of guinea pigs led to protection of 80–90% of animals challenged subcutaneously with 5–10 infectious doses (Aleksandrov *et al.*, 1958). A similar level of protection was seen in animals immunized with the same vaccine subcutaneously. In contrast, only half of aerogenically immunized animals challenged with 100 infectious doses of *B. melitensis* by aerosol were protected. Of 153 volunteers immunized aerogenically, only one had a febrile, systemic reaction, compared to 9 of 25 volunteers

immunized subcutaneously. Antibody titers were comparable after immunization by either route. Agglutination titers of 1:20 to 1:40 appeared in 20% of aerogenically immunized subjects as early as 7 days after vaccination (Aleksandrov *et al.*, 1958). At 30 days post-immunization, 96% were seropositive, with a median titer of 1:160. In other studies, of 1201 individuals vaccinated with the dry powder vaccine, systemic or respiratory reactions (laryngitis, tracheitis, bronchitis) occurred in 92 (7.6%). The systemic reactions, characterized by fever, slight lymphadenopathy, mucus membrane and facial hyperemia and minor defects in ventilation and blood oxygenation, occurred more commonly in previously sensitized individuals (Aleksandrov *et al.*, 1962), but were noted to be "harmless, of short duration" and "readily reversible." These studies suggested that administration of live vaccines by aerosol could obviate the local reactions caused by cutaneous vaccination, but could present a different set of problems due to local airway and systemic side effects.

In China, *B. abortus* 104M was used both subcutaneously and epicutaneously. This vaccine appears to be less attenuated than 19-BA. Like the PI vaccine, both 19-BA and 104M may have increased side effects in individuals who have been previously sensitized to *Brucella* (Elberg, 1981). Attempts to use the goat vaccine *B. melitensis* Rev1 for human vaccination were abandoned after studies in volunteers showed that the range between toxic and effective doses was too narrow (Pappagianis *et al.*, 1966; Spink *et al.*, 1962). These data suggest that vaccination of humans with live brucellae leads to protective immunity, but that the vaccines used to date are not sufficiently attenuated. In addition, subcutaneous or cutaneous vaccination may have side effects that can be more severe in previously infected persons.

3. Epidemiology

Brucella can infect humans by a variety of routes. Direct inoculation through the skin is an occupational hazard for slaughterhouse workers and veterinarians. Ingestion of unpasteurized dairy products is a common means of infection worldwide. Aerosolized organisms can infect via the conjunctiva, upper airway, or by infection of macrophages in the lower respiratory tract. While aerosolized bacteria are commonly recognized as the primary threat for biodefense purposes (Kaufmann *et al.*, 1997), they may also be an important cause of naturally acquired infection. Aerosol transmission has been implicated in infection of slaughterhouse workers (Buchanan *et al.*, 1974), laboratory workers after centrifuge tube breakage (Fiori *et al.*, 2000) and production of vaccines (Olle-Goig and Canela-Soler, 1987). *Brucellae* multiply to large numbers in placentas and pose a significant aerosol threat during abortion. Moreover, they can survive for months in soil (Elberg, 1981): aerosolization of infected dust has been suggested as a route of infection in campers in areas contaminated by infected livestock (Mousa *et al.*, 1988) and presumably in animal workers present when animals abort. These epidemiologic considerations need to be taken into account in development of animal models for vaccine development and testing, so proper routes of immunization and challenge can be used.

4. Host-pathogen Interactions in Primary Infection

Brucella survives in the host by a stealth strategy aimed at entering and replicating in mononuclear phagocytes. It produces no exotoxins to induce tissue damage, and has a number of features that minimize induction of danger signals, which might alert the host to its presence (Matzinger, 2002). Many of these features are found on the bacterial surface. Outer membrane lipopolysaccharide (LPS) has low endotoxic activity, so proinflammatory cytokines are not readily released in the early stages of infection when bacterial numbers are low. The long chains of O-polysaccharide (OPS) on the LPS of smooth bacteria fix sufficient serum complement to enhance opsonization (Eze *et al.*, 2000), but are resistant to complement-mediated lysis (Fernandez-Prada *et al.*, 2001). Inside phagocytes, bacteria foster phagosomal acidification (Porte *et al.*, 1999), inhibit the fusion of phagosomes with lysosomes (Naroeni *et al.*, 2001; Pizarro-Cerda *et al.*, 1998; Rittig *et al.*, 2001), remain in the phagosomes and replicate to enormous numbers inside their host cells. Phagosomal maturation in both HeLa cells and J774 murine macrophage-like cells and establishment of the endoplasmic reticulum replicative niche in HeLa cells are controlled by the VirB Type IV secretion apparatus (Comerci *et al.*, 2001; Delrue *et al.*, 2001). Following lysis of infected cells, the bacteria are ingested by other mononuclear phagocytes and the cycle of bacterial proliferation continues. With the development of an effective host response, presumably triggered by a sufficient bacterial load to provide a “danger” signal (Matzinger, 2002), bacterial proliferation is controlled and brucellae are gradually eliminated. The bacteria may, however, persist in their host cells for months or years, and recommence replication if the activity of immunological control mechanisms declines. Before disease is controlled, or perhaps as a consequence of host immune responses infection of mononuclear phagocytes or trophoblasts may lead to disease in almost any organ. In humans, in contrast to livestock, infection of fetus and placenta are or relatively minor importance, while systemic symptoms and local infections account for most morbidity.

A number of host cell activities, including production of reactive oxygen intermediates or nitric oxide (NO), cell-mediated cytotoxicity for infected macrophages, and T cell or T cell subset responsiveness have been shown to have antimicrobial consequences, but an absolute requirement for any one of these activities has not been described for control of primary infection. Moreover, recent work in our laboratory indicates that antibody plays no role in control of a primary *Brucella* infection in mice; the course of infection in B cell knockout mice challenged with *B. melitensis* 16M was identical to that in normal mice (M.Izadjoo, unpublished). On the other hand, certain aspects of the control of murine brucellosis have been established. The single most important factor is IFN-γ. Treatment of mice with anti-IFN-γ antibody exacerbates infection (Zhan and Cheers, 1993) and mice lacking the gene encoding IFN-γ succumb to uncontrolled infection (Murphy *et al.*, 2001). In addition, the acquisition of specific immunity is necessary for control of *Brucella*. Rag-1 mice, which lack B and T cells, but retain natural killer cells and other innate immune responses, allow progressive replication of *Brucella* over at least 20 weeks, while normal C57 animals control the organisms by 8 weeks (Izadjoo *et al.*, 2000). These studies, combined with the observations on B cell knockout mice, suggest that T cell-mediated immunity, with an unkown early contribution of innate immunity, is fundamentally responsible for clearance of *Brucella* after primary infection.

5. Host-pathogen Interactions in Secondary Infection

5.1. Pathogen Recognition and Host Response

While these studies indicate general requirements for recovery from infection, they do not sufficiently address the specific brucellacidal mechanisms that lead to elimination of the organism and ignore the special opportunities afforded to an immune host to protect itself. These opportunities encompass early recognition and enhanced effector mechanisms (whatever these may be) to discover and destroy the stealthy pathogen at an early stage, before it has multiplied sufficiently to cause disease. The first part of this action requires pathogen detection. Recognition of extracellular organsisms can occur via antibody directed against surface structures (predominantly LPS); LPS presented on the surface of infected macrophages (Forestier *et al.*, 2000) may also be a target for antibody. Recognition of intracellular organisms can also occur via T cells that see bacterial peptides on the surface of infected phagocytes in association with class I and class II histocompatibility antigens. In contrast to processes that control recovery from infection, which may operate after the organism has established itself throughout the mononuclear phagocyte system, these recognition activities can occur in the mucosa, submucosa, or in local draining lymph nodes, before widespread dissemination has occurred. In these locations, specific recognition mechanisms mediated both by immune cells and soluble factors interact with natural barriers. Modulation of host response in these areas early after infection may play a profound role in determining the subsequent course of infection and development of disease. Unfortunately, models to explore adaptive immunity are limited and their applicability to interactions of humans and *Brucella* is incompletely understood.

5.2. Animal Models of Adaptive Immunity

Development of models to dissect the interactions of both immune and nonimmune components of protection is complicated by at least two issues. First, there is great variability in the manifestations and pathogenesis of brucellosis among *Brucella* species and their mammalian hosts. Not only do different species of *Brucella* preferentially infect certain hosts, but the manifestations of disease may be quite different when the same strain of *Brucella* infects different hosts. For example, mice infected with *B. melitensis* 16M, a strain highly virulent for humans, do not show signs of distress, although they harbor virulent organisms at high numbers in their spleens for long periods after challenge (Crawford *et al.*, 1996). Goats infected with the same strain also show no systemic symptoms, but have a high frequency of abortion (Phillips *et al.*, 1997). On the other hand, nonhuman primates develop fever, bacteremia and weight loss when challenged via aerosol or conjunctiva with 16M (R.Borschel, unpublished). To complicate molecular approaches to vaccine development, genetically defined mutant strains may also behave differently in different hosts. PHE1, a *htrAcycL* mutant of *B. abortus* 2308, is slightly attenuated at early phases of infection in mice (Elzer *et al.*, 1994a). It is cleared more readily than the parent strain from goats, but causes abortion in these animals (Elzer *et al.*, 1998). In contrast, PHE1 has markedly increased clearance and does not cause abortion in cattle (Edmonds *et al.*, 2000). WR201, a *purEK* mutant of

16M, is attenuated in mice (Crawford *et al.,* 1996) and nonhuman primates, as described below. In goats, its degree of attenuation is less clear. After subcutaneous injection, it colonizes the local draining lymph nodes like 16M and is cleared from them at the same rate, but induces less intense inflammatory response and may have less propensity to cause disseminated infection (Cheville *et al.,* 1996). These observations suggest that small animal models will have some utility in screening potential vaccines, but nonhuman primate studies and early clinical trials will be crucial components of a successful vaccine program.

A second difficulty in model development is the choice of challenge route. Ideally, one should use a mucosal challenge route to model the entry point of *Brucella* in naturally acquired or deliberately delivered infection. Unfortunately, a large body of work examining pathogenesis and vaccine efficacy in laboratory animals has used direct injection of organisms via intravenous, intraperitoneal or subcutaneous routes. Bacteria injected by these routes bypass physical barriers and do not infect mucosal dendritic cells and macrophages, which are probably the first cellular targets of natural bacterial infection. Moreover, the tempo of disseminated infection is different, so bacteria arrive in the spleen and liver within one day of intraperitoneal or intravenous challenge, but may require a week to disseminate to these organs after intranasal challenge (Mense *et al.,* 2001). Shortening the time to dissemination and bypassing mucosal barriers may lead to inaccurate interpretation of vaccine efficacy. The historical failure to use mucosal challenge routes in laboratory animals is surprising, since these approaches have been used effectively in studies on livestock. In large food animals, conjunctival inoculation of brucellae leads to disseminated infection of mammary glands and lymph nodes in non-pregnant animals; in addition to these sites, the uterus and placenta of pregnant animals are infected (Corner *et al.,* 1987; Meador and Deyoe, 1986). Both conjunctival and oral challenge routes have proven useful to determine vaccine efficacy (Fensterbank *et al.,* 1982; Fensterbank *et al.,* 1985; Nicoletti and Milward, 1983; Plommet and Plommet, 1975). In other laboratory animal studies, conjunctival challenge of badgers (Corbel *et al.,* 1983) and beagles (Carmichael *et al.,* 1984) leads to disseminated infection.

5.3. Mechanisms of Vaccine-Mediated Immunity

Despite these two drawbacks, animal models, mostly focused on *B. abortus,* have provided insight into mechanisms that may play a role in protection against natural challenge infection in immune hosts. Perhaps surprisingly for an intracellular pathogen, *Brucella* appears to be susceptible to antibacterial antibody. Immunization of mice with killed, smooth strains of *B. abortus* or *B. melitensis* reduces the number of *B. abortus* CFU in spleen or liver when mice are challenged i.v. or i.p. (Montaraz and Winter, 1986; Pardon and Marly, 1976a; Pardon and Marly, 1976b). Cell lysate fractions that induce anti-LPS antibodies also reduce intensity of spleen and liver infection in animals challenged with smooth strains of *B. abortus* or *B. melitensis* i.v. or i.p. (Dubray and Bezard, 1980; Jacques *et al.,* 1991; Phillips *et al.,* 1989; Plommet and Plommet, 1989; Pugh *et al.,* 1990; Tabatabai *et al.,* 1992). These effects on the intensity of hepatic and splenic infection are most prominent 1–2 weeks after challenge. Studies using monoclonal antibodies confirmed that anti-OPS antibodies mediate these antibacterial effects (Limet *et al.,* 1987; Montaraz *et al.,* 1986). In contrast, monoclonal antibodies

directed against outer membrane proteins have little effect on spleen infection by smooth strains (Jacques *et al.*, 1992), but are highly effective against infection by *B. ovis* (Bowden *et al.*, 1995), a naturally rough organism that lacks surface OPS. The mechanism of the effect of anti-OPS antibody on transiently reducing infection of liver and spleen may reflect redistribution of organisms to lymph nodes and killing of bacteria in the nodes. Sulitzeanu demonstrated that active immunization with live or dead brucellae, passive transfer of immune serum, or pre-coating of bacteria with immune serum leads to redirection of i.p.-administered ^{131}I-labeled brucellae away from liver and spleen and into mesenteric lymph nodes (Sulitzeanu, 1959). When live bacteria are injected i.p. in passively immunized mice, however, the number of live bacteria in mesenteric lymph nodes is greatly reduced in immune mice compared to nonimmune animals (Sulitzeanu, 1965). In other studies, treatment with immune serum or immunization with live or dead brucellae restricted bacteria to the draining popliteal lymph node when low numbers of bacteria were injected subcutaneously into the footpads of mice (Pardon, 1977) or guinea pigs (Pardon and Marly, 1978), and inhibited the growth of bacteria in the node.

These data do not clarify whether antibody works solely by redirecting bacteria to a site that may have enhanced "natural" or innate antibrucella activity compared to other organs of the mononuclear phagocyte system, or if it sensitizes the bacteria to the antibrucella activity of the nodes or enhances lymph node antimicrobial capability. In vitro studies have not resolved this issue. Opsonization of brucellae by antibody or antibody plus complement enhances ingestion but does not inhibit the intracellular growth of bacteria in resting macrophages. On the other hand, opsonization enhances the antibrucella activity of macrophages activated by IFN-γ (Eze *et al.*, 2000; Gross *et al.*, 1998; Jones and Winter, 1992). It is possible that, in a lymph node, NK cells or T cells that respond to relatively nonpolymorphic antigens might provide sufficient IFN-γ to activate macrophages to inhibit the replication of opsonized brucellae, but this issue has not been addressed experimentally. It is likely, however, that an optimal protective response to *Brucella* challenge requires both anti-OPS antibody and specifically sensitized T cells that produce large amounts of IFN-γ.

A number of studies using passive transfer of immune cells or serum have demonstrated a role for cellular immune effectors as mediators of antibrucella activity. Pavlov *et al.* (1982) demonstrated that adoptively transferred $Ly1^+2^+$ (i.e., CD8) cells reduce the number of splenic bacteria following i.v. challenge with *B. abortus* strain 19. Araya found that both CD4 and CD8 immune T cells and serum obtained during the course of infection with strain 19 mediate approximately equivalent levels of protection against challenge with strain 19 in mice (Araya *et al.*, 1989). Plommet *et al.* (1986) demonstrated that spleen cells obtained from mice immunized with a peptidoglycan fraction of *Brucella* protect against challenge with virulent *B. abortus* 544. Furthermore, they showed that immune serum is at least as efficacious as immune cells, and serum and cells do not have additive or synergistic protective effects. Immune serum is less effective against *B. abortus* strain 2308 than against strain 19, while immune T cells preferentially protect recipients against challenge with the same strain used to infect donor mice (Araya and Winter, 1990). These studies suggest that immunization strategies that induce either antibody responses or sensitization of T lymphocytes will have anti-*Brucella* effects. Data from our own group, discussed later in this chapter, suggest that induction of both T

cell and antibody responses may be better than antibody alone for protection against intranasal challenges.

The success of a number of different vaccination strategies in livestock supports this concept. Early adjuvanted, whole, killed cell vaccines induced good antibody responses and provided effective protection. However, they also caused severe local reactions and had a limited duration of immunity. Although the ability of these vaccines to induce protein antigen-specific T cell responses is unknown, it is likely that the primary basis of protection lay in induction of anti-OPS antibody. Live, attenuated vaccines based on smooth bacteria (*B. abortus* strain 19 and *B. melitensis* Rev1) supplanted killed vaccines because of reduced side effects and longer-lasting immunity (Nicoletti, 1990). Like killed vaccines, these live vaccines elicit antibody that renders vaccinated animals positive in the serologic tests used to screen herds for brucellosis. They also induce lymphoproliferative responses to *Brucella* antigens that may contribute to vaccine efficacy (Kaneene *et al.*, 1978).

Recently, a highly attenuated rough organism, *B. abortus* strain RB51, has replaced strain 19 for cattle vaccination in the United States. RB51 is deficient in *wboA*, which encodes a glycosyl transferase, an enzyme required for synthesis of OPS. This vaccine elicits lymphoproliferative responses to *Brucella* protein antigens (Stevens *et al.*, 1996) but does not usually induce sufficient antibody to interfere with veterinary diagnostic tests for brucellosis. Since RB51 may express small amounts of OPS (Cloeckaert *et al.*, 2002) and may induce low levels of antibody, however, it is difficult to exclude the possibility that anti-*Brucella* antibody plays a role in protection of livestock immunized with it. Indeed, complementation of the *wboA* defect of RB51 leads to creation of a rough strain (RB51WboA) with unchanged survival, but which elicits antibody and enhanced antibrucella activity in mice (Vemulapalli *et al.*, 2000).

In mice, i.v. administration of live, attenuated vaccines derived from smooth or rough *Brucella* reduces spleen infection after i.v. challenge with smooth, virulent homologous or heterologous strains (Winter *et al.*, 1996). Smooth strains (Rev1 or 19) are more effective vaccines, even though persistence of rough and smooth strains in the mice is similar (Winter *et al.*, 1996). In these same studies, RB51, given i.p., was less effective, probably because it persisted for a shorter period of time; killed vaccines were only marginally effective. These data further support the notion that anti-LPS antibody and T-cell-mediated immunity cooperate for maximal vaccine efficacy.

6. Nonhuman Primate Models and Studies with Live, Attenuated Strains of *B. melitensis*

As part of his work with the Mediterranean Fever Commission, Shaw (1907) reported the first studies of nonhuman primates challenged with *Brucella*. His finding that monkeys were susceptible to intranasal infection with *B. melitensis* only after repeated exposures led to the incorrect conclusion that monkeys had reduced sensitivity to infection. In later experiments, Fleischner and Meyer (1920) found that *B. abortus* and *B. suis* could cause disease monkeys similar to that described by Shaw with *B. melitensis*, although monkeys still required multiple applications of bacteria to establish an infection and some were never infected.

Huddleson and Hallman (1929), using freshly isolated organisms instead of multipassage laboratory isolates, were able to establish acute brucellosis in rhesus macaques. *B. abortus* was the least infective, with only 2 of 8 animals developing brucellosis, while all animals receiving *B. suis* and *B. melitensis* became infected. Infected animals developed positive serology and organisms were isolated from blood. At necropsy, cultures of the lung, liver, spleen, kidneys, and heart blood also yielded bacteria. Although fever was frequently observed, it was difficult to evaluate because only morning and afternoon measurements were taken. The authors concluded that all three *Brucella* species that cause brucellosis in humans can also establish disease in rhesus macaques. *B. abortus* was the least virulent, followed by *B. melitensis*, then by *B. suis*, the most virulent. Aerosol challenge models were later developed and applied to monkeys. Henderson (1952), challenged rhesus macaques with *B. melitensis* strain 6015 and found that the ID_{50} was 1.3×10^3 organisms (95% confidence interval=$1.2–1.5\times10^3$ organisms) (Elberg *et al.*, 1955). These studies laid the foundation for testing of potential human vaccines.

B. melitensis Rev1, which is now the vaccine of choice to prevent caprine brucellosis, was originally developed circuitously as a candidate vaccine for humans. An avirulent, naturally occurring mutant of *B. melitensis* that was dependent upon streptomycin for growth (Herzberg and Elberg, 1953) was first suggested as an ideal vaccine candidate, since it should be avirulent in vaccinees, who would lack the antibiotic. The streptomycin-dependent mutant strain was developed from a single colony of *B. melitensis* strain 6056 and selected for growth in the presence of high concentrations of streptomycin. Only 2 per 1×10^8 streptomycin-dependent organisms reverted to streptomycin susceptibility in vitro. Guinea pigs inoculated with 1×10^{11} dependent organisms did not develop brucellosis up to 4 weeks after injection. Mice remained infected for 2 weeks, but had largely eliminated the organism from their spleens by 4 weeks. Administration of 1×10^6 bacteria intravenously or 1×10^{10} cells subcutaneously to rhesus monkeys led to bacteremia 10 days later, but no bacteremia at 14 and 25 days (Herzberg and Elberg, 1953).

To determine more accurately the ability of the streptomycin-dependent mutant to survive in monkeys, 13 rhesus macques were inoculated subcutaneously with 1×10^{10} CFU of the streptomycin-dependent mutant and sacrificed up to 45 days later for culture of tissues (Elberg *et al.*, 1955). At 14 days, but not thereafter, organisms were recovered from the inguinal lymph node draining the injection site and from spleen, liver and axillary lymph node in all animals. Bacteria were cleared from most sites by 21 days. No organisms were recovered from blood cultures obtained weekly for 45 days. None of the mutant organisms recovered from the tissues had reverted to streptomycin independence. A 3–4 day febrile response that began within 72 hours post-injection was seen in animals that received live mutant organisms but not in those that received killed organisms. Two animals were reinoculated with 1×10^{10} CFU of the mutant strain 21 days after the initial injection and organisms were recovered only from the inguinal lymph nodes at 11 days and only from the spleen at 21 days. This experiment indicated that primary infection limits the proliferation and dissemination on rechallenge with a homologous strain of *Brucella*.

Initial enthusiasm for the streptomycin-dependent mutant vaccine was supported by avirulence, antibody production and protection in mice, although there was little

protection in guinea pigs (Herzberg and Elberg, 1953). This enthusiasm was soon tempered, however, when mice (Herzberg and Elberg, 1955) and rhesus monkeys (Elberg *et al.,* 1955) inoculated with steam-killed organisms also demonstrated appreciable serological titers to *Brucella* and were equally, but only partially, protected by live and killed bacteria. This finding led the investigators to conclude that the vaccine mutant must replicate in the host in order to confer complete immunity. Herzberg and Elberg also noted that some of the streptomycin-dependent *B. melitensis* strains that had reverted to streptomycin independence and sensitivity to killing by streptomycin were attenuated for growth and protective against challenge infection in guinea pigs and mice (Herzberg and Elberg, 1953; 1955). After it was found to be attenuated and immunogenic in goats (Elberg and Meyer, 1958), it became the vaccine of choice for these animals. The nature of the mutation(s) in Rev1 is still unknown.

Elberg and Faunce (1962) subsequently examined the attenuation and protective efficacy of Rev1 in *Cynomolgus phillippinensis* monkeys. Rev1 administered subcutaneously to monkeys at 1.8 X 10^9 CFU led to bacteremia that persisted for up to 4 weeks, infection of spleen and liver for up to 4 weeks, and infection with less than 10 CFU/lymph node for up to 8 weeks. In a second series of experiments, animals were given 10^4,10^6 or 10^8 CFU. The frequency of bacteremia was greatest (5/5 and 4/5 animals) in the two higher dose groups, compared to 2/5 animals in the lowest dose group. Interestingly, the duration of bacteremia was longer in the lowest dose group (60 and 62 days) compared to the highest dose group (8/9 animals with bacteremia from 20–34 days, 1 with bacteremia for 44 days). Spleen, liver and deep cervical, inguinal and axillary nodes were consistently positive for up to 44 days in the second series of experiments, with some animals positive in the nodes for up to 117 days. Monkeys in the first series and the high dose vaccinees in the second series also developed fever.

Monkeys from the first series of experiments were challenged with strain 6015 administered subcutaneously at graded doses ranging from 1×10^3 to 1×10^6 CFU. Unvaccinated controls received challenge doses of either 10^2 or 10^3 CFU. When examined 43–52 days after challenge, all 9 control monkeys had heavy infection in liver, kidney, spleen and nodes. In contrast, only 1 of 20 immunized animals had bacteria in liver, kidney or spleen. No bacteria were recovered from 11 of 20 immunized animals, and these were present in low numbers in one or two lymph nodes. One animal had persistent infection of multiple nodes with the vaccine strain (Elberg and Faunce, 1962).

Monkeys from the second series were exposed to the challenge strain via the respiratory route. The aerosol challenge dose consisted of either 1×10^8 strain 6015 for the vaccinates or 1×10^4 for the controls. Even using a large challenge dose, significant reduction in the challenge organisms was observed. More importantly, even when the vaccine dose was reduced 10,000 fold there was only a slight reduction in the clearance capacity (Elberg and Faunce, 1962). These important studies demonstrated that immunization with a live, attenuated vaccine could protect primates against both subcutaneous and aerosol challenge with virulent organisms, but also reflected the potential of Rev1 to persist in the lymph nodes.

Chen and Elberg (1970) further characterized immune response and protection with orally administered Rev1 in *Cynomolgus* monkeys. Animals given 3.3×10^7 CFU by force feeding oral capsules did not become bacteremic, while those given 3.3×10^9, and 3.3×10^{11} did. All except the lowest dose resulted in anti-*Brucella* antibody. After

subcutaneous challenge with 1.6×10^3 CFU of 6015, challenge strain organisms were recovered from 1/2 animals in the high and low dose groups and 0/2 in the middle dose group. In another study, 3 monkeys were immunized subcutaneously with 5×10^9 CFU of Rev I. Two of the 3 became bacteremic. Animals were challenged subcutaneously with 6015 12 weeks after immunization. Six weeks after challenge, no challenge organisms were recovered at necropsy from tissues of any of the 3 monkeys. An additional 3 animals were given 2 doses of Fraction I (a soluble protein extract of heat killed, dried Rev1) emulsified 1:1 with adjuvant 65 (86% peanut oil, 10% mannide monooleate, and 4% aluminum monostearate). Compared to live Rev1, Fraction I elicited lower antibody titers and showed no protective efficacy. These studies suggested that vaccination with live organisms was effective, but vaccination with a product that elicited antibody was not. Interpretation of these data is complicated by the virulence of the live vaccine and the low antibody titers elicited by injection of the bacterial fraction.

In a subsequent study (Chen and Elberg, 1973), these same authors examined pre-immunization with Fraction I followed by live vaccination with lower doses of Rev1, administered intradermally or cutaneously. Only 1 of 20 animals so immunized developed Rev1 bacteremia. In challenge studies on immunized aimals, no organisms were recovered from 4 of 5 animals challenged with 925 CFU of 6015. In a second experiment, no organisms were recovered from 7 of 8 animals challenged with 6625 CFU and none were recovered from 5 of 8 animals challenged with 20,600 CFU. The 3 animals with positive cultures in the latter group had lower intensity of infection compared to nonimmunized controls. The authors proposed that a two-stage immunization strategy such as that used in their studies might overcome the difficulties of vaccine virulence.

These vaccine studies in nonhuman primates provided important benchmarks for vaccine safety and efficacy. First, they demonstrated, as inferred from human studies, that killed vaccines have little efficacy, even though they may induce antibody. Second, they established systems to evaluate safety of live, attenuated vaccines and demonstrated that live Rev1 conferred impressive protection against challenge, consistent with the Soviet field experience with strain 19 human vaccine. Unfortunately, since studies of Rev1 in volunteers described above showed that it was even more virulent than the Soviet live, attenuated vaccine, Rev1 was discarded as a potential candidate.

7. Development of New Models and Genetically Defined, Live, Attenuated Vaccine Candidates

7.1. Murine Intranasal Challenge Model

In 1992, renewed concern about the biowarfare threat of brucellosis prompted additional studies focused on developing a genetically defined, live, attenuated vaccine that would protect against aerosol infection with *Brucella*. To avoid the interpretive difficulties raised by the use of nonmucosal challenge routes, we first developed a murine intranasal challenge model using *B. melitensis* 16M. Administration of 10^3 CFU of 16M intranasally to anesthetized BALB/c mice leads to disseminated infection of liver and spleen in 50% of animals by 2 weeks. A dose of 10^4 CFU leads to 100% infection of

lungs, but no apparent illness and no pneumonia (Mense *et al.*, 2001). Bacteria are gradually eliminated from the lungs; approximately 60% of animals still have pulmonary infection after 8 weeks (Izadjoo *et al.*, 2004). At this dose, 16M disseminates to cause 90–100% splenic infection, which persists without clinical evidence of disease for months. Splenomegaly and increased splenic white pulp and marginal zones occur, as well as a mononuclear cell hepatitis.

7.2. Construction of WR201 and Testing in Mice

The confinement of brucellae to phagosomes, where amino acids and nucleotides are limited, suggested that creation of mutations in genes encoding biosynthetic pathways for nutrients might lead to reliably attenuated live vaccines. Our general approach in the creation of new live vaccine candidates was to attenuate *Brucella melitensis*, one of the most virulent biovars for humans, by introducing defined nonreverting mutations into targeted genomic loci. At the start of our effort in the early 1990s, little was known about the contributions of individual genes to *Brucella* pathogenesis and *Brucella* genomes were yet to be sequenced. Since our mission was to develop candidate vaccines against brucellosis as expeditiously as possible, we chose genes as attenuation targets based on the experience of vaccinologists working with other live attenuated bacterial pathogens such as *Salmonella*. The strategy was simple: clone *Brucella* homologs of selected attenuation targets, introduce sizeable deletions into these cloned genes, then exploit innate homologous recombination to exchange deleted alleles with the wild type loci in the genome of *Brucella melitensis* strain 16M. The first mutant developed with this rationale targeted the de novo purine biosynthesis pathway. *B. melitensis* WR201 was made from 16M by replacement of most of *purE*, an intergenic region, and the first 7 bp of *purK* with a kanamycin resistance cassette (Drazek *et al.*, 1995). WR201 shows the expected purine auxotrophy when cultured on minimal medium. Moreover, in contrast to its parent 16M, WR201 fails to grow in human monocyte-derived macrophages, while 16M increases approximately 100-fold in 72 hrs (Drazek *et al.*, 1995).

WR201 is also markedly attenuated for growth in BALB/c mice. After i.p. injection, it initially replicates in the animals, with increasing numbers of CFU in the spleen for up to 3 days. The number of organisms in the spleen then begins to decline and bacteria are nearly completely cleared from liver, lungs and spleens by 8 weeks (Crawford *et al.*, 1996). Mice immunized i.p. with WR201 develop anti-LPS antibody and antigen-specific production of IL-2 and IFN-γ, but not IL-4, by cultured spleen cells (Hoover *et al.*, 1999b). These data suggest that immunization with this mutant leads to a Th1 immune response. In accord with this observation, intraperitoneal immunization of mice with WR201 reduces dissemination of intranasally administered 16M and slightly enhances clearance of the virulent organism from the lungs (Hoover *et al.*, 1999b). At a challenge dose of 10^4 CFU of 16M, protective efficacy against dissemination ranged from 50–70%. Recently, WR201 was found to be slightly more attenuated and protective when given orally to mice (Izadjooet *et al.*, 2004). Parallel studies examined the ability of vaccines that elicit antibody against LPS to protect in this model. LPS prepared from both *B. melitensis* and *B. abortus* was given intranasally to mice as a noncovalent complex with outer membrane protein from *Neisseria meningitidis* group B, an excellent adjuvant for polysaccharide antigens. This LPS-GBOMP vaccine elicits both systemic and mucosal

antibody (Van De Verg *et al.,* 1996) against *Brucella* LPS. Anti-LPS IgG, primarily of IgG1 and IgG3 subclasses, persists for at least 7 months in blood. Like animals immunized with WR201, mice immunized with LPS-GBOMP vaccine are protected from dissemination to liver and spleen after intranasal challenge with 16M (Bhattacharjee *et al.,* 2002). In contrast to those immunized with the live vaccine, however, they do not clear the challenge organisms more rapidly from their lungs. Passive transfer of *Brucella*-immune serum to mice challenged intranasally recapitulates these findings (M.Izadjoo, unpublished). These studies indicated that vaccines that elicit antibody alone, in animals with an intact immune system, may be sufficient for inhibition of dissemination, but may not enhance destruction of bacteria at the site of entry. These data do not exclude a participatory role for non-antibody-mediated responses, however. Indeed, administration of *Brucella*-immune serum to RAG-1 knockout mice, which lack mature B and T cells, fails to modify the course of infection following intranasal or intraperitoneal challenge with 16M (M.Izadjoo, unpublished). These studies indicate that antibody must cooperate with cellular immune components to protect mice against respiratory challenge.

7.3. Testing of WR201 in an Aerosol Challenge Model in Rhesus Macaques

The encouraging findings with WR201 in the mouse model suggested that this live, attenuated vaccine should be tested in nonhuman primates. Building on the previous experience with brucellosis in nonhuman primates and the ready availability of immunological reagents for *Macaca mulatta*, we initiated studies aimed at developing aerosol and mucosal challenge models using *B. melitensis* 16M. The first experiment (R.Borschel, unpublished) was designed to test the ability of 16M to cause acute brucellosis in rhesus macaques. Studies in 5 pairs of animals established an ID50 of approximately 10^2 CFU as measured by bacteremia. The day of onset of bacteremia was inversely related to the aerosol dose. Interestingly, 2 challenge controls, housed upwind of the other animals in a room insert with directional airflow, also had evidence of infection. One control animal had a single positive blood culture and the other developed significant antibody titers. It is likely that these infections represent contamination from the fur of monkeys exposed to aerosols, despite thorough air washing of fur after challenge (Kruse and Wedum, 1970).

Following the initial studies, 4 additional animals were challenged via aerosol with 1×10^7 organisms. Animals were monitored telemetrically using temperature recording devices implanted subcutaneously (R.Borschel, unpublished). All became bacteremic within 14 days; 2 animals were positive by 7 days. Fever developed in 2 animals beginning by 10 days. The other 2 animals became febrile by 35 days post-exposure. Histopathologic studies on the animals from these first two experiments showed inflammation in liver, kidney, spleen testis and epididymis of animals exposed to 16M (Menseet *et al.,* 2004). Spleens were enlarged, with increased ratio of white to red pulp. This ratio was proportional to the challenge dose. In addition to histologic evidence of hepatitis, serum gamma glutamyl transferase and alkaline phosphatase levels rose during the course of infection in challenged animals.

To determine whether WR201 was attenuated in monkeys as well as in mice, rhesus macaques were given 10^{12} CFU of 16M or WR201 orally by gavage, followed for 4

weeks, then necropsied (R.Borschel, unpublished). Animals given 16M became ill, decreased their food intake, and lost weight. Animals given WR201 appeared clinically normal. Serial quantitative blood cultures disclosed more than 10-fold higher CFU of 16M than WR201. Blood cultures remained positive until necropsy in animals given 16M, but became negative after 3 weeks in those given WR201. At necropsy, animals given 16M had extensive infection of spleen and lymph nodes, which had colony counts 30-fold higher than counts in nodes from animals given WR201.

In another study (R. Borschel, unpublished), monkeys were given WR201 at the same dose and route and challenged via aerosol with 10^7 CFU of 16M 9 weeks later. Four monkeys were necropsied one week before challenge and were found to have a mean of 30 CFU of WR201 in lymph nodes. All 4 nonimmunized monkeys challenged with 16M became bacteremic and febrile; none of 4 immunized animals did so. At necropsy 8 weeks after challenge, 16M was recovered from nodes and other tissues of nonimmunized animals, but no 16M were recovered from immunized monkeys. Surprisingly, one colony of WR201 was recovered from a testis in one animal and one colony recovered from a lymph node of another animal.

These data indicate that WR201, while highly attenuated and protective against aerosol challenge with 16M in monkeys, may not be sufficiently attenuated for administration to humans. It is also possible, however, that the prolonged tissue persistence we observed may be due to the high oral vaccine dose. A lower dose may result in less vaccine persistence and permit use of WR201 in future trials.

7.4. Implications of Experience with WR201

Three encouraging conclusions emerged from the murine and nonhuman primate studies with WR201. First, purine auxotrophy of *Brucella* is associated with a substantial degree of attenuation in two different animal species. Second, administration of this live vaccine leads to a combined humoral and cellular immune response to *Brucella*. Third, the vaccine provides sterile immunity and protection from disease when monkeys are challenged by aerosol at approximately 10^5 times the ID50. These conclusions further suggest that purine auxotrophy may be an excellent foundation for development of live, attenuated *Brucella* vaccines. Deletion of purine synthesis genes may be a more effective basis for attenuation in *Brucella* than in other facultative intracellular bacteria because of differences in rate of bacterial replication and location in the host. For example, *Shigella,* which is a rapid-growing organism, is not confined to the phagosome, but replicates in the cytoplasm. *Shigella purE* mutants are fully virulent (Formal *et al.,* 1971). *Salmonella,* like *Brucella,* is confined to phagosomes once ingested by macrophages. In contrast to *Brucella,* however, *Salmonella,* like *Shigella,* are fast growers. Hypoxanthine auxotrophic mutants of *S. typhimurium* are avirulent when injected into mice at low numbers, but not at high numbers (McFarland and Stocker, 1987). Presumably, at high numbers, salmonellae can grow fast enough extracellularly to overwhelm the immune system before the bacteria are ingested and trapped inside the barren phagosome. *Brucella,* on the other hand, has little chance of dividing before it is ingested and sequestered in a phagosome. These unique characteristics of *Brucella* may permit development of a vaccine based on purine or other metabolite auxotrophy.

7.5. Development and Testing of Rough Mutants and Rough Purine Auxotrophs

We initially believed that an engineered live attenuated *Brucella* vaccine for humans that lacked OPS would be valuable to minimize vaccine-induce seropositivity and maintain the usefulness of anti-LPS serologic tests. Winter *et al.* (1996) showed that transposon interruption of the *wboA* locus in *B. abortus, B. melitensis* and *B. suis* resulted in a rough phenotype and attenuation in mice. That group also demonstrated protection of mice against intraperitoneal challenge with virulent *Brucella* after immunization with these rough *wboA* mutants. We replaced the genomic *wboA* locus in strain 16M with an allele of the gene with 607 bp removed using internal *Cla*I sites and replaced with a chloramphenicol resistance cassette. This rough *wboA* deletion mutant, designated WRR51, was taken up more readily than smooth bacteria, but, like WR201, failed to replicate in human macrophages. Intraperitoneally administered WRR51 was distributed in spleens, livers and lungs in numbers trending lower but not significantly different from those of strain WR201, which was included as a benchmark of attenuation. However, the *wboA* mutant did not exhibit the spike of replication in the spleen at one week seen with both strain WR201 and virulent control strain 16M (Crawford *et al.,* 1996). Strain WRR51 persisted in these organs for a comparable duration to strain WR201, though the latter persisted in the spleen in low numbers at 12 weeks in one experiment, while strain WRR51 was repeatedly cleared from the spleen before 12 weeks. Strain WRR51 was also eliminated from livers and lungs by four weeks, while strain WR201 was still present at these time points (M.Nikolich, unpublished).

The *purEK* deletion was introduced into strain WRR51 to create a more attenuated rough, purine auxotrophic mutant, WRRP1. Not only did WRRP1 fail to replicate in human macrophages, it actually decreased in viable count by nearly one log over 72 hours in multiple experiments (M.Nikolich, unpublished). Moreover, this dual mutant was severely attenuated in the mouse, persisting in spleen, liver and lungs in very low numbers (over three logs fewer CFU in spleens than strain WR201 at three days) and consistently cleared from these organs in less than two weeks. In the mouse intranasal challenge model, strain WRRP1 provided no measurable protection against dissemination to the spleen, while strain WRR51 provided only a modest protective effect (20% protection versus the 70% protection provided by strain WR201 in the same model). These results indicated that the presence of the O-side chain component of LPS might be essential to elicit protective immunity. These data are consistent with the notion that the success of strain RB51 as a cattle vaccine may partially depend on its low level expression of this immunogen.

7.6. Development and Testing of *wboA*-Complemented Rough Purine Auxotrophs as a Vaccine Delivery Platform

The severe attenuation exhibited by the *wboA purEK* dual mutant WRRP1, while unpromising as a live human vaccine in itself, presented an interesting potential for further development as a vaccine platform. We believed we could exploit the crippling nature of the combination of mutations in strain WRRP1 to maintain replicative plasmids within mammalian vaccinees by including *trans* complementation of the rough mutation on the plasmid. This mechanism to provide an in vivo selection for a replicative plasmid

could be used to efficiently deliver a variety of plasmid-encoded antigens into host antigen presenting cells, including protective antigens from other pathogens (particularly intracellular pathogens), cancer antigens or even homologous antigens to enhance protection against *Brucella*. We demonstrated that this vaccine platform concept is practicable by expressing GFP in strain WRRP1 on a replicative *wboA*-complementing plasmid (M.Nikolich, unpublished). This complemented strain, designated WRRP1cg, was smooth, green, and behaved like WR201 in cultured human macrophages. In two different experiments, when WRRP1cg was introduced orally into male mice at 10^{11} CFU, it persisted in the spleens for six weeks at lower numbers than typically seen for strain WR201 introduced by the same route. The characteristic spike in replication at one week in the spleen was also observed with strain WRRP1cg. Strain WRRP1cg was found in testis and epididymis only in the first week. Indeed, overall systemic dissemination of strain WRRP1cg appeared to be significantly reduced in comparison with strain WR201. Uncomplemented WRRP1 introduced in the same manner in these experiments persisted at barely detectable levels in the spleen up to two weeks, and was recovered from the lungs, but no other tissues, only at three days.

These experiments indicate that *wboA* complementation of WRRP1 can be used as a means to maintain an expression plasmid in vivo that can be used to express heterologous proteins. Approximately 98% of the WRRP1cg colonies recovered from mice in these experiments fluoresced green, indicating their GFP expression, and retained a smooth phenotype. The small subset of colonies that did not manifest these phenotypes also had lost ampicillin resistance encoded on the plasmid, indicating that some plasmid loss occurred even with the in vivo selection. We confirmed that the plasmid was also gradually lost from strain WRRP1cg in vitro, and was eventually lost completely after serial passage in broth culture over 45 days. Perhaps this gradual loss of plasmid, resulting in reversion of the strain to the severely attenuated, rough purine auxotrophic WRRP1, accounts for the reduced persistence and dissemination of WRRP1cg in mice relative to WR201. This slight enhancement of attenuation is a fortuitous, but desirable attribute, since we believe additional attenuation of this genetic background may enhance safety for humans. This complementation approach may not only provide a useful platform for heterologous antigen presentation, but may also be a highly advantageous strategy in itself for live vaccination against brucellosis.

7.7. Development and Testing of *hfq* Mutants

In further studies, we collaborated with Dr. Roop's laboratory to develop and test strains with deletions in *hfq*, which encodes the *Brucella* homolog of Host Factor I, an RNA-binding protein involved in the regulation of genes involved in stationary phase survival. Robertson, *et al.* noted that deletion of *hfq* significantly attenuates *B. abortus* in murine macrophages and mice (Robertson and Roop, 1999). *B. melitensis hfq* mutants are attenuated in mice and goats (Roop *et al.*, 2003). We introduced the same mutation into 16M in our laboratory and found the resulting strain, designated MNPH1, to be at least as inhibited in its ability to survive in human macrophages as was strain WR201 (M.Nikolich, unpublished). Moreover, MNPH1 was slightly more attenuated than WR201 after oral introduction into BALB/c mice, with more rapid clearance from the spleen, liver and lungs, but was also less immunogenic. While administration of a single

oral dose of 10^{10} or 10^{11} CFU of WR201 protected mice against intranasal challenge with 16M, two doses of 10^{11} CFU of MNPH1 were required for similar efficacy (M.Izadjoo, unpublished). When tested in nonhuman primates, MNPH1 was immunogenic and partially protective against conjunctival challenge with 16M. In addition, it did not disseminate to the male reproductive organs (R.Borschel, unpublished). We do not know the basis of its reduced immunogenicity and protective efficacy, which are less than expected on the basis of its persistence in experimental animals. It is possible that interruption of a global regulatory gene like *hfq* leads to reduced expression of bacterial antigens that are important for generation of an effective host immune response. If so, global regulatory genes may be less attractive targets than genes with narrower effects.

7.8. Additional Mutants with Purine Auxotrophy

Early clinical trials will be an important component of further research efforts with recombinant live, attenuated vaccines for humans. Although we anticipate that the nonhuman primate model will have more predictive power than murine or caprine models, its actual value will remain unknown until well-defined strains can be directly compared in all these systems. Moreover, definition of in vitro immune correlates of protection by comparison of human and nonhuman primate or other models will be crucial steps in advancing development of live vaccines. Because of these uncertainties, we are developing additional candidates by introducing secondary mutations that retain smoothness into the *purEK* background of strain WR201 (M.Nikolich, unpublished). We constructed MNP54, an unmarked variant of strain WR201, using allelic exchange with the *Bacillus subtilus* levansucrase-encoding *sacB* as a counterselectable marker and have added a number of secondary mutations to the MNP54 background. One of the promising dual mutants generated by this approach has a secondary deletion mutation in *hfq* and is designated MNPH3. This *purEK hfq* double mutant appeared to be at least as impaired in its ability to replicate in human macrophages as either strain MNP54 or strain MNPH1. Interestingly, MNPH3 was no more attenuated than MNPH1 in mice. It may be safer, however, due to the second, “backup” mutation. These attenuation results emphasize that creation of multiple deletions may not result in reduced survivability. At present, there is no obvious method to determine which combinations of mutations will be additive, antagonistic or indifferent for affecting survival of *Brucella*. It is likely that numerous empiric attempts will be required to develop optimally attenuated candidates. Creation of these strains may also permit the design of algorithms to predict interactive effects.

7.9. Other *Brucella* Mutants

7.9.1. Mass Screening Approaches

Fortunately, numerous additional potential targets for deletion have been described in *Brucella*. The powerful technique of signature-tagged mutagenesis has been used to identify mutants of *B. suis*, *B. abortus*, and *B. melitensis* that are attenuated for survival in mononuclear phagocytes or mice. Foulongne *et al.* (2000) detected mutants of *B. suis* with reduced survival in THP1 and HeLa cells. Mutations were found in 14 different genes, including the VirB Type IV secretion system (3 genes), LPS biosynthesis,

nucleotide biosynthesis, and metabolism of nitrogen and glucose metabolism. Hong *et al.* (2000) found that 13 *B. abortus* mutants with defects in genes encoding aromatic amino acid biosynthesis, LPS biosynthesis and the VirB region were not recovered 2 weeks after injection into mice, but a mutant with a defect in glycine dehydrogenase, an enzyme induced during stationary phase, survived for a period between 2 and 8 weeks. Lestrate *et al.* (2000) found 18 attenuated mutants of *B. melitensis,* including one with a mutation in *virB* and others with mutations in transporters, amino acid and DNA metabolism, a two-component regulatory system, and a stress protein. Interestingly, no mutants were described with defects in LPS synthesis.

Two efforts have used mini-Tn5 transposon libraries to detect mutations that impair entry and replication in cultured target cells. Kohler *et al.* (2002b) screened a library of *B. suis* in the human macrophage-like cell line THP-1 and identified 133 mutants with defects in 59 different genes. Of these, a relatively large number were involved in amino acid synthesis and others were involved in the pathways found by the signature tagged mutagenesis technique. This study was supported by results obtained by Kim *et al.* (2003) who screened a mini-Tn5 transposon library of *B. abortus* for entry and replication in HeLa cells. They identified 44 mutants, most of which were in the *virB* operon or metabolism of purines and pyrimidines.

7.9.2. Extensively Characterized Attenuated Mutants

These mass screening approaches have provided a wealth of genes to examine more fully both for studies of pathogenesis and for development of attenuated vaccine candidates. Table 1 shows more extensively characterized single gene deletion mutants that have reduced survival of *Brucella* in macrophages or animals. Attenuating mutations represent genes encoding LPS biosynthesis, the VirB pathway, synthesis of purines and aromatic compounds, regulators and others. The LPS mutants are highly attenuated, but the requirement for anti-OPS antibody as an important contributor of an effective immune response may limit their utility as human vaccines. VirB may be an attractive target for vaccine use because of its marked attenuating effects; it will be interesting to see whether mutants in this operon have reduced immunogenicity. The studies by Tibor, *et al.* (Tibor *et al.*, 2002) on *omp10* mutant 544D10 are interesting, because they show dissociation between the ability of this strain to survive in mice and to replicate intracellularly in vitro. This finding underscores the limitations of screening methods in predicting in vivo behavior of vaccine candidates.

Table 1. Attenuated *Brucella* mutants

Parent Strain*	**Mutant Strain**	**Mutation**	**Gene product function**	**Attenuation**	**Anti-*Brucella* effect, model**	**References**
LPS mutants						
BM, BS	VTRM1, VTRS1	*wboA*	glycosyltransferase required for OPS synthesis	BALB/c mice	i.p. challenge of	(Winter *et al.*, 1996)

					BALB/c mice with BA,BS, BO, BM	
BM	VTRM1	*who A*	glycosyltransferase required for OPS synthesis	goats	abortion, goats	(Elzer *et al.*, 1998)
BA2308	RA1	*wboA*	glycosyltransferase required for OPS synthesis	mice		(McQuiston *et al.*, 1999)
BM 16M	B3B2		perosamine synthetase, OPS synthesis	mice, not bovine macrophages		(Godfroid *et al.*, 1998)
BA2308	CA180, CA353, CA533, CA613	various *lps* operon	OPS synthesis	BALB/c mice		(Allen *et al.*, 1998)
BA2308	B2211	*pgm*	phophoglucomutase, LPS and other glucose polymer synthesis	mice, HeLa cells		(Ugalde *et al.*, 2000)
BA2308 (several)		*per, wbkA, wa**, manB*$_{core}$	synthetases required for OPS and core LPS synthesis	mice	i.p. challenge of BALB/c mice with BA, BO	(Monreal *et al.*, 2003)
Auxotrophic mutants						
BM16M	WR201	*purEK*	purine synthesis	human monocyte-derived macrophages; cleared from mice 8 weeks	intranasal challenge of BALB/c mice with 16M	(Drazek et al., 1995; Hoover *et al.*, 1999a)
BS		*aroC*	aromatic compounds	murine macrophages, mice		(Foulongne *et al.*, 2001)
VirB mutants						
BA		*virB4*	Type IV secretion system	murine macrophages, mice		(Watarai *et al.*, 2002)
BS1330		*virB*	Type IV secretion system	THP1 cells, human monocytes, mice		(O'Callaghan *et al.*, 1999)
BA2308		*virB*	Type IV	HeLa cells, mice		(Sieira *et al*

			secretion system			2000)
BA		*dnaK*	stress protein	murine macrophages, mice		(Kohler *et al.*, 2002a; Kohler *et al.*, 1996)
Other mutants						
BA2308	KL7	*bacA*	cytoplasmic membrane transport protein	murine macrophages, mice		(LeVier *et al.* 2000)
BA2308	BvI129	*cgs*	cyclic beta(1–2) glucan synthesis	HeLa cells, mice		(Inon de Iannino *et al.*, 1998)
BA19	BAI129	*cgs*	cyclic beta(1–2) glucan synthesis	HeLa cells, mice	i.p. challenge of BALB/c mice with BA	(Briones *et al.*, 2001)
BA2308		*bvr*	2-component regulatory system	macrophages, HeLa cells, mice		(Sola-Landa *et al.*, 1998)

Parent Strain*	***Mutant Strain***	***Mutation***	***Gene product function***	***Attenuation***	***Anti-Brucella effect, model***	***References***
BA2308	Hfq3	*hfq*	HF-I, stationary phase stress resistance	murine resident peritoneal macrophages; BALB/c mice		(Robertson and Roop, 1999)
BA		*hemH*	ferrochetalase	J774 cells, mice		(Almiron *et al.*, 2001)
BA2308	PHE1	*cycL, htrA*	cytochrome C maturation (CycL), stress protein (HtrA)	murine macrophages, bovine neutrophils and macrophages, cattle; minimally attenuated in mice, goats		(Edmonds *et al.*, 2000; Elzer *et al.*, 1994b; Elzer *et al.*, 1996)
BM16M	RWP5	*cycL, htrA*	cytochrome C maturation (CycL), stress protein (HtrA)	goats, minimal in mice	abortion (goats), not colonization	(Phillips *et al.*, 1997; Phillips *et al.*, 1995)
BA	BA25,BM25	*omp25*	Omp25	bovine	abortion	(Edmonds *et*

BM, BO	BO25			macrophages, trophoblasts, mice, pregnant cows, goats	(goats, BM25)	*al.*, 2001; Edmonds *et al.*, 2002a; Edmonds *et al.*, 2002b)
BA544	BA544D10	*omp10*	Omp, outer membrane lipoprotein	mice		(Tibor *et al.*, 2002)
BA544	BA544D19	*omp19*	Omp, outer membrane lipoprotein	bovine macrophages, HeLa cells, mice		(Tibor *et al.*, 2002)

*BM=*B. melitensis;* BS=*B. suis;* BA=*B. abortus;* BO=*B. ovis*

7.9.3. Non-attenuated Mutants

In a number of cases, gene interruption does not lead to attenuation. Deletions of genes encoding stress protein HtrA(Phillips and Roop, 2001; Roop *et al.*, 2001; Tatum *et al.*, 1994) carbohydrates *(chvE* or *gguA)* (Alvarez-Martinez *et al.*, 2001), periplasmic protein BP26 (Boschiroli *et al.*, 1997), bacterioferritin (Denoel *et al.*, 1997), response regulator protein FeuP (Dorrell *et al.*, 1998) erythritol catabolism (Sangari *et al.*, 1998)immunodominant protein P39 (Tibor *et al.*, 1998) the siderophore 2,3 dihydroxybenzoic acid (Bellaire *et al.*, 1999) the nitrogen response regulator NtrC (Dorrell *et al.*, 1999) UDP-galactose-4-epimerase *(galE)* (Petrovska *et al.*, 1999), cytochrome bc(1) complex (Ko and Splitter, 2000b), and ATP binding cassette (ABC) transporters (Ko and Splitter, 2000a) have little or no attenuation when tested in vivo or in mononuclear phagocytes. Deletion of the Lon protease from *B. abortus* leads to transient attenuation, similar to that seen with *cycL htrA* mutants, but no effect on long-term persistence (Robertson *et al.*, 2000). Deletion of *clpA*, which encodes a molecular chaperone, from *B. suis*, leads to enhanced persistence in vivo (Ekaza *et al.*, 2000).

8. Molecular Approaches to Enhance Immunogenicity

It is possible that MNPH1, complemented WRRP1, or other satisfactorily attenuated deletion mutants will not elicit a sufficiently protective immune response. Additional molecular approaches to boost immunogenicity by overexpressing protective antigens or coexpression of adjuvant immunomodulators appear promising (Vemulapalli *et al.*, 2002). Enhancement of immunogenicity may allow use of more attenuated strains that provide a wider safety margin but better immunogenicity. Construction of mutants that overexpress homologous antigens, however, is not a trivial task. The function and quantity of expressed protein, location of protein in the bacterium (e.g., accumulation in cytosol or secreted) and further translocation of proteins within the host cell may not only have unpredictable effects on vaccine strain survival in vitro or in vivo, but may also influence immunogenicity. Proteomic or in silico predictive methods that identify

strongly immunogenic proteins based on recently available complete genome sequences may provide preliminary targets for overexpression.

9. Summary and Outlook

Brucellae are highly infectious organisms that cause significant morbidity in humans. Genetically uncharacterized live vaccines have been shown to be protective, but not sufficiently attenuated. A number of molecular approaches have been used recently to develop genetically defined strains that are attenuated for growth in macrophages or in a variety of animal models; some of these elicit antibrucella immune activities. The explosion in knowledge that will occur as a result of complete genome sequencing should greatly enhance our understanding of pathogenetic mechanisms in brucellosis. This understanding will not only provide more targets for deletion of genes to make additional live vaccine candidates, but will identify proteins that may be useful either as components of subunit vaccines or for overexpression to enhance immunogenicity of live vaccines. Indeed, the number of potential targets already exceeds our ability to evaluate them realistically. A crucial component of live vaccine development will be early performance of clinical trials to assess the ability of animal models to predict attenuation of candidate vaccines. In the absence of such information, many candidates will be tested and touted at great expense, but little actual progress will be made toward fielding a vaccine. Once satisfactory benchmarks of attenuation and good in vitro correlates of protection are established, the burgeoning genomic and proteomic information can be used more effectively to engineer strains with improved ratios of efficacy to toxicity.

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Chapter 18
Alternative Ovine Brucellosis Vaccine: Experiences with Drug Delivery Systems

Carlos Gamazo*, Maite Estevan, Maribel Murillo, M.Jesús Grilló, Clara M.Marín, Montserrat Barberán, M.Mar Goñi, José M.Blasco, and Juan M.Irache

From: *Brucella:* Molecular and Cellular Biology. Edited by: Ignacio López-Goñi and Ignacio Moriyón

Abstract

It is generally assumed that live vaccines are better than inactivated or acellular vaccines, as they are less expensive and induce a more persistent immunity. However, the microencapsulation of antigens in biodegradable polymers may turn the balance in favour of acellular vaccines. In this chapter, we describe the properties of drug-vaccine delivery systems based on the use of such polymers, and their application in the control of experimental brucellosis. We have demonstrated that micelles of major membrane antigens from *Brucella ovis* (HS extract) can be microencapsulated in poli-(ε-caprolactone), and that this preparation (HS-PEC) induces a protective effect against a challenge against experimental brucellosis in mice and rams. The resulting microparticles had diameter sizes of less than 3 μm and contained significant amounts of the unaltered antigenic complex, which was subsequently released in pulses from the microparticles. These microparticles were injected orally or subcutaneously in B ALB/c mice in order to observe the protection conferred against experimental infection with the virulent strains *B. abortus* 2308 or *B. ovis* PA. The results showed that subcutaneous administration of HS-PEC microparticles eliciting high amounts of IFN-γ and IL-2 but low quantities of IL-4, and protected mice against any of the challenge strains used. Such protection was similar to that provided by the reference live attenuated *B. melitensis* Rev 1 vaccine. Similarly, oral immunization was also able to protect mice challenged with *B. ovis*. Additional research was performed in rams, where HS-PEC was innocuous and, in contrast to Rev 1 immunized animals, did not induce

antibodies against smooth lipopolysaccharide. To establish the protective value of this rough subcellular vaccine, rams were challenged with *B. ovis*, and found to have a similar protection level to than that conferred by Rev 1. In conclusion, protection against experimental infection in mice and rams after one single shoot, and its potential for mucosal vaccination, suggest that HS-PEC microparticles may represent a serious alternative to the conventional attenuated anti-brucellosis vaccines.

* For correspondence email cgamazo@unav.es

1. Safety Versus Efficacy in Vaccination

Nowadays, the importance of vaccination in the control of infectious diseases is unquestionable; the progress that has been made in human and animal health during the last few decades have guaranteed that. However, when it comes to evaluating the cost/benefits of a vaccine, we need to take into account not only that the vaccine prevents the illness, but in addition, that it should not cause negative effects for the immunized animal or for the handler. Live attenuated vaccines are still by far the most utilized for their efficiency with respect inactivated (bacterins) and acellular ones, but is it necessary to run the risk of using live vaccinal strain? Are there any safer alternatives?

The knowledge we have about genomics which allows us to selectively knock out specific virulent genes, a procedure that is, without doubt, the safest since the methods of empirical attenuation developed by Pasteur and Koch at the end of the nineteenth century. Nevertheless, in the application of such a rational approach, we can obtain attenuated strains that retain some residual virulence. The causes can be varied. For example, we still do not know precisely which genes and/or the mechanisms of regulation are required for virulence; the mutant can acquire these or other genes from other bacteria or through phages, and in this way revert to its original virulence. On the other hand, quantitatively, the attenuation should not be excessive, since it could result in the generation of an avirulent strain instead of an attenuated one. The avirulent strain would be eliminated rapidly by the host immune system. Another problem relating to the use of an attenuated vaccine is its potential release into the environment where it could recover its virulence in other hosts.

Due to the concerns outlined above, the restrictions for the use of attenuated, genetically modified *Brucella* organisms in vaccination have become very strict. In addition, the Centre for Disease Control and Prevention have tightened regulations for the possession of biological agents that have the potential to pose a severe threat to public health and safety (CDC's Select Agent Program includes *Brucella*). Thus the use of mutants containing antibiotic resistance genes as a selectable markers will be greatly restricted if not prohibited in the future (and will require approval by the Health and Human Services Secretary after consultation with experts). The CDC states that "an entity may not conduct experiments utilising recombinant DNA that involves the deliberate transfer of a drug resistance trait to select agents that are not known to acquire the trait naturally, if such acquisition could compromise the use of the drug to control diseases agents in humans, veterinary medicine, or agriculture" (42 CFR Part 73.10,

Select Agent Program, CDC). This ruling will certainly have a significant impact on performing studies with genetically modified *Brucella* organisms in the U.S.A.

In conclusion, in the essential discussion about the quantitative cost/benefits of a vaccine, on the cost side we should project not only the economic costs derived from its production (investigation and development included), but also the costs derived from the negative effects of its use, for example, the residual virulence in the host. In the of case *Brucella*, the cost/benefit ratio would not favour attenuated vaccines.

On the other hand, given that most of the vaccines currently available were produced before 1970, it is very likely that the majority of vaccines used in the veterinary practice would not pass the strict control measures required for the use of a biological product. For instance the manufacturing process should be validated perfectly and the final product should pass rigorous quality controls. To overcome these problems current research efforts are directed towards the development of new vaccines containing perfectly characterized antigens, that are established and safe, and have a composition and preparation methods that are rigorously controlled. However the new vaccines in this biotechnology age suffer, in general, from poor immunogenicity necessitating the use of adjuvants. These adjuvants have a variety of functions, such as the facilitation of vaccine passage through cellular barriers and the stimulation of the cells of the immune system. Microparticles may be considered as adjuvants that can accomplish these requisites, and given their capacity to interact with mucosal cells, they are even able to induce a response at the mucosal level. We will review this type of adjuvant later in this chapter. First however, it is necessary to review the problem of ovine brucellosis and its control (Bale, 1992; Bowersock *et al.*, 1999).

2. Control of Ovine Brucellosis

Currently, the most universal system of prophylaxis used against ovine brucellosis involves subcutaneous inoculation of the vaccine *B. melitensis* Rev 1 (Jiménez de Bagüés *et al.*, 1989). Rev 1 is a live attenuated *B. melitensis* streptomycin resistant strain derived from a virulent *B. melitensis* isolate which became streptomycin-dependent (Elberg *et al.*, 1957). Since its introduction to the marketplace, patent free, in the 1960s, the benefit derived from its use is incalculable, not only protecting the ovine livestock and goats against *B. melitensis*, but, in addition, providing protection against *B. ovis* in ovine livestock (Elberg *et al.*, 1957; Alton *et al.*, 1967; Fensterbank *et al.*, 1985). Both pathogens produce big economic losses in a number of countries around the world, due to their ability to cause infertility problems and abortions in livestock. Nevertheless, Rev 1 is an attenuated strain not free from virulence. It produces abortions and infertility in some of the inoculated animals and it is also virulent for man (Alton *et al.*, 1967; Fensterbank *et al.*, 1985; Blasco *et al.*, 1993; Bardenstein *et al.*, 2002), with the additional problem that Rev 1 is resistant to streptomycin. In addition, let us not forget that *B. melitensis* is one of the most virulent bacterial species for man and that it has the potential to be used as a biological weapon; knowledge of this use could arouse fear in the majority of the population, who may be ignorant of the fundamentals and significance of biological attenuation (Young, 1983; Kaufmann *et al.*, 1997). Finally, another drawback to the use of the Rev 1 vaccine is its long persistence in the immunized animal.

Although this vaccine offers a good protective immunity in the host by inducing an elevated level of circulating antibodies (against the smooth lipopolysaccharide, LPS-S), these antibodies persists for many years after vaccination, making the differentiation between the infected animals and those which were vaccinated impossible. For all of these reasons, the use of Rev 1 is prohibited in those countries where *B. melitensis* infection has been eradicated. Consequently, the need for a better vaccine for brucellosis eradication clearly exists, but in spite of the progresses made in recent years, "the ideal vaccine" for ovine vaccination is not yet available.

According to the World Health Organization division for emerging and other communicable diseases, surveillance and control agencies in 1997, the ideal live vaccine has to comply, at least, the following requisites: (i) it must be innocuous for the vaccinated animal including the prevention of abortion or sterility, (ii) it has to provide long-term protection with a single vaccination, (iii) it must minimise the long-term production of antibodies, which may interfere with the current serodiagnosis tests of field infections, (iii) it should not be transmittable to other animals or contaminate meat and milk products, (iv) it must be non-pathogenic for humans, (v) it has to be biologically stable. Rev 1 clearly does not comply with at least four of these conditions. Therefore, our objective was to develop an alternative vaccine, and we have considered the use of a subcellular extract (avoiding problems related to virulence), obtained from a rough strain (avoiding false positives in serodiagnosis). In order to induce protective immunity after a single inoculation, we will discuss the use of "Drug Delivery System" as adjuvants to encapsulate the rough antigenic extract.

3. Acellular Vaccines Against Brucellosis

Various authors have used acellular extracts of *Brucella*, derived from both smooth-and rough-strains, as potential protective antigens (used principally in animal laboratories) (Sterne *et al.*, 1971; Winter, 1983; Montaraz *et al.*, 1986; Winter *et al.*, 1988; Jacques *et al.*, 1991; Dzata *et al.*, 1991a, 1991b; Tabatabai *et al.*, 1992; Blasco *et al.*, 1993; Stevens *et al.*, 1994; Tabatabai *et al.*, 1994; Pugh *et al.*, 1994; 1996). Equally, they have used recombinant proteins (Bowden *et al.*, 1995; Oliveira *et al.*, 1996; Al-Mariri *et al.*, 2001; Velikovsky *et al.*, 2002;), synthetic peptides (Stevens *et al.*, 1994), DNA vaccines (Kurar *et al.*, 1997; Cespedes *et al.*, 2000; Rosinha *et al.*, 2002; Leclerq *et al.*, 2002), and idiotypic antibodies mimicking the O-chain (Beauclair *et al.*, 1990). We have opted to use subcellular extracts directly; these were derived from a rough strain, *Brucella ovis*. An advantage of using such extract is that it avoids the problem of obtaining sero-positive reactions to the S-LPS i.e. as is currently found in vaccinated animals.

Brucella ovis is a stable rough form which lacks of the O-polysaccharide side chains characteristic of the smooth strains of *Brucella*, but contains an outer membrane protein (Omp) profile similar to other members of the genus. In a previous study, it was demonstrated that the pattern of Omps obtained for different *B. melitensis* and *B. ovis* strains from different geographical origin were very alike (Gamazo *et al.*, 1989). Along with the fact that they do not present antigenic diversity (97% of their amino acids are conserved) (Cloeckaert *et al.*, 1996), and that these Omps are strongly immunogenic, we hypothesised that a subcellular vaccine containing an outer membrane complex of *B. ovis*

might be effective in protecting against infections by both rough *B. ovis* and smooth *B. melitensis* species (Gamazo *et al.,* 1989). In fact, as mentioned previously, the smooth vaccine strain *B. melitensis* Rev 1 protects sheep against the rough-type *B. ovis*. Other data in favour of the use of Omps is that the complex of these proteins with LPS is an excellent diagnostic tool for *B. ovis* infection. Thus, the majority of the male sheep infected by *B. ovis* or *B. melitensis* possess elevated levels of circulating antibodies against the Omps (Riezu-Boj *et al.,* 1990; Zygmunt *et al.,* 1994; Kittelberger *et al.,* 1995).

The procedure to obtain an extract enriched in the outer membrane components was based on one originally described by Myers *et al.* (1972). These researchers used an extract from *B. ovis* obtained after heat treatment in saline conditions (HS) for the diagnosis of lambs infectedwith *B. ovis*. Later, this extract was characterized as an antigenic complex containing the following main components: (i) Omps, (ii) rough LPS and (iii) phospholipids, being specifically enriched in the Omp of group 3 (Omp25 or Omp3a, and Omp31) and lipoproteins Omp10, Omp16, Omp19. These proteins are exposed on the surface of the bacteria (Riezu-Boj *et al.,* 1986; Gamazo *et al.,* 1989; Murillo *et al.,* 2001), and it was reported that monoclonal antibodies against these Omps (Omp 16,19 and 31 kDa) provided great protection against *B. ovis* infection (Bowden *et al.,* 1995). With all these data in mind, our proposal was to use the HS of *B. ovis* as the antigenic component in a subcellular vaccine.

It should be mentioned however that the exploitation of this new generation of vaccines, including the subcellular vaccines, is currently limited. This is principally due to the poor immunogenicity of these non-living vaccines which require booster doses to be administered in order to induce protective immunity. In addition, immunity against *Brucella* requires cell-mediated mechanisms (Cheers, 1984; Araya *et al.,* 1989; Murillo *et al.,* 2001), in particular, Th1 immune. responses characterized by production of IFN-γ (Zhan *et al.,* 1993; Murphy *et al.,* 2001). Therefore, in addition to the subcellular antigenic components, it is critical that suitable adjuvants are used in order to get the appropriate immune response. The selection of the correct adjuvant to tailor the right cell-mediated immune mechanisms requires a good knowledge of its potential, briefly summarized below (Bowersock *et al.,* 1999; Hunter, 2002).

4. Adjuvants and Microparticles

An adjuvant is any substance which increases the immune response (antibody/ cellular mediated) to the antigen with which it is mixed. Depending on the type of adjuvant, one or another branch of the immunity will be stimulated. The adjuvants act principally by means of three mechanisms:

(i) Forming an antigen depot in the site of administration, from which the antigen would be released during a variable period of time (e.g., aluminium based salts). These compounds mainly stimulate the production of antibodies by the induction of Th2-lymphocytes, characterized by the production of IgG1 and IgE. The mechanism of action seems to be due at least in part for the presence of an excess of free alumin; this forms a depot that will induce the recruitment and activation of many immune cells to the site of inoculation. However, this “favourable” local inflammation may cause a

granulome, or even eosinophilia to be created (Gupta *et al.*, 1995; Cooper, 1995). In addition, these adjuvants cannot be lyophilised and they may produce allergic reactions after a reimmunization. Despite these drawbacks, the only adjuvants currently approved for use in man are these aluminium derivatives.

(ii) Activating cells of the innate immune system, driving and focusing an acquired immune response. These are called "Immunostimulatory Adjuvants", and often contain pathogen associated molecular patterns (PAMP). Some examples are monophosphoryl lipid A (MPL), muramil dipeptide (MDP), trehalose dimycolate (TDM), or CpG DNA (Woodard *et al.*, 1980a; 1980b; Winter *et al.*, 1983; Montaraz *et al.*, 1986; Dzata *et al.*, 1991b; Tabatabai *et al.*, 1992; Blasco *et al.*, 1993; Pugh *et al.*, 1994). These adjuvants are also capable of stimulating cell mediated immunity (via Th1). However, their high economic cost is a major drawback.

(iii) Targeting associated antigens into antigen presenting cells (APC), including macrophages and dendritic cells. They are categorized as "Vaccine Delivery Systems", and are generally particulated: emulsions, pluronic micelles, microparticles, ISCOMs and liposomes.

Several of these adjuvants have been tested previously in mice (MDP, ISCOM, etc) and rams (Freund, MDP) (Blasco *et al.*, 1993; Jiménez de Bagüés *et al.*, 1994; Gamazo, *et al.*, 2003). For example, an emulsion of HS with pluronic L121 in squalene combined with MDP was used to immunize rams against an experimental infection by *B. ovis* (Blasco *et al.*, 1993). A copolymer emulsion of L121 forms fibrous structures at water conforming droplets. Their hydrophobic surfaces are highly effective in adsorbing HS and preserving conformational epitopes, thus inducing strong antibody responses. MDP, which stimulates a cellular mediated immune response, should be included since it will interact synergistically with the copolymers in the induction of a balanced antibody and CMI against the incorporated antigens (Takayama *et al.*, 1991). In addition to HS, other subcellular extracts were studied (HS free of LPS, outer membrane vesicles and cell envelopes) with the same L121-MDP adjuvant. Control groups such as unvaccinated rams, commercial bacterins and Rev 1 were also included. Each ram vaccinated with the acellular extracts was revaccinated subcutaneously 18 weeks after first immunization. A challenge infection was performed with a virulent strain of *B. ovis* at the 31st week after the second immunization, and the animals were slaughtered 12 weeks later. After sacrifice, the liver, spleen, testes and epididymis (separately), vesicular glands, bulbourethral glands, ampullae and iliac, submaxillary, parotid, retropharyngeal, precrural and prescapular lymph nodes were taken for bacteriological analyses. Bacteriological results indicated that HS was as effective as Rev 1 (60% protection) at inducing protection; besides, anatomopathological studies indicated that the lesions observed in the rams vaccinated with HS were less severe than those in the Rev 1 vaccinated group (Blasco *et al.*, 1993). These were very promising results, but the potential acceptance of these preparations is limited. This is mainly due to the undesirable local inflammatory reactions elicited by the adjuvant, its high cost, and the need for revaccination; all of these factors making widespread use in field applications unsuitable.

Therefore the development of particulate vaccine delivery systems as adjuvants (e.g. microparticles) became an attractive alternative. Microparticles are relatively new systems for drug and vaccine delivery; their development has been very rapid during the past few years. These particles consist of solid spherical particles of greater than one

micrometer diameter that form a continuous network, or matrix system (of either a polymeric or macromolecular material) in which the substance to be encapsulated is dispersed in the molecular form, solid dispersion (Okada *et al.,* 1995; Hanes *et al.,* 1997). Among the many advantages that these systems offer in bio-medicine applications, the following are considered noteworthy (Couvreur *et al.,* 1993):

(i) Increase the stability of the material (drugs, antigens, etc.) incorporated during the manufacturing process, transport and storage of the active product.
(ii) Protection of the encapsulated active product against its chemical or enzymatic inactivation in the environmental conditions of the organism.
(iii) Improve the transport of the biologically active molecule to areas of the body in which the molecules have to produce its beneficial action, including the ability to interact with MALT and systemic immune cells. For instance, these formulations are potential carriers for oral vaccine delivery due to their protective effects on the encapsulated antigens and their ability to be taken up by the Peyer's patches in the gut (Powell, 1996). They are also delivered avidly and efficiently to phagocytic cells, and it has been demonstrated that dendritic cells and macrophages serve efficiently as antigen-presenting cell to T and B cells for the carried-antigens (Audran *et al.,* 2003; Thiele *et al.,* 2001).
(iv) Prolonged time of residence of the loaded drug in the organism (interesting for those molecules with a high clearance and a low biological half-life, such as protein compounds) controlling its release from the pharmaceutical formulation.

Microparticles can be prepared with natural or synthetic biodegradable materials. The former group includes proteins (albumin, collagen, gelatine) and polysaccharides. The latter group include hyaluronic acid, alginic acid, chitosan, poly-esters of hydroxic acid, poly-orthoesters, poly-alkylcarbonates, poly-aminoacids, poly-anhydrides, poly-acrylamides and poly-alkyl-cyanoacrylates. Nevertheless, the most frequently used materials in the manufacturing of microparticles are the polyesters, with the poly-lactioco-glycolic acid (PLGA) copolymers being amongst the primary candidates because of their excellent tissue compatibility, biodegradability and regulatory approval. Improved vaccines by microencapsulation of antigens in PLGA have been demonstrated for a number of antigens (Johansen *et al.,* 2000). Poly(ε-caprolactone) (PEC) is another biocompatible and biodegradable polyester polymer that degrades slowly and does not generate an acidic environment unlike the PLGA copolymers (Baras *et al.,* 2000). Other advantages of PEC includes its hydrophobicity, stability and low cost. At this point, we will summarize our own experience on the use of different formulations of microparticles formed with PLGA and PEC to deliver *B. ovis* HS antigens to generate a protective immune response against brucellosis after a single injection.

5. Microencapsulation of the HS Antigenic Extract from *Brucella ovis*

The manufacturing procedures for the microparticles formation may be classified into three groups: physicochemical, chemical, and mechanical methods. The selection of the appropriate manufacturing procedure depends not only on the

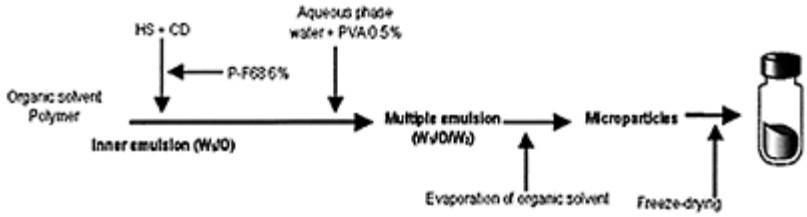

Figure 1. Scheme of the solvent evaporation method used to encapsulate the HS antigenic extract in microparticles (Murillo *et al.*, 2001; Murillo *et al.*, 2002a). CD: β-cyclodextrin; PVA: poly vinyl alcohol; P-F68: Pluronic® F68.

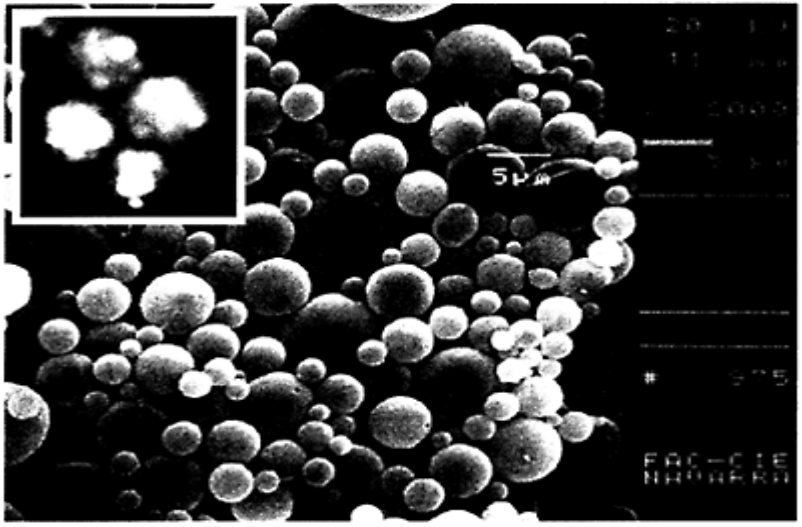

Figure 2. Microphotography of the HS-PEC formulation obtained by SEM. The size of microparticles range from 0.5 to 7.0 µm. The upper figure shows an image obtained by optical microscopy of flourescein loaded microspheres in order to reveal their internal structure.

physical and chemical properties of the active substance/polymer pair, but also on the final characteristics of the resulting microparticles (granulometry, internal structure, drug loading, release profile, wetting capability,…); and, the administration route should be taken into account, since microparticles are suitable for vaccination via intramuscular, subcutaneous or oral routes. Although other methods have been used for the microencapsulation of the antigenic extract HS from *B. ovis* (Blanco *et al.,* 2003), only the results obtained with the solvent evaporation method will be discussed in this chapter.

HS was successfully microencapsulated by a solvent evaporation method in either PEC or PLGA (75:25) polymers containing different pharmaceutical auxiliaries such as β-cyclodextrin and other excipients [Pluronic® F68, polyvinil pyrrolidone (PVP) or Tween 20]. Figure 1 summarises the foundation of this process. In brief, the method is based on the previous formation of an aqueous solution of the antigenic extract mixed with β-cyclodextrin in the selected excipient (Pluronic® F68, PVP or Tween 20). A primary emulsion (inner emulsion, w_1/o) is then obtained by mixing it with the polymer (PLGA or PEC) previously dissolved in methylene chloride. The multiple emulsion ($w_1/o/w_2$) is formed when the inner emulsion is added to a second aqueous phase containing polyvinil alcohol (PVA) as a particle stabilizer. The subsequent evaporation of the organic solvent causes the microparticles formation. This process, as well as the adequate selection of pharmaceutical auxiliaries, enabled us to obtain smooth and spherical microparticles (Figure 2).

First of all, we studied the influence of polymer type (PLGA or PEC) and different pharmaceutical auxiliaries on the physicochemical characteristics and *in vitro* properties of the resulting microparticles containing the HS-cyclodextrin mixture. Cyclodextrins (CDs) constitute a large family of cyclic carbohydrates derived from starch. They differ from one to another by the number of glucopyranose units in their structure. The parent CDs contain six, seven and eight glucopyranose units, and are referred to as alpha, beta and gamma CDs, respectively. The overall shape of CDs is that of as a truncated cone, hydrophilic on the outside and relatively hydrophobic on the inside. These are able to form inclusion complexes with a wide range of substrates in aqueous solution. This property of CDs led us to using them to reduce the very low water solubility and tendency of the HS extract to generate irreversible aggregates in aqueous media (Murillo *et al.,* 2002b). The first observation was that regardless of the excipient used, PEC microparticles had a significantly lower mean size than PLGA particles. On the other hand, the size of microparticles prepared in the presence of Pluronic® F68 was always smaller than those prepared with either PVP or Tween 20 (see Table 1). This is possibly a result of the influence of the high hydrophilic/lipophilic value of the Pluronic® F68 surfactant on the capacity of the emulsifier to stabilise the inner aqueous phase and, thus, reducing the size of the resulting microparticles (Hilbert *et al.,* 1999; Benoit *et al.,* 1999; Lin *et al.,* 2001a). In any case, overall, the diameters of the PEC microparticles were in the range of 1–3 μm, the optimal size to be captured either by monocyte-macrophage, dendritic cells, and Peyer's patches cells (Moore *et al.,* 1995; Conway *et al.,* 2001), making it potentially useful for oral administration.

Another characteristic considered important in the screening for the most appropriate formulation was the antigen loading per microparticle. High amounts

Table 1. Physico-chemical characteristics of HS-loaded microparticles: size (µm) and antigen loading (µg/mg microparticles). Data express the mean±SD. HS was microencapsulated by a solvent evaporation method in either PEC or PLGA (75:25) polymers containing different pharmaceutical auxiliaries (β-cyclodextrin and one of the following: Pluronic® F68, polyvinyl pyrrolidone or Tween 20)

Polymer	Surfactant	Size (µm)	HS Loading (µg/mg)
PEC	Pluronic 6%	1.3±0.5	5.2±1.5
	PVP 5%	1.7±0.9	6.4±0.1
	Tween 20 4%	2.0±0.2	5.4±0.2
PLGA (75:25)	Pluronic 6%	2.8±0.2	3.8±0.7
	PVP 5%	3.5±0.3	1.9±0.3
	Tween 20 4%	3.7±0.2	10.8±0.2

of polymer may induce local inflammatory reactions (Yeh *et al.,* 2002), therefore, high antigen loading per microparticle would be preferred. In our work, none of the excipients tested significantly modified the HS loading by PEC microparticles (Table 1). In contrast, the type of excipient inside PLGA microparticles highly influenced the HS loading. This phenomenon could be associated with the lower hydrophobicity of PLGA, and, thus, with its lower precipitation rate comparing with that of PEC (Cao *et al.,* 1999).

As mentioned above, an important issue in the selection of microparticles as immunoadjuvants is their potential for use as controlled delivery systems i.e. vaccine is released over a period of time. The type of amphiphilic polymers and even the excipient used may modulate the antigen release profile (Rojas *et al.,* 1999; Tobio *et al.,* 1999; Sánchez *et al.,* 1999; Lin *et al.,* 2001b; Morita *et al.,* 2001). For instance, the high hydrophilicity of PVP has been related to the capacity of this excipient to migrate to the outer water phase during the solvent evaporation process (Morita *et al.,* 2001), which might accelerate the release of HS by forming aqueous channels. On the contrary, Tween 20 appears to be able to reduce the number of aqueous channels between the internal aqueous droplets as well as those communicating with the external medium, reducing the burst effect. Pluronic® F68 has been widely employed to prevent the possibility of irreversible interactions between proteins and the PLGA polymers (Blanco *et al.,* 1998; Tobio *et al.,* 1999). These properties agree well with the *in vitro* release behaviour of HS from the different formulations under our own experimental conditions (Murillo *et al.,* 2002a).

The release profiles of HS from the different microparticle formulations assayed in this study are represented in Figure 3. The presence of PVP in both PLGA and PEC microparticles induced a large initial burst release of HS in 24 h amounting to 100% and

70%, respectively, of the antigen load. In contrast, the burst release from microparticles containing Tween 20 was lower; only, between 18% (for PLGA microparticles) and 35% (for PEC microparticles) of the loaded HS was released in the first 24 h. The association of Tween 20 to PLGA microparticles allowed the production of a second release pulse in days 2 and 3, and a third pulse in day 7, from which the HS was released in a continuous way, at least for 2 more

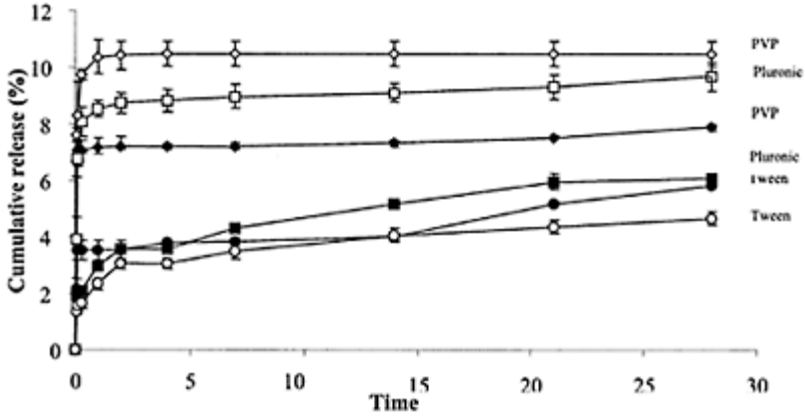

Figure 3. Cumulative *in vitro* release of HS from poly(ε-caprolactone) (dark symbols) and poly(lactide-co-glycolide) microparticles (open symbols). Pluronic® F-68 (□), PVP (Δ), Tween 20 (O).

weeks. However, in the case of PEC microparticles, an initial burst effect followed by a lag phase of at least two weeks was observed. When using Pluronic® F68 with PLGA polymer, the burst effect consisted of about 80% of the loaded antigen while in the case of PEC microparticles the burst effect contained only 20% and was followed by a second pulse on day 2 and a third pulse on day 7, followed by a continuous release for 2 weeks. The first pulse, correlated with the presence of approximately 20% of HS bound to the surface of the microparticles, is considered to exert a major and dominating influence on the intensity of the immune response following s.c. injection. The later slow release of the remaining antigens (around 40% of the HS antigenic complex remained inside the particles after 28 days of incubation) would keep stimulating the immune system. Therefore, the HS released from microparticles containing Pluronic® F68 was the most closely related to the antigen release by live bacteria in that it mimicks the effect of multiple doses through time.

A problem related with the microencapsulation of antigens is the loss of biological activity. As mentioned previously, encapsulation techniques involve several steps (use of organic solvents, shear induced stress during emulsification, freeze-drying,…) that may damage the antigens, thereby causing denaturation (Johansen *et al.*, 1998; Li *et al.*, 2000;

Diwan *et al.*, 2001). To prevent the antigen instability during the water-solvent evaporation method, conjugation of proteins with polyethylene glycol or "pegylation" may be used (Diwan *et al.*, 2001). Other substances such as albumin, trehalose and cyclodextrins have been used to diminish the detrimental effect of the microencapsulation process (Yeh *et al.*, 1996; Blanco *et al.*, 1998; Rojas *et al.*, 1999; Tobio *et al.*, 1999; Sánchez *et al.*, 1999; Johansen *et al.*, 2000; Lin *et al.*, 2001a; Morita *et al.*, 2001). Thus, we used β-cyclodextrins that, in combination with the other excipients, was able to preserve the antigenicity of the HS components, as it was demonstrated by SDS-PAGE and immunoblotting analysis (Figure 4). These results confirm the potential of these microparticulated HS formulations for immunization.

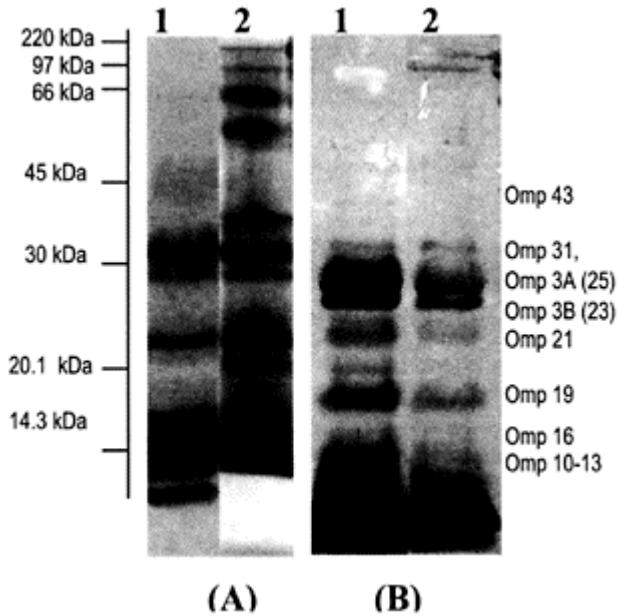

Figure 4. Comparative analysis of the HS, free vs. microencapsulated. Panel A: SDS-PAGE and silver staining of free HS (1) and HS released from the HS-PEC formulation (2); load was the equivalent to 12 µg HS/well. Panel B: Western blot analysis with a pool of sera from *Brucella ovis* infected rams against (1) free HS, and (2) HS released from HS-PEC microparticles containing Pluronic® F68.

Microparticles can be recognized as foreign materials by antigen presenting cells (APCs) (Tabata *et al.*, 1988a; 1988b), therefore, we compared the properties of several microparticle formulations containing the antigenic extract HS on cellular interaction and activation of J774 monocytes. It has been demonstrated that polymers, surfactans and hydrophilic surface agent (PVA) have great influence on phagocytosis of microparticles by macrophages. The hydrophilicity of microspheres resulting from the nature of the polymer and/or excipients used led to a decrease of phagocytosis intensity (Tabata *et al.*, 1990a; 1990b; Lacasse *et al.*, 1998). In our study, neither the antigen loading nor the excipient used in the internal aqueous phase affects the internalisation of microparticles by macrophages (Figure 5). In contrast, significant differences were found depending on the polymer used, with a higher uptake of PEC-microparticles compared to PLGA, probably due to the higher hydrophobicity of PEC (Illum *et al.*, 1982; Ahsan *et al.*, 2002).

In addition to drug delivery, the ingestion of microparticles may result in the activation of macrophages and, subsequently, an enhancement of its properties as APCs (Artursson *et al.*, 1997). The participation of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) in the regulation of the immune system is widely documented (Babior *et al.*, 1984). PEC-Pluronic® microparticles were the most active NO inducers from all the pre-selected formulations. This was considered as another key aspect, and a point of reference, in the selection process of the more appropriate formulation.

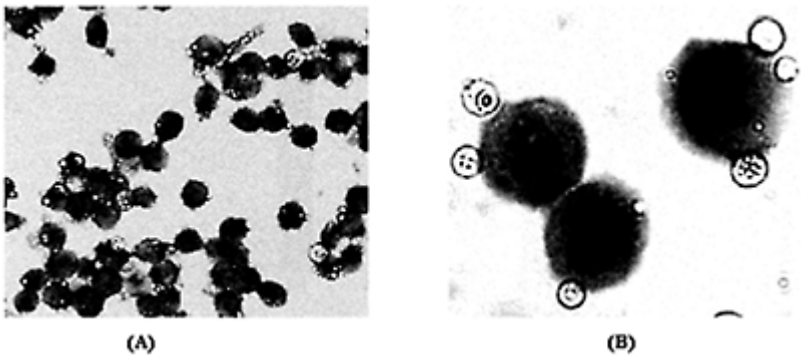

Figure 5. Uptake of microparticles by cells from the macrophage cell line J774. Panel A shows a representative picture of the uptake of microparticles at the begining of the assay, and panel B one hour later.

6. Cell Mediated Immunity and Protection

In addition to the strong interaction of PEC-microparticles with macrophages and enhanced NO production mentioned above, these particles were found to induce strong

IFN-γ production following immunization in contrast to using HS-PLGA microparticles (Murillo *et al.*, 2002b) (Figure 6a). *Brucella*, as an intracellular bacterium, induces a Th1 cytokine profile during infection in murine, ovine and human hosts. Therefore, T lymphocyte activation that promotes IL-2 and IFN-γ cytokines upon vaccination appears to be a decisive cell-mediated mechanism to provide resistance to *Brucella* infection (Araya *et al.*, 1990; Fernandes *et al.*, 1995; Fernández-Lago *et al.*, 1996; Agranovich *et al.*, 1999). It is well documented that the antigen type by itself may influence Th-cell differentiation towards a preferential Th1 or Th2 pathway. In fact, it has been shown elsewhere that heat-inactivated whole *B. abortus* cells or LPS from *Brucella* promotes the production of IL-2 and IFN-γ, but not IL-4, making these good potential carriers for vaccine delivery in situations requiring a strong Th1-like response for protection against even xeno-infections (Zaitseva *et al.*, 1995; Lapham *et al.*, 1996; Vemulapalli *et* al., 2000). In our hands, the free HS complex from *B. ovis* as well as the isolated R-LPS induced the release of high levels of IFN-γ when incubated with naïve splenocytes confirming their immunopotentiating properties toward Th1 pathway (Murillo et al., 2002b).

Besides the antigen itself, many other interlocking factors may contribute to the Th1/Th2 crucial decision for effective immunity. For example factors such as the delivery vehicle used, and the antigen presenting cells (APCs) are important. Professional APCs have the capacity to very efficiently internalise foreign particulate material such as microparticles and bacteria. However, in comparison to highly efficient phagocytes such as macrophages, DCs display significantly reduced phagocytosis activity, and in consequence, an optimised microparticle formulation with respect phagocytosis may therefore benefit APC process (Kiama *et al.*, 2001; Thiele *et al.*, 2001). Therefore, when the antigen is bound to the surface of microparticles and internalised by phagocytosis, antigen presentation is significantly improved compared to the uptake of soluble antigen up-regulating cytokine gene transcription (Kovacsovics-Bankowski *et al.*, 1995; Shen *et al.*, 1997; Venkataprasad *et al.*, 1999; Peter *et al.*, 2001; Walter *et al.*, 2001; Faraasen *et al.*, 2003; Thiele *et al.*, 2003a; 2003b). In conclusion, microparticles carrying antigens are able to affect the type of immune response.

To study how the microparticulate adjuvant may alter the intensity and cytokine profile after primary immunization, mice were inoculated with two different formulations containing either PEC or PLGA as the polymers, using the subcutaneous and oral inoculation routes, maintaining constant the amount of HS encapsulated. Although PEC or PLGA formulations encapsulated similar amounts of HS, subcutaneous administration of HS-PEC microparticles was able to activate the Th1 pathway eliciting a high IFN-γ and IL-2 release (Figures 6a and 6b). In contrast, HS-PLGA microparticles elicited a Th2 response (Figure 6c). PLGA being more hydrophilic than PEC, would show a lower affinity of the particles towards antigen presenting cells and thus diminishing the Th1 activation and activating the Th2 pathway instead (Torché *et al.*, 2000). These results are in agreement with the higher *in vitro* macrophage interaction and activation of HS-PEC microparticles with respect HS-PLGA ones (Murillo *et al.*, 2001).

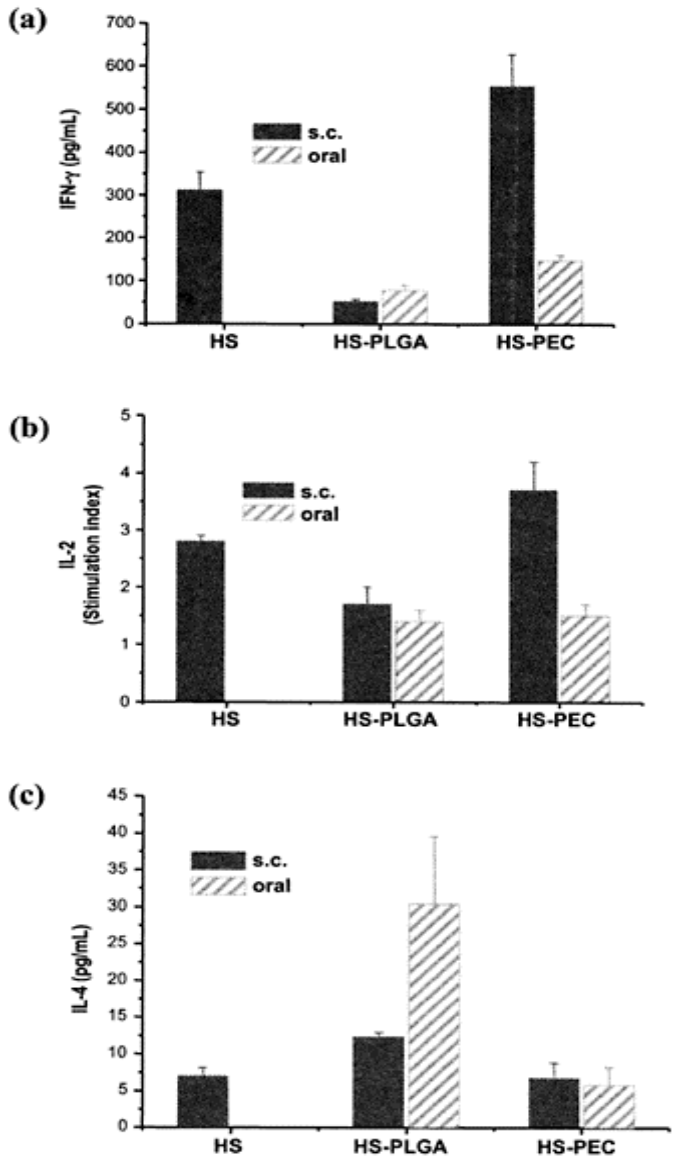

Figure 6. Immunogenicity (cell-mediated immunity) of HS, free and microencapsulated, after sc admnistration in BALB/c mice. Levels of IFN-γ (a), IL-2 (b) and IL-4 (c) secreted by splenocytes from mice

immunized with free or encapsulated HS, after in vitro stimulation with HS (100 µg/mL).

In addition to the polymer used, the route of immunization also plays an important role in the nature of immune response induced. The subcutaneous route has been the most investigated in terms of new controlled release vaccines. However, these have also been used for oral delivery due to the fact that they can be taken up by the Peyer's patches in the gut (Eldridge *et al.,* 1989; Jani *et al.,* 1992). Different approaches for achieving gastrointestinal bioadhesion of colloidal particles have been based on the use of non-swellable and hydrophobic polymers. In this case, the adhesion is mainly due to the inherent tendency of these small particles to develop intimate contact on mucosal surfaces (Ponchel *et al.,* 1998). The polyester microparticles described in this study are below 3 µm, a size suitable for M-cells uptake. Therefore we studied the bioadhesive capacity of the microparticles developed. Our results demonstrated that in contrast to PLGA, PEC microparticles interacted better with the Peyer's patches in the gut. These results are in agreement with the levels of IFN-γ released after oral immunization of mice with HS-PEC microparticles. In contrast oral immunization with PLGA did not show significant Th1-cytokine levels, and only the level of IL-4 was significant (Figure 6c). These results confirm the advantages of using PEC instead PLGA as the matrix to microencapsulate HS from *B. ovis* (Murillo *et al.,* 2001).

When HS-PEC was injected subcutaneously in BALB/c mice, protection against experimental infection with virulent *B. abortus* 2308 and *B. ovis* PA reference strains was elicited ($P<0.01$) (Figure 7). Moreover, this protection was similar ($p>0.05$) to that conferred by *B. melitensis* Rev 1 reference vaccine (Murillo *et al.,* 2001). Thus, the potent induction of IFN-γ response by the HS-PEC microparticles is correlated with the high level of protection induced (Murillo *et al.,* 2001). Some promising results were obtained after oral immunization of mice. When the oral route was used with HS-PEC microparticles significant levels of IFN-γ were released. In contrast, oral immunization with HS-PLGA did not show significant cellular-cytokine levels, and only IL-4 release was significant. Both the HS-PEC and the living attenuated Rev 1 vaccine, given orally, induced significant protection ($p<0.05$) against *B. ovis* infection (Murillo *et al.,* 2001).

However, the relevance of mice as models for ruminant brucellosis is uncertain. In consequence, additional research was performed in rams in order to establish the protective value of the innocuous rough subcellular vaccine presented in this chapter. We used four month-old rams randomised divided them into groups

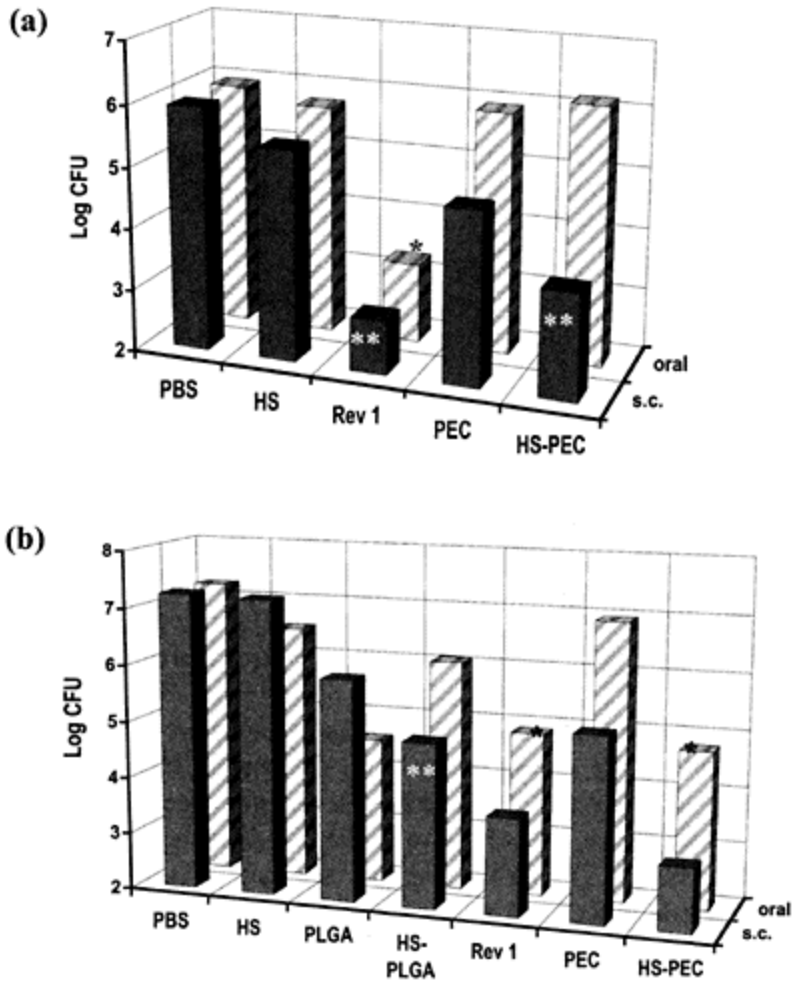

Figure 7. Protection conferred against experimental infection with the virulent *B. abortus* 2308 (a) and *B. ovis* PA (b) reference strains in BALB/c mice. Results are expressed in Logs CFU/spleen. PBS: control unvaccinated mice; Rev 1: reference vaccine; PEC: empty PEC microparticles; HS-PEC: HS loaded PEC microparticles; PLGA: empty PLGA microparticles; HS-PLGA: HS

loaded PLGA microparticles. (*) $p<0.05$; (**) $p<0.01$ vs. the unvaccinated control group.

to compare the efficacy of the Rev 1 vaccine against the test vaccine HS-PEC at two different doses of HS (0.1 and 0.8 mg), and a control unvaccinated group was also included. Each ram was immunized subcutaneously once, and challenged with a virulent strain of *B. ovis* seven months later, to be slaughtered two months after challenge. Our first objective was to confirm that the vaccinal antibodies do not interfere with the interpretation of conventional serological tests for diagnosis of ovine brucellosis caused by *B. melitensis*. As the HS from *B. ovis* lacks the O-polysaccharide chain of LPS, no significant positive serological responses could be expected in *B. melitensis* diagnostic tests (based on detection of antibodies against O chain, the immunodominant component of smooth *Brucella*). The negative seroagglutination (Rose Bengal test) obtained with the sera from vaccinated rams confirmed it. After necropsy, organs and lymph nodes were cultured in the search for *B. ovis* colonization. Briefly, results indicated that HS-PEC provided a similar protection ($p<0.05$) against *B. ovis* in rams to that conferred by the Rev 1 reference vaccine. Rev 1 was able to protect all the animals, and a dose-response effect was observed in the rams immunized with HS-PEC microparticles. Thus, with the higher dose of HS-PEC only two animals became infected (in contrast with the lower dose that was similar to control unvaccinated group). These results suggest performing additional studies in rams with higher dose of antigens.

7. Additional Considerations and Concluding Remarks

Microparticles have also been used for oral delivery due to their ability to be taken up by the Peyer's patches or by other specific areas of the mucosa (Powell, 1996). The delivery of vaccine antigens to mucosal surfaces is of particular interest, because it can generate immunity at the major portals of pathogen entry. One must take into account the fact that *Brucella* cells accede the host through the *mucosae*, and consequently, in order to offer the highest level of protection, the site of immunization should be parallel to the area of infection, as it has been suggested for other pathogens (Childers *et al.*, 1990; Rubas *et al.*, 1990). Vaccination strategies based on mucosal immunization can also be safer, minimize adverse effects and make administration easier. The microparticles used in this work have a mean size below 3 μm which is the optimum size to be taken up by the Peyer's patches (Eldridge *et al.*, 1989). Besides, in general, the fact that the more hydrophobic a microparticle, the better the absorption by the Peyer's patches (Powell, 1996); this is another factor that favours the use of HS-PEC microparticles for oral administration. Actually, our results demonstrated that the loading of HS within PEC microparticles enabled us to concentrate 3-fold more HS antigens in the Peyer's patches region than with PLGA carriers. The high bioadhesive capacity of this formulation suggests the need of further investigations to determine its efficacy against smooth species when used at higher doses in oral regimes.

There is also much emphasis on combining vaccine antigens together into single multivalent formulations. Thus the WHO has long recognised the practical advantages in

the development of multi-vaccines capable of inducing long-lasting immunity against several pathogens following a single dose, thus reducing costs and increasing compliance (Lloyd, 2000). Many such combined vaccines are being evaluated by industry and some are already licensed. In this connotation, multivalent vaccine manufacture is one of the most prominent examples of the use of modern pharmaceutical technology. Thus, the technology of microencapsulation discussed in this chapter offers the possibility of the production of vaccines containing diverse antigens from different species.

In summary, microparticulate antigen delivery systems are of special interest as stable carriers and effective adjuvants for the delivery of vaccines. The attractive properties of HS-PEC microparticles as an interesting anti-*Brucella* vaccine candidate are based on the following characteristics: i), the formulation protects the encapsulated substances from its eventual degradation during storage and/or biological conditions; ii), lets a sustained release profile, avoiding repeated administrations; iii) likely for mucosal vaccination; iv), vigorous activator of macrophages; v), the induced antibody response does not interfere in *B. melitensis* diagnostic tests; vi), modulates the immunogenicity of HS componentes towards a Th1 immune response; vii), induces a significant protection against experimental infection in mice and rams after one single dose; viii), avirulent and innocuous.

It is clear that to establish the protective value of the HS-PEC vaccine additional research must be performed in other susceptible hosts in addition to but rams and sheep. *Brucella* spp. may infect a broad range of animals, including wild animals frequently in contact with domestic livestock, that may act as reservoirs of the disease, hence, any potential vaccine against brucellosis should also be tested in those animals. In addition, the innocuousness of this rough subcellular vaccine makes it suitable for use even in human hosts.

Acknowledgements

This work was supported by grants from the “Ministerio de Educación y Cultura de España, CICYT” (Grants BIO097–0777-C03 and AGL2000–0299-C03) and Programme “Redes Temáticas de Investigación Cooperativa del FIS—Promoción de las Regiones (Objetivo 2, 2000–2006) (Red temática de Investigación en Brucellosis, Ref. No. G03/201), from Spain.

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Brucella

Molecular and Cellular Biology

Publication of the complete genome sequences of several different *Brucella* species has provided researchers with the opportunity to take a more directed approach to study this organism's physiology, virulence, and genetic diversity. In this book highly acclaimed *Brucella* scientists comprehensively review all of the most important advances in the *Brucella* post-genomic era providing for the first time a coherent picture of *Brucella* molecular and cellular biology.

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ISBN 1-904933-04-1

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