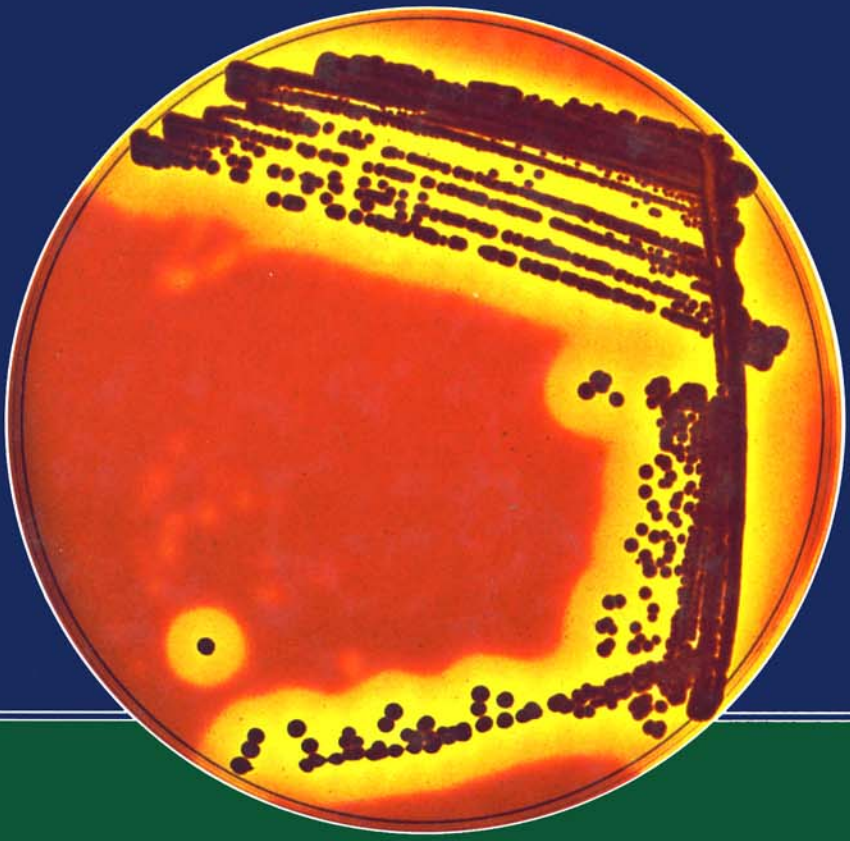
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Advances in MICROBIAL PHYSIOLOGY

EDITED BY ROBERT K POOLE



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Advances in
MICROBIAL PHYSIOLOGY

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Preface

Physiology is the study of the functioning of an organism or any of its parts, and of the processes that occur in living cells. Its origins are ancient. From AD 161, Galen promulgated and developed Aristotle's views on human physiology, particularly on the respiratory and circulatory systems, and developed a physiological scheme that informed medicine throughout the West for centuries. (Indeed, so basic was Galen's scheme that, in university dissection studies, the works of Galen were read aloud by the Reader and the relevant parts of the anatomy were pointed out by the Demonstrator. Many countries retain these academic titles.) Physiology was for many centuries dominated by a holistic view, that is, the belief that the whole is more than the sum of the parts.

At the beginning of the twentieth century there emerged a strong, 'mechanistic' view that all phenomena of life could be reduced to the laws of physics and chemistry. This stand was challenged by biologists who sought to find relationships *between* parts of an organism and who believed that any study of the parts of an organism in isolation was seriously flawed. In fact, a similar view had been taken by Claude Bernard half a century earlier, but the application of new physiological methodologies (to the nervous system) resulted in a more rigorous, holistic view that was to exert a powerful influence for many decades. Twentieth century biology has been characterized by the welding together of hitherto disparate strands. Biochemistry has re-unified the holistic and mechanistic views. It has, on the one hand, revealed remarkably intricate details of sub-cellular organelles and molecules and, on the other, unveiled the crucial relationships between cell function and structure. The development of biochemistry and an appreciation of multiple domains of organization, both temporal and spatial, in living organisms has been instrumental in the development of *microbial* physiology.

The small size of microorganisms means that physiological study of individual cells has, until recently, been technically almost impossible. This apparent set-back is actually also one of microbiology's greatest assets: since microbial populations of a single species or strain reflect to a large extent the properties of each cell in the population, it becomes possible to observe physiology and biochemistry of cells through a study of the behaviour and capabilities of populations that are easy to grow and manipulate. This ability to produce relatively large quantities of cells for analysis, coupled with emerging techniques for analysis of metabolites, macromolecules, nutrients and microbial products allowed microbial physiology and biochemistry to race ahead in the post-War period. Development had also been stimulated

by the need to understand large-scale culture of fungi for antibiotic production, and by Monod's landmark monograph on growth kinetics. It is perhaps these aspects of microbiology that, in the eyes of many, epitomize microbial physiology.

Advances in Microbial Physiology was first published in 1967. Under the pioneering editorship of Professor Tony Rose, with the collaboration at various times of John Wilkinson, Gareth Morris and Dave Tempest, the series has become immensely successful and influential. The Editors have always striven to interpret microbial physiology in the broadest possible context and have never restricted the contents to 'traditional' views of whole cell physiology. Coverage of 'holistic' topics or whole cell studies such as ion fluxes, stress responses and motility have gone hand-in-hand with detailed biochemical analyses of individual transport systems, electron transport pathways and many aspects of metabolism. In agreeing to assume editorial responsibility for *Advances in Microbial Physiology* after Tony Rose's untimely death, I am acutely aware of the high standards set by his commissioning and editing. Indeed, in preparing this first 'new' volume, I have been able to draw entirely on reviews commissioned by my predecessor. I intend that *Advances in Microbial Physiology*, although within new covers, will continue to publish topical and important reviews and that physiology will be interpreted as widely as in the past to include all material that contributes to our understanding of how microorganisms and their component parts work. In physiology, as in all aspects of microbiology, genetics and molecular biology play increasingly important roles and I hope that this will be adequately reflected in the pages of this series. At the time of writing, there has been much excitement in the popular press and in broadcasts, over the reported sequencing of the genomes of two free-living eubacteria. This is certainly a remarkable achievement and those working on *Escherichia coli*, yeast, and other microorganisms may not be so far behind in publishing their complete sequences. However, from the point of view of microbial physiology, these feats are starting points, not ultimate achievements. The imaginative use of the sequence data in helping to answer the question 'how do microorganisms work?' is the real challenge for microbial physiology.

The success of this series depends, of course, on submission of timely and authoritative reviews on as broad a range of topics as possible. Suggestions for contributions are most warmly welcomed.

I should like to thank the contributors for making this volume possible and the staff at Academic Press, particularly Léona Daw, for their help and guidance. I am particularly indebted to my wife, Joy, for the considerable help she has given me in some of the least interesting, but most important, aspects of this work.

Robert K. Poole

Cellulose Hydrolysis by Bacteria and Fungi

P. Tomme, R. A. J. Warren and N. R. Gilkes

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Abbreviations: CBD, cellulose-binding domain; CBHI and CBHII, cellobiohydrolases I and II, respectively; Cel and Cen, common abbreviations for cellulases; Cip, cellulosome-integrating protein from *Clostridium* spp; DS, duplicated segment on cellulases from *Clostridium* spp. involved in protein–protein interactions; Fn3, fibronectin type III modules; RD, receptor domains on scaffolding proteins from *Clostridium* spp. that recognize the duplicated segment on cellulases; Xyn, common abbreviation for xylanases.

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1. INTRODUCTION

Plants synthesize about 4×10^9 tonnes of cellulose annually (Coughlan, 1990) but this material does not accumulate because fungi and bacteria efficiently degrade all plant biomass to provide themselves with energy and carbon, ultimately recycling carbon dioxide into the ecosystem. Fundamental studies on cellulolytic microorganisms were once motivated by the need to prevent fungal attack of cotton fabric in the tropics (Mandels and Reese, 1964) but, more recently, by the potential for producing fermentable sugars from cellulosic biomass (Saddler, 1993). Although biomass conversion schemes are not economically viable at present, interest in enzymatic cellulose hydrolysis remains strong, not only because of the potential of cellulases in other biotechnological applications but also because satisfactory answers to even rather basic questions about the mechanisms of hydrolysis continue to elude us. Recent advances in our understanding of the structural and functional organization of individual cellulases, their regulation, and the ways in which the multiple enzyme components of cellulolytic systems cooperate, are the main focus of this review, but frequent reference is also made to other β-1,4-glycanases. Consideration of related enzymes such as chitinases and amylases can help us to understand how cellulases work; also, cellulose nearly always occurs in close association with plant cell wall matrix polysaccharides so that enzymes such as xylanases are intimately involved in the attack of cellulose *in vivo*. We begin with an overview of cellulose structures because, although chemically simple, cellulose is more than a homopolymer of β-1,4-linked glucose units. An appreciation of its complex physical organization and its interactions with other plant cell wall components is central to our understanding of the mechanisms of cellulase action.

2. CELLULOSE STRUCTURES

All cellulose is produced biosynthetically, mostly by algae and higher plants but also by certain bacteria, marine invertebrates, fungi, slime moulds and amoebae (Richmond, 1991). Synthetic complexes on the outer surface of the plasma membrane produce polymers of β-1,4-linked glucosyl residues, with degrees of polymerization ranging from about 100 to greater than 10 000, which coalesce into crystalline microfibrils. Despite its chemical simplicity, the diversity of origin and subsequent treatment to which cellulosic biomass is subjected during purification result in a complex range of physical forms of cellulose. Consequently, the description of cellulosic substrates includes such qualities as size, shape, porosity, surface area, association with non-cellulosic components, molecular conformation and crystallinity, all of which are relevant to the process of enzymatic hydrolysis.

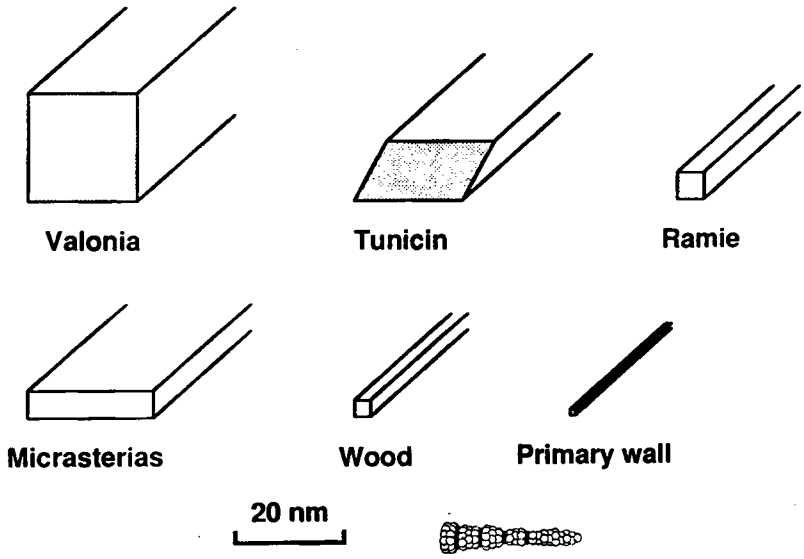


Figure 1 Comparison of the size and shape of various cellulose microfibrils. *Valonia* and *Micrasterias* are green algae; ramie, wood and primary cell wall microfibrils are representative of cellulose from higher plants; tunicin is so-called "animal cellulose", isolated from the ascidian tunic (H. Chanzy, unpublished). The low-resolution structure for *C. fimi* CenB determined by small angle X-ray scattering analysis (Meinke *et al.*, 1992) is included for size comparison.

The majority of cellulose is produced as a component of plant cell walls. These are often described as a composite of cellulose microfibrils embedded in an amorphous matrix, similar to reinforced concrete (Preston, 1974). The microfibrils have a structural role in the cell wall, imparting strength and contributing to its size and shape. Different organisms produce microfibrils of characteristic dimensions: microfibrils from giant unicellular algae such as *Valonia* spp. have a lateral dimension of about 20 nm and contain hundreds of cellulose chains; in contrast, those from the primary walls of higher plants are much smaller (Chanzy, 1990) (Fig. 1). Matrix materials consist of hemicelluloses that are further associated with pectins and proteins in primary plant cell walls and with lignin in secondary cell walls; the exact composition varies between organisms and with cell differentiation. Covalent lignin–carbohydrate bonds involving ester or ether linkages to hemicelluloses are well documented (Jeffries, 1990) but covalent attachment to cellulose is less certain. In secondary cell walls, the hemicelluloses consist mainly of xylans, substituted with a variety of side chains, and mannans and glucomannans (Puls, 1993). In most primary plant cell walls, xyloglucans form the interface between the microfibrils and the wall matrix but in some monocots, such as

maize, this position is occupied by glucuronoarabinoxylans (Carpita and Gibeaut, 1993) (Fig. 2). It is cellulose in this complexed form that cellulolytic microorganisms typically encounter under natural circumstances and which they must hydrolyze, either individually or as part of a consortium. This is why these organisms usually produce an array of polysaccharide hydrolases such as xylanases and mannanases, sometimes together with enzymes capable of lignin degradation, in addition to enzymes that hydrolyze β-1,4-glucosidic bonds in cellulose.

The concept of the plant cell wall composed of microfibrils embedded in a matrix (Fig. 2) is useful, but it is misleading to consider this composite as a classical two-phase system comprised of a crystalline cellulose phase and an amorphous phase including all other components (Atalla, 1993). In a true two-phase system, material at the phase boundary is an insignificant proportion of the total, but cellulose microfibrils from most plants are sufficiently small (Fig. 1) that chains exposed on the surface are a large proportion of the total. From this perspective, we see that polymer chains at the boundary of microfibrils, intimately associated with the various matrix molecules, form a significant proportion of the substrate presented to organisms that hydrolyze cellulose in plant cell walls. Recent data indicate hemicelluloses can become occluded within and between crystalline cellulose domains during the synthesis of cellulose microfibrils by the bacterium *Acetobacter xylinum*; these data imply that the relationship between cellulose and hemicellulose in the cell walls of higher plants is much more intimate than was previously thought (Atalla *et al.*, 1993). It is possible that molecules at the cellulose–hemicellulose boundaries and those within the crystalline cellulose domains require different enzymes for efficient hydrolysis. If so, this may help to explain why cellulolytic microorganisms typically synthesize a range of different cellulases with apparently overlapping specificities and why some xylanases carry substrate-binding domains with affinity for cellulose (see section 3.3). Consideration of the phase boundary is also relevant to the preparation of cellulose samples for structural analysis and as substrates for cellulases because their removal may cause patterns of aggregation that are not representative of the native state.

Native cellulose occurs in the form of cellulose I, one of several possible crystal lattice types, in which the individual cellulose chains are organized in parallel orientation in the direction of the long axis of the microfibril. Cellulose II is another lattice type produced by alkali treatment (mercerization) of cellulose I; many accounts describe the organization of chains in cellulose II as antiparallel. In these and other possible lattice types (celluloses II, III_I, III_{II}, IV_I and IV_{II}), the cellulose chains are arranged in layered sheets but the various types differ in the organization of hydrogen bonds between adjacent sheets (Marchessault and Sundararajan, 1983; Sarko, 1986). Many discussions of enzymatic cellulose hydrolysis simply consider the various forms of native cellulose as existing between two extremes: crystalline and

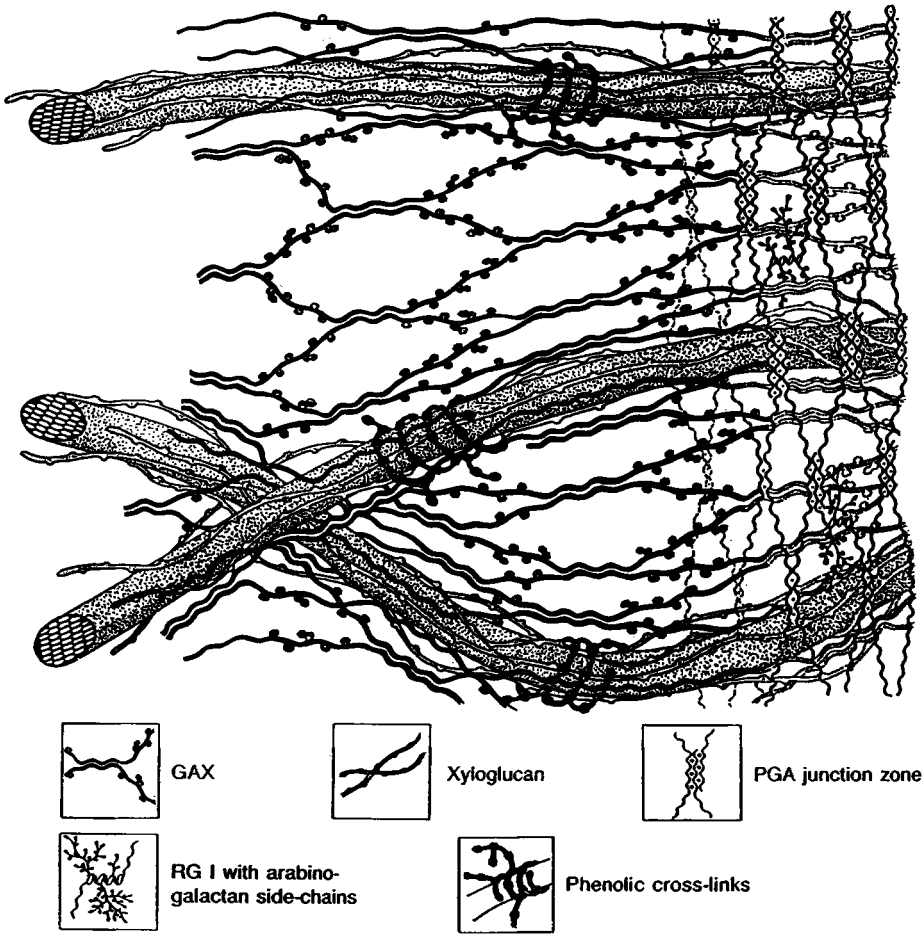


Figure 2 Model of a portion of the primary cell wall from grasses to illustrate some of the complexities faced by micro-organisms that hydrolyze plant biomass. The model, based on analyses of coleoptiles from maize and related monocots, shows a single stratum of cellulose microfibrils just after cell division; several such strata coalesce to form a cell wall. The microfibrils are interconnected with glucuronoarabinoxylans, "wired" to the microfibrils by phenolic cross-links. In the walls of many other higher plants, interconnections are formed by xyloglucan molecules, hydrogen-bonded to the microfibril surfaces. The relationship between cellulose microfibrils and hemicellulases may be even closer than this representation implies, as discussed in the text. Further complexities are introduced by the deposition of protein and lignin after cell elongation ends. (Reproduced from Carpita and Gibeaut, 1993, with permission. Copyright 1993 BIOS Scientific Publishers Ltd, Blackwell Science Ltd and the Society for Experimental Biology.)

amorphous. In this view, cotton and cellulose from bacteria (*Acetobacter* spp.) provide examples of highly crystalline cellulose while cellulose that has been swollen in phosphoric acid (Walseth cellulose) is regarded as amorphous. Common analytical substrates derived from bleached commercial wood pulps, such as Avicel, are seen as a blend of these two forms. Recent studies emphasize that this simple picture of cellulose structure is incomplete and that progress in determining how cellulases act requires a more sophisticated description of native cellulose structures and the way these are modified during sample preparation. The brief discussion that follows attempts to summarize a view of the substrate that reconciles current structural data and is relevant to enzymatic hydrolysis. Many of the concepts are those presented by Atalla *et al.* and readers are referred to original articles for more detailed information (Atalla, 1989, 1990, 1993; Atalla and VanderHart, 1984, 1989).

Cellulose structures can be defined in terms of three organizational levels (Atalla, 1993). The primary level is concerned simply with covalent bonds. It is generally accepted that cellulose is a homopolymer of β-1,4-linked anhydroglucose units although the existence of a small proportion of branch residues is still admissible; if present, they could require specialized enzymes for hydrolysis. The secondary level describes molecular conformation, i.e. the spatial relationship of the repeat units to one another while the tertiary level defines the association of molecules into ordered aggregates such as crystalline lattices. The secondary and tertiary levels of organization of most polymeric systems are not usually considered independently because only one secondary structure is compatible with a given tertiary structure, but there are at least two stable secondary structures for cellulose: k_I and k_{II}; these differ in the arrangements of their intramolecular hydrogen bonds but are able to coexist within a particular lattice because they occupy very similar regions of space. Therefore, cellulose I and II are different lattice types that can accommodate two types of secondary structures; k_I is the predominant structure in cellulose I while k_{II} is predominant in cellulose II (Atalla and VanderHart, 1984).

More recently, Raman spectroscopy, ^{13}C nuclear magnetic resonance (NMR) and microdiffractometry studies have indicated that cellulose I actually contains two distinct crystal lattices: cellulose Iα, the so-called "algal-bacterial" type, with triclinic symmetry, and cellulose Iβ, the "cotton-ramie" type, with monoclinic symmetry (Atalla, 1990, 1993; Sugiyama *et al.*, 1993). The two forms differ in the organization of their intermolecular hydrogen bonds (Atalla and VanderHart, 1989). Cellulose Iα and Iβ occur in characteristic proportions in celluloses from different sources. Iα predominates in cell walls of primitive organisms such as *Acetobacter* and some algae (e.g. *Laminaria*, *Cladophora* and *Valonia* spp.) that synthesize cellulose from linear terminal complexes. The cell walls of other algae and higher plants, such as *Chara* spp. and cotton, are rich in Iβ and are associated

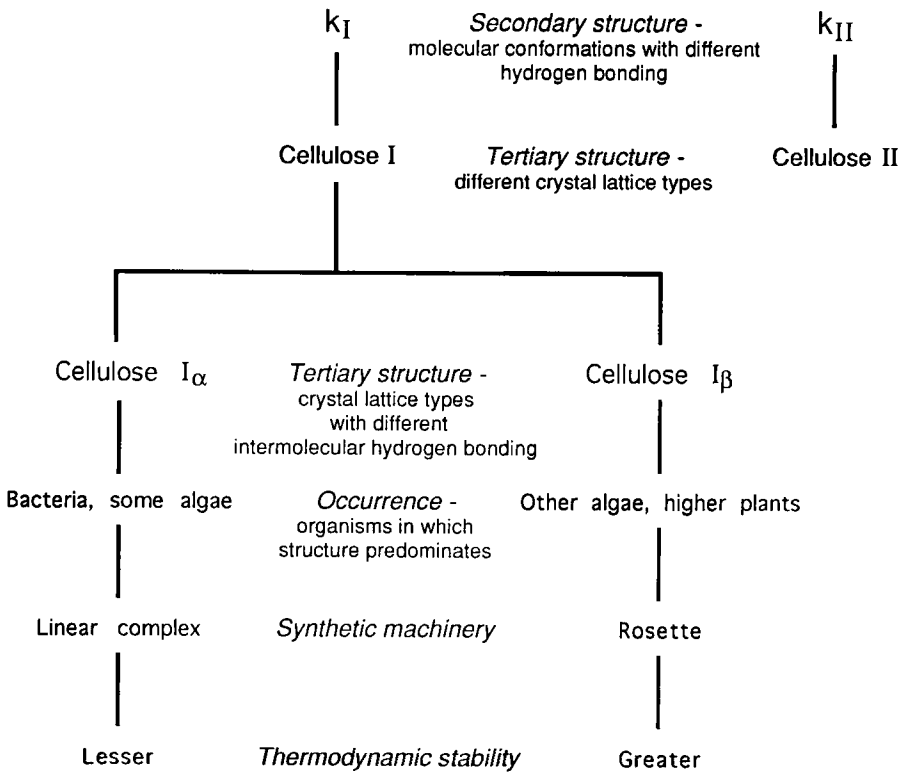


Figure 3 Summary of structures and properties of celluloses I and II. (Adapted from Coughlan, 1992.)

with biosynthetic complexes in the form of rosettes (Atalla, 1989, 1990; Atalla and VanderHart, 1989). Animal cellulose from tunicates is entirely Iβ (Belton *et al.*, 1989) but there is no known source of pure cellulose Iα. Microdiffraction studies on algal cellulose led to the conclusion that the Iα and Iβ forms alternate along the length of microfibrils (Sugiyama *et al.*, 1991) but studies on *Acetobacter* cellulose suggest that Iα and Iβ domains coexist throughout the cross-section of microfibrils (Hackney *et al.*, 1995). Cellulose Iα is thermodynamically less stable and is converted into Iβ by annealing (Yamamoto *et al.*, 1989). These concepts are summarized in Fig. 3. The antiparallel organization of cellulose II, proposed on the basis of X-ray crystallographic data (Blackwell, 1982), has also been questioned: energy minimization studies, for example, indicate that both parallel and antiparallel orientations are plausible (Sakthivel *et al.*, 1989).

Mechanistic studies must eventually address how cellulases hydrolyze both cellulose Iα and Iβ. Cellulose Iα is more susceptible than cellulose Iβ to attack by cellulase mixtures from *Trichoderma* sp. (Sugiyama *et al.*, 1993).

Preliminary studies suggest that CBHI preferentially hydrolyzes cellulose Iα while CBHII prefers cellulose Iβ (M. Ishihara, personal communication). Therefore, the dimorphism of cellulose I may also be related to the diversity of β-1,4-glucanases secreted by cellulolytic microorganisms. The geometry of the crystal lattice is also relevant: modelling studies predict that particular sites on the crystal surface are more susceptible to attack (Henrissat *et al.*, 1988) and there is some evidence that cellulases, or their isolated cellulose-binding domains (CBDs), adsorb to specific faces or corners of *Valonia* cellulose microcrystals (Chanzy *et al.*, 1984; Gilkes *et al.*, 1993) (see section 3.3). The extent of crystallinity of purified cellulose is a function of the preparative and analytical methods employed but it is clear that cellulose microfibrils are not uniformly crystalline: imperfections in packing or mechanical damage result in a proportion of substrate in which the lattice is disordered or paracrystalline (Kulshreshtha and Dweltz, 1973; Scallan, 1971). Models describing enzymatic hydrolysis usually focus on these so-called amorphous regions where hydrogen bonding interactions are perturbed as the primary sites of attack (see section 4.2).

It is clear from the above discussion that cellulose microfibrils integrated into the plant cell wall are the normal substrate for cellulolytic organisms in natural environments, but biochemical studies always use cellulose in some modified form to enable or simplify analyses. Purified preparations of insoluble, unsubstituted plant celluloses are used as close approximations to native crystalline cellulose but soluble, unsubstituted, mixed linkage glucans (e.g. barley β-glucan), soluble, substituted celluloses (e.g. carboxymethylcellulose), as well as soluble cellooligosaccharides and glucosides, are often used to investigate specific aspects of cellulase action. Methods for preparing various cellulosic substrates together with procedures for assaying cellulase activities have been compiled and discussed in detail (Wood and Kellogg, 1988).

Avicel, filter paper or cotton are most often used to study the hydrolytic properties of cellulases or their adsorption to substrate because they are considered representative of highly crystalline cellulose I and are readily available. However, all are rather heterogeneous and undefined substrates with a significant proportion of non-crystalline material and have complex morphologies that confuse analysis (Atalla, 1993; Marshall and Sixsmith, 1974). Alkali extraction during preparation of Avicel from bleached wood pulps results in some conversion to cellulose II. Obviously, data obtained with such materials should be interpreted with great caution: for example, enzymatic studies in which only a small proportion of the total substrate is attacked do not necessarily describe the hydrolysis of cellulose I. Ramie fibre appears to be a better choice because of its simpler morphology. *Acetobacter* and tunicate cellulose also have simple morphologies and are suitable substrates to differentiate the behaviour of enzymes on cellulose Iα and Iβ, respectively (Atalla, 1993). The other cellulose lattice types (celluloses II,

III_I, III_{II}, IV_I and IV_{II}) are potentially useful substrate analogues to investigate enzyme action (Weimer *et al.*, 1991); celluloses II and IV_I may have physiological significance because of their possible occurrence in some plant species (Chanzy *et al.*, 1979; Kuga and Brown, 1991). Cotton or Avicel that has been swollen in concentrated phosphoric acid (Walseth, 1971) is often regarded as a convenient source of amorphous (i.e. non-crystalline) cellulose, but its structure is unclear and recent accounts describe it as a low crystallinity form of cellulose II; amorphous cellulose prepared by regeneration from a solution in dimethyl sulphoxide–sulphur dioxide–ethylene diamine is a preferable alternative (Atalla, 1993).

3. STRUCTURE AND FUNCTION OF CELLULASES AND RELATED POLYSACCHARIDE HYDROLASES

3.1. General Aspects

The efficient hydrolysis of cellulose requires the interaction of several hydrolases and the enzymes produced by cellulolytic microorganisms typically include an array of endoglucanases and exoglucanases (cellobiohydrolases). This section describes recent developments in our understanding of the structure and function of the individual enzymes. Cellulase systems are always associated with other, related polysaccharide hydrolases, particularly xylanases, because of the close relationship of hemicelluloses with cellulose in the plant cell wall (see section 2), and additional insights are drawn from consideration of these enzymes. More detailed information on xylanases is contained in several recent reviews (Coughlan and Hazlewood, 1993; Gilbert and Hazlewood, 1993; Hazlewood and Gilbert, 1993b; Thomson, 1993).

It is now apparent that most cellulases and xylanases are composed of a catalytic domain joined to one or more ancillary domains or modules, frequently by a recognizable linker sequence (Gilkes *et al.*, 1991b); only a few microbial cellulases and the cellulases produced by higher plants (e.g. *Phaseolus vulgaris* EgI, Table 1) have just a catalytic domain (Fig. 4). This modular organization is shared by other enzymes that hydrolyze insoluble polymeric carbohydrates, such as amylases and chitinases (Coutinho and Reilly, 1994; Flach *et al.*, 1992), and the various arrangements of domains seen in these enzymes appear to have evolved by a process of domain shuffling (Guisseppi *et al.*, 1991; Warren *et al.*, 1986). The occurrence of two domains in certain fungal and bacterial cellulases was first demonstrated by the analysis of products following limited proteolysis; the linker regions of many cellulases are particularly sensitive to proteolysis, presumably because they are exposed, and the individual domains are released as discrete

Table 1 Classification of cellulase and xylanase catalytic domains into structurally related families according to amino acid sequence similarities.

Family[a]	Organism[b]	Enzyme	Accession no.[c]
A1	*Aspergillus aculeatus* (KSM510)	Man	L35487
	Bacillus polymyxa	Egl	M33791/M33840/A35136
	Caldocellum saccharolyticum	CelB[f]	X13602
	Cellulomonas fimi	CenD	L02544
	Cellulomonas flavigena	ORFA[g]	Al-Tawheed (1988)
	Clostridium thermocellum	CelB	X03592
	Clostridium thermocellum	CelG	X69390
	Pseudomonas fluorescens	EGC	X61299
	Trichoderma reesei	Man	L25310
	Xanthomonas campestris	EngXCA	M32700
A2	*Bacillus circulans*	EG3A	Hansen (1989)
	Bacillus sp.	CelB	Z33876
	Bacillus sp. (1139)	End1	D00066/M15743
	Bacillus sp. (KSM-64)	End	M84963
	Bacillus sp. (KSM-635)	End2	M27420
	Bacillus sp. (N-4)	CelA	M14729
	Bacillus sp. (N-4)	CelB	M14781
	Bacillus sp. (N-4)	CelC	M25500
	Bacillus subtilis (BaG10)	End	M38634
	Bacillus subtilis (BSE616)	End1	D01057
	Bacillus subtilis (CK2)	Cel	X67044
	Bacillus subtilis (N-24)	End2	M28332
	Bacillus subtilis (PAP 115)	End1	X04689/Z29076
	Bacillus subtilis (DLG)	End2	M16185
	Butyrivibrio fibrisolvens (A46)	CelA	M37031
	Clostridium acetobutylicum	Egl	M31311
	Clostridium josui	CelA	D16670
	Erwinia carotovora	CelV	X76000/X79241

	Erwinia chrysanthemi	CelZ	Y00540
	Ruminococcus albus (F-40)	EGIV	D16315
	Streptomyces lividans (66)	CelA	M82807
	Thermomonospora fusca	E5	L01577
A3	*Candida albicans*	Exg	X56556
	Clostridium thermocellum (F1)	EG C307	D00945
	Clostridium thermocellum	CelC	M19422
	Fibrobacter succinogenes	Egl3	M29047
	Ruminococcus flavefaciens	CelA	X51944
	Saccharomyces cerevisiae	EXG1	M34341
	Saccharomyces cerevisiae	EXG	X59259/S52935
	Saccharomyces cerevisiae	Lam	X59259
	Unidentified bacterium	Cel	U12011
A4	*Bacillus lautus*	CelB	M33762
	Bacteroides ruminocola	Egl	M38216
	Butyrivibrio fibrisolvens (H17c)	End1	X17538
	Caldocellum saccharolyticum	ManA[e]	L01257
	Caldocellum saccharolyticum	CelC[f]	Bergquist *et al.* (1993)
	Clostridium cellulolyticum	CelCCA	M93096/M32362
	Clostridium cellulolyticum	CelCCD	D90341
	Clostridium cellulovorans	EngB	M37506
	Clostridium cellulovorans	EngD	M37434
	Clostridium longisporum	CelA	L02868
	Clostridium thermocellum	CelE	M22759
	Clostridium thermocellum	CelH	M31903
	Neocallimastix patriciarum	CelB	Z31364
	Prevotella ruminicola AR20	End	S22458
	Prevotella ruminicola	Xyn	M83379
	Ruminococcus albus (8)	CelA	L04563
	Ruminococcus albus (AR67)	EGA	L10243
	Ruminococcus albus (F-40)	Egl1	M30928
	Ruminococcus albus (SY3)	CelA	X54931

Table 1—continued

Family[a]	Organism[b]	Enzyme	Accession no.[c]
	Ruminococcus albus (SY3)	CelB	X54932
	Ruminococcus flavefaciens (17)	EndA	S55178
	Streptomyces lividans	ManA	M92297
	Xanthomonas albilineans	EG	L26543
A5	*Cryptococcus flavus*	EG	D13967/S45137
	Humicola insolens	EGII	X76046
	Pseudomonas solanacearum	Egl	M84922
	Robillarda sp. (Y-20)	Egl	JX0131
	Sclerotinia sclerotiorum	EG2[d]	S13663
	Trichoderma reesei	EGII	M19373
B	*Agaricus bisporus*	Cel3a	L24519
	Agaricus bisporus	Cel3b	L24520
	Cellulomonas fimi	CbhA	L25809
	Cellulomonas fimi	CenA	M15823
	Cellulomonas flavigena	ORFB[g]	Al-Tawheed (1988)
	Fusarium oxysporum	B homologue	L29377
	Humicola insolens	CBHII	Schou *et al.* (1993)
	Humicola insolens	EGVI	Schou *et al.* (1993)
	Microbispora bispora	CelA	P26414
	Phanerochaete chrysosporium ME446	CBHII	Tempelaars *et al.* (1994)
	Streptomyces halstedii	Cel1	Z12157
	Streptomyces sp. (KSM-9)	CasA	L03218
	Trichoderma reesei	CBHII	M16190/A03821/M55080
	Thermomonospora fusca	E2	M73321
	Thermomonospora fusca	E3	Spezio *et al.* (1993a)
C	*Fusarium oxysporum*	C homologue 1	L29378
	Fusarium oxysporum	C homologue 2	L29379
	Humicola insolens	CBHI	Schou *et al.* (1993)

	Humicola insolens	EGI	Schou *et al.* (1993)
	Humicola grisea	CBHI	X17258
	Neurospora crassa	CBHI	X77778
	Penicillium janthinellum	CBHI	X59054
	Phanerochaete chrysosporium	CBHI	M22220
	Phanerochaete chrysosporium	CBHI.1	Z22528
	Phanerochaete chrysosporium	CBH1-1	X54411/S40817
	Phanerochaete chrysosporium	CBH1-2	X54411/S40817
	Phanerochaete chrysosporium	CBH1-3	Covert *et al.* (1992b)
	Phanerochaete chrysosporium	CBH1-4	L22656
	Phanerochaete chrysosporium	CBH1-5[d]	Z11730
	Phanerochaete chrysosporium	CBH1-6[d]	Z11731
	Trichoderma koningii (G-39)	CBHI	X69976
	Trichoderma longibrachiatum	CBHI	X60652
	Trichoderma reesei	CBHI	P00725
	Trichoderma reesei	EGI	M15665
	Trichoderma viride	CBHI	X53931
D	*Acetobacter xylinum*	EglA	M96060
	Bacillus circulans	Bgc	X52880
	Bacillus sp. (KSM-330)	Endo-K	M68872
	Cellulomonas uda	Egl	M36503
	Clostridium cellulolyticum	CelCCC	M87018
	Clostridium josui	EglB	D16670
	Clostridium thermocellum	CelA	K03088
	Erwinia chrysanthemi	CelY	M74044
E1	*Butyrivibrio fibrisolvens*	CedI	X55732
	Fibrobacter succinogenes (S85)	EGB	L14436
	Cellulomonas fimi	CenC	X57858
	Clostridium cellulolyticum	CelCCE[d]	M87018
	Clostridium thermocellum	CelD	X04584
	Clostridium thermocellum	Cbh3	X80993

Table 1—continued

Family[a]	Organism[b]	Enzyme	Accession no.[c]
	Pseudomonas fluorescens	EglA	X12570
	Streptomyces reticuli	Cel1	X65616
	Thermomonospora fusca	E1	L20094
E2	*Arabidopsis thaliana*	Egl	U17888
	Caldocellum saccharolyticum	CelA[e]	L32742
	Caldocellum saccharolyticum	CelC[e]	Bergquist *et al.* (1993)
	Cellulomonas fimi	CenB	M64644/M33026
	Clostridium cellulolyticum	CelCCG	M87018
	Clostridium cellulovorans	CelC	M95180
	Clostridium josui	ORF2[g]	D16670
	Clostridium thermocellum	CelF	X60545
	Clostridium thermocellum	CelI	L04735
	Clostridium stercorarium	CelZ	X55299
	Dictyostelium discoideum	CelA	M33861
	Fibrobacter succinogenes (AR1)	$EndA_{FS}$	M58520
	Glycine max	Egl1	U00730
	Glycine max	Egl2	U00731
	Lycopersicon esculentum	Cel1	U13054
	Lycopersicon esculentum	Cel2	U13055
	Persea americana	Egl	M17634/X59944
	Persea americana	Cel1	X55790
	Persea americana	Cel2	X55790
	Phaseolus vulgaris	Egl	M57400
	Prunus persica	Egl	Z23119
	Sambucus nigra	Egl	X74290
	Thermomonospora fusca	E4	M73322/L20093
F	*Actinomadura* sp.	XynII	U08894
	Aspergillus niger (=kawachii)	XynA	D14847

	Bacillus sp. (C-125)	XynA	D00087
	Bacillus stearothermophilus 21	XynA	Z29080/D28121
	Bacteroides ovatus	Xyn	U04957
	Butyrivibrio fibrisolvens (H17c)	XynB	X61495
	Butyrivibrio fibrisolvens	XynA	A37755
	Caldocellum saccharolyticum	CelB[e]	X13602
	Caldocellum saccharolyticum	XynA	M34459
	Clostridium stercorarium (F-9)	XynB	D12504
	Cellulomonas fimi	Cex	L11080/M15824
	Clostridium thermocellum	XynX	M67438
	Clostridium thermocellum	XynY	X83269
	Clostridium thermocellum	XynZ	M22624
	Cryptococcus albidus	Xyn	X12596
	Filobasidium floriforme	Xyn	JS0734
	Fusarium oxysporum	Xyn	L29380
	Magnaporthe grisea	Xyn	L37530
	Neocallimastix patriciarum	XylB	X76919/S71569
	Penicillium chrysogenum	Xyl	M98458
	Pseudomonas fluorescens	XynA	X15429
	Pseudomonas fluorescens	XynB	X54523
	Ruminococcus flavefaciens	XynA[f]	Z11127
	Streptomyces lividans	XlnA	M64551
	Streptomyces thermoviolaceus	XynI	A43937
	Thermoanaerobacter saccharolyticum	XynA	M97882
	Thermoascus aurantiacus	Xyn	S16922
	Thermophilic bacterium (rt8.84)	XynA	L18965
	Thermotoga maritima	XynA	Z46264
G	*Aeromonas caviae*	XynA	D32065
	Aspergillus awamori	Xyn	X78115
	Aspergillus niger	XynA	A19535
	Aspergillus kawachii	XynC	D14848/S45138
	Aspergillus tubigensis	XynA	L26988

Table 1—continued

Family[a]	Organism[b]	Enzyme	Accession no.[c]
	Aureobasidium pullulans	XynA	U10298
	Bacillus sp.	XynS	X59058
	Bacillus sp.	XynY	X59059
	Bacillus circulans	Xyn	X07723
	Bacillus pumilus	XynA	X00660
	Bacillus stearothermophilus	XynA	U15985
	Bacillus subtilis	Xyn	M36648
	Cellulomonas fimi	XynD	X76729
	Chainia sp.	Xyn	Bastawde *et al.* (1991)
	Clostridium acetobutylicum	XynB	M31726
	Clostridium stercorarium	XynA	D13325
	Cochliobolus carbonum	Xyn1	L13596
	Fibrobacter succinogenes	XynC[e]	U01037
	Fibrobacter succinogenes	XynC[f]	U01037
	Humicola insolens	Xyl1	X76047
	Magnaporthe grisea	Xyn22	L37529
	Neocallimastix frontalis	Xyn1	X82266
	Neocallimastix frontalis	Xyn2	X82439
	Neocallimastix patriciarum	XylA[e]	X65526
	Neocallimastic patriciarum	XylA[f]	X65526
	Nocardiopsis dassonvillei	XynII	PQ0202
	Ruminococcus flavefaciens	XynA[e]	Z11127
	Ruminococcus flavefaciens	XynB[e]	Z35226
	Ruminococcus flavefaciens	XynD[e]	S61204
	Schizophyllum commune	Xyn	A44597
	Schizophyllum commune	Xyn	Shareck *et al.* (1991)
	Streptomyces lividans	XlnB	M64552
	Streptomyces lividans	XlnC	M64553
	Streptomyces sp. (36a)	Xyn	Shareck *et al.* (1991)

	Stretptomyces sp. (EC3)	Xyn	X81045
	Streptomyces thermoviolaceus	XynII	Tsujibo *et al.* (1992)
	Thermomonospora fusca	XynA	U01242
	Trichoderma harzianum	Xyn	A44593
	Trichoderma reesei	Xyn1	X69573/S51973
	Trichoderma reesei	Xyn2	X69574/S51975
	Trichoderma viride	Xyn	A44594/A44595
H	*Aspergillus aculeatus*	Egl	X52525
	Aspergillus kawachii	Egl	D12901
	Aspergillus oryzae (KBN616)	Egl	Kitamoto *et al.* (1993)
	Erwinia carotovora	CelS	M32399
	Humicola insolens	EGIII	Schou *et al.* (1993)
	Streptomyces lividans	CelB	U04629
	Streptomyces rochei (A2)	EglS	X73953
	Trichoderma reesei	EGIII	Ward *et al.* (1993)
I	*Bacillus* sp.	Man	M31797
	Clostridium thermocellum	EglH	M31903
	Bacteroides ruminocola	Egl	M38216
J	*Bacillus lautus*	CelA	M76588
	Caldocellum saccharolyticum	ManA[f]	L01257
	Ruminococcus flavefaciens	CelB	U08621
K	*Fusarium* sp.	K homologue	L29381
	Humicola insolens	EGV	Schou *et al.* (1993)
	Pseudomonas fluorescens	EGB	X52615
	Trichoderma reesei	EgV	Z33381
L	*Caldocellum saccharolyticum*	CelA[f]	L32742
	Cellulomonas fimi	CbhB	L38827
	Clostridium cellulolyticum	CelCCF	M87018
	Clostridium cellulovorans	P70	Doi *et al.* (1993)

Table 1—continued

Family[a]	Organism[b]	Enzyme	Accession no.[c]
	Clostridium josui	ORF1[g]	D16670
	Clostridium thermocellum (ATCC27405)	CelS	L06942
	Clostridium thermocellum (YS)	S8	Morag *et al.* (1993)
	Clostridium stercorarium (NCIB11745)	Avicelase II	Bronnenmeier *et al.* (1991)

[a]Families are classified according to Gilkes *et al.* (1991b); subtypes (Béguin, 1990) are designated by additional numbers. The alternative family nomenclature (Henrissat and Bairoch, 1993; Henrissat, personal communication) is as follows: A, 5; B, 6; C, 7; D, 8; E, 9; F, 10; G, 11; H, 12; I, 26; J, 44; K, 45; L, 48.
[b]Strain designations are given in parentheses.
[c]GenBank, SWISS-PROT, EMBL or PIR database accession numbers are given if available; literature references are cited in other cases.
[d]Partial sequence data only.
[e]N-terminal catalytic domain.
[f]C-terminal catalytic domain.
[g]Translated open reading frame.

functional units (Gilkes *et al.*, 1988; Tomme *et al.*, 1988; Van Tilbeurgh *et al.*, 1986). Domains in new enzymes can now usually be recognized by comparison of their primary structures with those of other enzymes whose structural and functional organizations have been defined; functions can then be analyzed by expression of appropriate gene fragments, followed by biochemical analysis. The ancillary domains and modules identified in cellulases and xylanases include cellulose-binding domains (CBDs), the "duplicated segments" in enzymes from *Clostridium* spp., S-layer-like modules, and the fibronectin-type III (Fn3) modules typical of *Cellulomonas fimi* cellulases; there are also various regions of unknown function.

The majority of fungal endoglucanases and cellobiohydrolases are composed of a catalytic domain and a C-terminal CBD, joined by a linker rich in proline and hydroxy amino acid residues (Gilkes *et al.*, 1991b). Some fungal β-1,4-glycanases have an N-terminal CBD; others, notably those from *Neocallimastix* spp., have no CBD but contain C-terminal repeated sequences of undefined function (Black *et al.*, 1994). The bacterial enzymes show much more diversity: they range from small, single domain cellulases to very large multidomain enzymes containing two CBDs, or even two catalytic domains, joined by various linker sequences. The structural linkage of two catalytic domains can be considered as a strategy for enzyme regulation, as discussed in section 5.3.

3.2. Catalytic Domains

3.2.1. *Structural Families of Catalytic Domains*

The catalytic domains of nearly all cellulases and xylanases can be classified into one of a few distinct families on the basis of amino acid sequence similarities, although the degree of similarity is often low. The original classification of 21 cellulases and xylanases into six enzyme families used a comparative technique called hydrophobic cluster analysis (Henrissat *et al.*, 1989). In general, structurally related proteins contain hydrophobic cores which retain a common fold and peripheral regions with more divergent structures (Chothia and Lesk, 1986). The success of hydrophobic cluster analysis can be attributed to its ability to detect regions corresponding to folds that are highly conserved because they are part of the catalytic core. The number of cellulases and xylanases with known primary structures has now grown to over 200 and, in most cases, new enzymes can be classified into established families by more conventional amino acid sequence comparison techniques that detect characteristic sequence motifs. These motifs include those containing the aspartic and glutamic acid residues directly involved in catalysis, and comparison with well-characterized enzymes allows these residues to be identified in new enzymes (Tull *et al.*, 1991; Wang *et al.*,

1993a). A recent classification of several types of glycosyl hydrolases, including chitinases, amylases and β-1,3-glucanases, in addition to cellulases and xylanases, describes 45 enzyme families (Henrissat and Bairoch, 1993). An updated classification of cellulase and xylanase catalytic domains using available sequence data is contained in Table 1; related cellodextrinases, β-1,3(4)-glucanases and β-1,4-mannanases are included.

The number of cellulases and xylanases with known three-dimensional structures is still relatively small but the predicted folding similarity within families has been confirmed in families B and G (Table 2). Families of cellulases and xylanases generally have distinct folds, as discussed in section 3.2.4. Despite the wealth of comparative data already available, new families of cellulases may yet be discovered: family L (Table 1) is a new family of cellulases which has recently emerged (see section 4.3.1).

Family C presently contains only fungal enzymes; the known members of families D and L are all from bacteria; however, other families contain both bacterial and fungal enzymes (families A, B, F, G, H and K) or prokaryotic and plant enzymes (family E), suggesting that lateral transfer of β-1,4-glycanase genes has occurred (Gilkes *et al.*, 1991b). Furthermore, within a given family, sequence-based taxonomy is not in accordance with the accepted bacterial phylogeny, again implying that these genes are being spread by extensive horizontal transfer (Bork and Doolittle, 1992). This is particularly obvious in those families containing genes from both Gram-positive and Gram-negative organisms (e.g. families A and D). A good example is the high sequence similarity between endoglucanase Y from the Gram-negative bacterium *Erwinia chrysanthemi* and an endoglucanase from *Cellulomonas uda*, a Gram-positive bacterium: the G+C content of the *celY* gene matches that of the *E. chrysanthemi* chromosome (58 *vs.* 57 mol % respectively), but there is a large discrepancy between the G+C content of the *C. uda egl* gene (61%) and the rest of the *C. uda* chromosome (72%), suggesting recent transfer of the gene to *C. uda* (Guisseppi *et al.*, 1991).

Figure 4 Schematic representation of single and multidomain cellulases and xylanases showing the diversity of structural and functional organization. The lengths of the domains and modules are proportional to the number of amino acid residues. Catalytic and cellulose-binding domain families are indicated by letters and numbers; Family 16 is a family of β-1,3(4)-glucanases (Henrissat and Bairoch, 1993). CfiCenA, *Cellulomonas fimi* endoglucanase A (Wong *et al.*, 1986); CfiCenB, *Cellulomonas fimi* endoglucanase B (Meinke *et al.*, 1991a); CfiCenC, *Cellulomonas fimi* endoglucanase C (Coutinho *et al.*, 1991); CfiCbhB, *Cellulomonas fimi* cellobiohydrolase B; CfiXynD, *Cellulomonas fimi* xylanase D (Millward-Sadler *et al.*, 1994); CthCelD, *Clostridium thermocellum* endoglucanase D (Joliff *et al.*, 1986); RflXylD, *Ruminococcus flavefaciens* xylanase D (Flint *et al.*, 1993a); TreEGIII, *Trichoderma reesei* endoglucanase III (Ward *et al.*, 1993); TsaXynA, *Thermoanaerobacterium saccharolyticum* BA-RI xylanase A (Lee *et al.*, 1993).

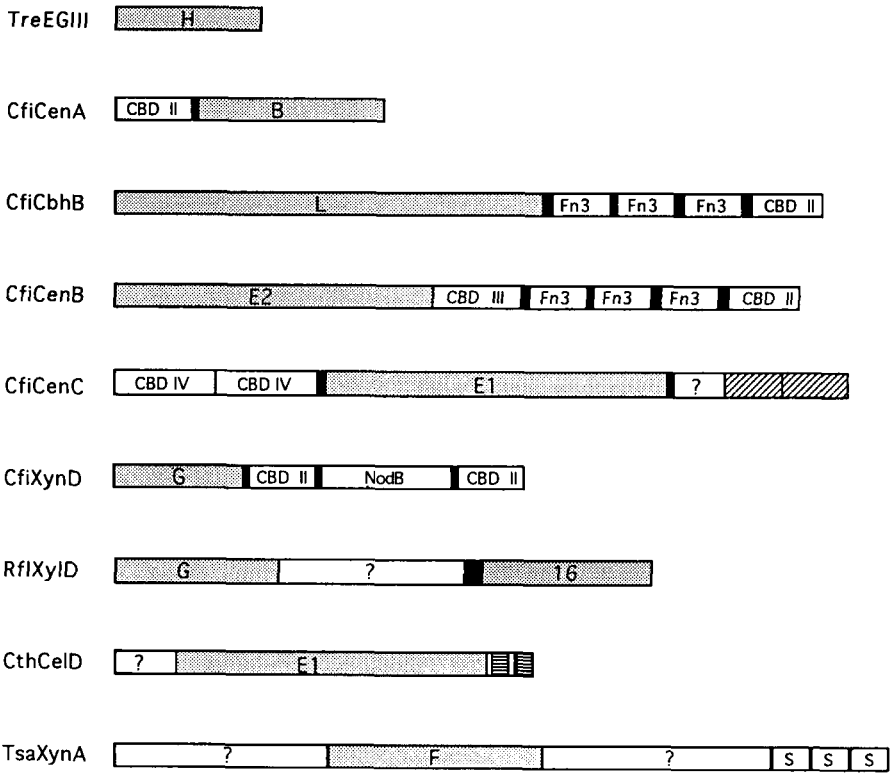

TreEGIII
H
CfiCenA
CBD II
B
CfiCbhB
L
Fn3
Fn3
Fn3
CBD II
CfiCenB
E2
CBD III
Fn3
Fn3
Fn3
CBD II
CfiCenC
CBD IV
CBD IV
E1
?
CfiXynD
G
CBD II
NodB
CBD II
RfIXyID
G
?
16
CthCelD
?
E1
TsaXynA
?
F
?
S
S
S
Catalytic domain (letters refer to cellulase family)
CBD
Cellulose-binding domain (numbers refer to CBD family)
Fn3
Fn3 module
Duplicated segment
NodB
Nodulation B-like module
C-terminal repeat
S
S-layer-like module
?
unknown function
Linkers

Table 2 Catalogue of cellulases and xylanases for which X-ray crystallographic analysis has been reported or is in progress.

Family[a]	Organism	Enzyme	Reference
H	*Aspergillus aculeatus*	Egl[b]	Okada (1991)
–	*Aspergillus oryzae*	Xyn	Golubev *et al.* (1993)
G	*Bacillus pumilus*	XynA[b]	Arase *et al.* (1993)
G	*Bacillus circulans*	Xyn[b]	Campbell *et al.* (1993)
G	*Bacillus* sp. strain D3	Xyn	Pickersgill *et al.* (1993)
A3	*Candida albicans*	Exg	Cutfield *et al.* (1992)
F	*Cellulomonas fimi*	Cex[b]	White *et al.* (1994)
A4	*Clostridium cellulolyticum*	CelCCA	Roig *et al.* (1993)
A3	*Clostridium thermocellum*	CelC	Dominguez *et al.* (1994)
E1	*Clostridium thermocellum*	CelD[b]	Juy *et al.* (1992)
F	*Clostridium thermocellum*	XynZ	Souchon *et al.* (1994)
C	*Humicola insolens*	EGI	Davies *et al.* (1992)
K	*Humicola insolens*	EGV[b]	Davies *et al.* (1993)
F	*Pseudomonas fluorescens*	XynA[b]	Harris *et al.* (1994)
F	*Streptomyces lividans*	XynA[b]	Derewenda *et al.* (1994)
F	*Thermoascus aurantiacus*	Xyn	Viswamitra *et al.* (1993)
B	*Thermomonospora fusca*	E2[b]	Spezio *et al.* (1993b)
G	*Trichoderma harzianum*	Xyn[b]	Campbell *et al.* (1993)
C	*Trichoderma reesei*	CBHI[b]	Divne *et al.* (1994)
B	*Trichoderma reesei*	CBHII[b]	Rouvinen *et al.* (1990)
C	*Trichoderma reesei*	EGI	Divne *et al.* (1993)
H	*Trichoderma reesei*	EGIII	Ward *et al.* (1993)
G	*Trichoderma reesei*	XYNI[b]	Törrönen and Rouvinen (1995)
G	*Trichoderma reesei*	XYNII[b]	Törrönen *et al.* (1994)

[a]Families are classified according to Gilkes *et al.* (1991b); Subtypes (Béguin, 1990) are designated by additional numbers. The alternative family nomenclature according to Henrissat and Bairoch (1993) is as follows: A, 5; B, 6; C, 7; E, 9; F, 10; G, 11; H, 12; K, 45.
[b]Crystal structure solved.

3.2.2. *Functional Families of Catalytic Domains*

Similarities in folding patterns and overall conservation of active site topology within a given enzyme family can now be predicted with confidence (Zvelebil and Sternberg, 1988). Therefore, it is anticipated that all members of a particular family will use the same reaction mechanism (see section 3.2.3) and have related substrate specificities. The substrate specificities of 15 cellulases, representing six enzyme families, are in general agreement with the established structural classification (Claeyssens and Henrissat, 1992). Small differences in the hydrolysis patterns of small soluble chromophoric and fluorophoric substrates shown by members of some families (e.g. A and E) are consistent with the existence of subtypes; however, at least

three families contain enzymes with clearly different substrate specificities. Family D contains both β-1,4-glucanases and β-1,3(4)-glucanases; family H contains members with β-1,4-mannanase and β-1,4-glucanase activity; family A contains β-1,4-glucanases, β-1,3-glucanases, β-1,4-mannanases, cellodextrinases and at least one β-1,4-xylanase (Table 1).

Other families show more subtle differences in substrate specificity: for example, Cex from *C. fimi*, is a β-1,4-xylanase, like the other enzymes in family F (Gilkes *et al.*, 1991a), but it also has activity on aryl β-1,4-cellobioside and insoluble cellulose (Gilkes *et al.*, 1984) while other family F xylanases, such as *Neocallimastix patriciarum* XylB (Black *et al.*, 1994) have no detectable activities on cellulose or the aryl-β-glucosides. Obviously, substrate specificities within cellulase families do differ. Comparison of two β-glucanases from barley (Varghese *et al.*, 1994) shows that both enzymes have a similar size, approximately 50% sequence identity, and share superimposable α/β-barrel folds; the two catalytic glutamic acid residues and the surrounding sequence regions are completely conserved. However, the two enzymes have quite distinct substrate specificities: one enzyme hydrolyzes β-1,3 linkages in β-(1,3) and β-(1,3)-β-(1,6)-glucans; the other is active only on the β-(1,4)-linkages in β-(1,3)-β-(1,4)-glucans. These differences are attributed to a few small amino acid substitutions at the end of the substrate-binding groove. Presumably similar considerations apply to related cellulases and xylanases and differences in substrate specificity probably arose by divergent evolution from a common ancestor, through a small number of amino acid substitutions. Families B and C contain both endo-β-(1,4)-glucanases and exo-β-(1,4)-glucanases (cellobiohydrolases). Endo–exo specificity can be attributed to differences in the distribution of surface loops as discussed in section 3.2.4.

Although substrate specificities within families differ, current data show that all members of a given family share the same stereoselectivity (Gebler *et al.*, 1992a; Schou *et al.*, 1993). This finding provides strong circumstantial evidence that family members share similar active site topologies with the same basic catalytic mechanism (see below). Five families (A, C, F, G and H) catalyze hydrolysis with retention of anomeric configuration, six (B, D, E, J, K and L) with inversion (Fierobe *et al.*, 1993; Gebler *et al.*, 1992a; Meinke *et al.*, 1994; Schou *et al.*, 1993; Shen *et al.*, 1994); there are no data available yet for family I.

3.2.3. *Catalytic Mechanisms*

All glycosyl hydrolases catalyze stereoselective hydrolysis: the configuration about the anomeric centre is either inverted (single displacement reaction) or retained (double displacement reaction) upon hydrolysis of the glycosidic linkage. Details of both reaction mechanisms are shown in Fig. 5. Retaining enzymes, but not inverting enzymes, also show transglycosylation activity

(Sinnot, 1990). The catalytic residues in both inverting and retaining enzymes can be tentatively identified by chemical modification (Bray and Clarke, 1994; Macarron *et al.*, 1993; Tomme *et al.*, 1992) or site-directed mutagenesis (Baird *et al.*, 1990; Belaich *et al.*, 1992; Lee *et al.*, 1993; Lüthi *et al.*, 1992; Navas and Béguin, 1993; Py *et al.*, 1991; Tomme *et al.*, 1991), preferably in conjunction with three-dimensional structural analysis (Chauvaux *et al.*, 1992; Ko *et al.*, 1992; Wakarchuk *et al.*, 1994). A more precise technique has been used to define the nucleophile in retaining enzymes. The glycosyl–enzyme intermediate formed during catalysis by these enzymes can be trapped using a mechanism-based inhibitor (e.g. 2′-4′-dinitrophenyl 2-deoxy-2 fluoro-*D*-cellobioside) and the nucleophile identified. This approach allowed unequivocal identification of the nucleophiles in *Agrobacterium faecalis* β-glucosidase (Withers *et al.*, 1990), *C. fimi* Cex (Tull *et al.*, 1991), *Escherichia coli* β-galactosidase (Gebler *et al.*, 1992b), *Clostridium thermocellum* endoglucanase C (Wang *et al.*, 1993a) and *Bacillus subtilis* xylanase (Miao *et al.*, 1994). Mechanism-based inhibitors cannot be used to identify the nucleophile in inverting enzymes because the reaction does not proceed via a glycosyl–enzyme intermediate (Fig. 5).

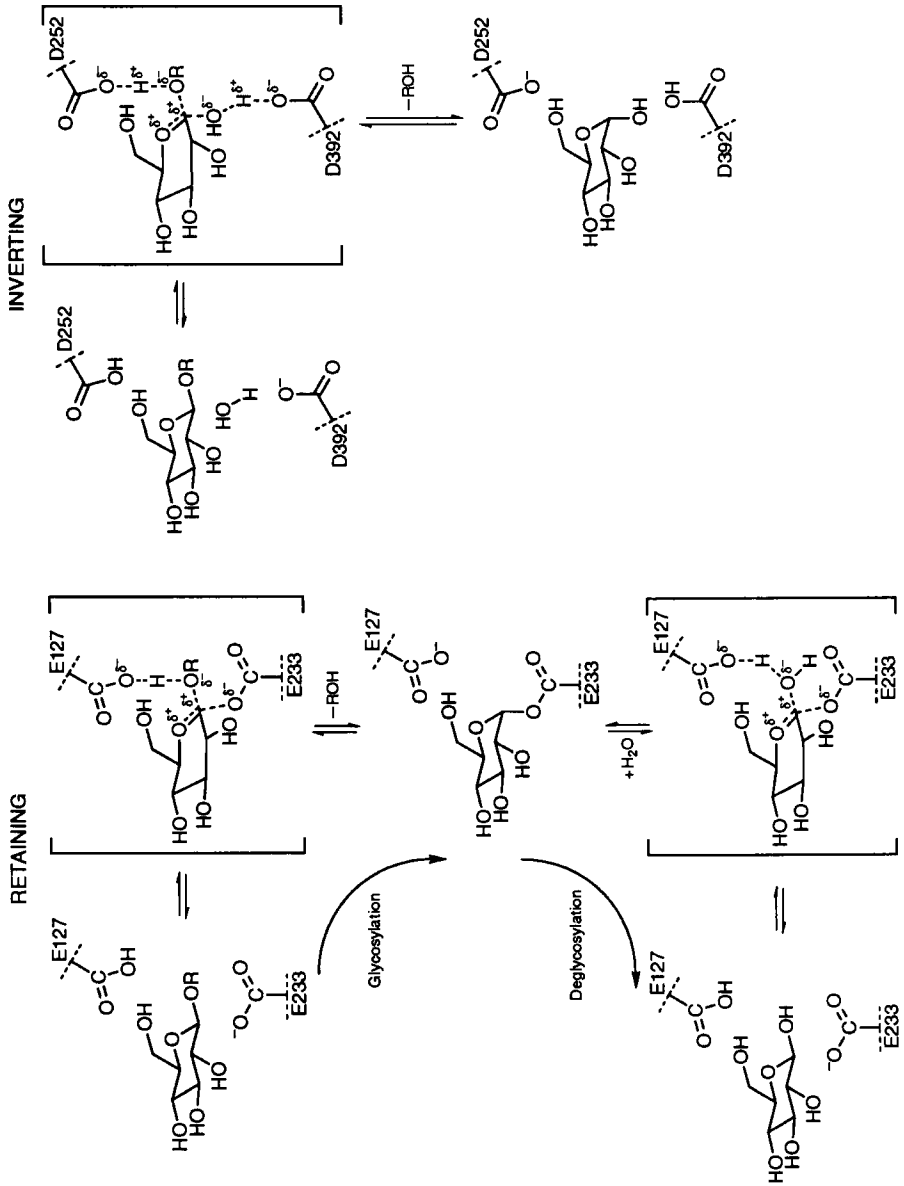
The general acid–base catalyst in Cex (Glu 127) was recently identified by a novel approach that involves a combination of site-directed mutagenesis of conserved glutamic and aspartic acid residues with detailed kinetic analysis of hydrolysis in the presence of sodium azide (MacLeod *et al.*, 1994). The approach relies upon the ability of an azide molecule to replace the acid–base catalyst when it is mutated to an alanine or glycine residue and should be applicable to any cloned cellulase or xylanase in which putative catalytic residues can be identified by sequence comparison.

3.2.4. *Three-dimensional Structures of Cellulase and Xylanase Catalytic Domains*

X-ray crystallographic analysis of about 20 cellulases and xylanases has been reported or is in progress (Table 2). Although few three-dimensional structures have been solved, the number is steadily increasing. Small

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Figure 5 Reaction mechanisms of retaining and inverting β-1,4-glycanases; the numbered catalytic residues are those of Cex (MacLeod *et al.*, 1994; Tull and Withers, 1994; Tull *et al.*, 1991) and CenA (H. Damude, unpublished) from *C. fimi*. In the retaining mechanism, the nucleophile (Glu 233) participates in the formation of a glycosyl–enzyme intermediate; formation and hydrolysis are catalyzed by the general acid/base (Glu 127). In the inverting mechanism, the nucleophile (Asp 392) promotes the ionization of a water molecule which attacks the anomeric centre directly, in a general acid–base (Asp 252)-catalyzed reaction. Both reactions proceed via oxocarbonium ion-like transition states.


INVERTING
RETAINING
D252
D392
E127
E233
−ROH
+ROH
+H2O
Glycosylation
Deglycosylation

cellulases and xylanases can be crystallized as intact enzymes (e.g. Campbell *et al.*, 1993) but crystallization of the multidomain cellulases has proven difficult, probably because the linker regions connecting the domains are flexible; to avoid this problem the catalytic domains of these enzymes are crystallized in isolation (e.g. Bedarkar *et al.*, 1992). Currently three-dimensional structures have been solved for enzymes from families B, C, E, F, G, H and K (Table 2). The evolutionary relationship between the folding patterns of glycosyl hydrolases remains to be established. Representatives of families B, C, E, F and K have distinct folds but the three-dimensional structures determined for *Aspergillus aculeatus* endoglucanase 1 (family H) and *Bacillus pumilus* XynA (family G) show a folding similarity that is not easily detected by comparison of primary structures (Okada, 1991; Törrönen *et al.*, 1993).

The first catalytic domain structure to be solved was that of *T. reesei* exocellobiohydrolase II (CBHII) from family B. This comprises a central α/β barrel structure, similar to that of triose phosphate isomerase; the barrel contains five α-helices and seven β-strands (Rouvinen *et al.*, 1990). The active site is enclosed by two extended surface loops to form a tunnel about 20Å in length and containing at least four subsites, A–D. Catalysis proceeds by inversion of configuration with bond cleavage between subsites B and C. The tunnel-shaped active site is thought to restrict bond cleavage of long cellulose polymers to the non-reducing ends. Although *T. reesei* cellobiohydrolases are reported to have some endoglucanase activity (Ståhlberg *et al.*, 1993a), this is difficult to explain unless the surface loops move when the enzyme encounters the substrate. As predicted by amino acid sequence comparison, the three-dimensional structure of the family B endoglucanase 2 (E2) catalytic domain from *T. fusca* is very similar to that of CBHII, but there is a significant difference in the organization of the active site (Spezio *et al.*, 1993b). The C-terminal surface loop of CBHII is absent in E2 while the N-terminal loop is present in E2 but is pulled back, so that it no longer covers the active site; as a result the E2 active site is shaped like a groove rather than a tunnel. This provides a rational explanation for the endo activity of E2. Consistent with this explanation, the regions corresponding to the CBHII surface loops are absent in all the family B endoglucanases but present in the other two known family B exocellobiohydrolases; in *C. fimi* CbhA, these regions are even longer than those in CBHII (Meinke *et al.*, 1994).

The three-dimensional structure of the catalytic domain of CBHI, the family C *T. reesei* cellobiohydrolase, has now also been solved (Divne *et al.*, 1994). It comprises four short α-helices and 15 antiparallel β-strands that stack to form a curved β-sandwich sheet The active site of CBHI contains seven subsites, A–G, and the proposed location of bond cleavage is between subsites B and C. The CBHI active site is also tunnel-shaped but about twice as long as that of CBHII. It is tentatively proposed that CBHI cleaves cellulose polymers from the reducing end, in accordance with results obtained

using small soluble substrates (Vrsanka and Biely, 1992) and that the chain enters from subsite G. The relatively long tunnel is thought to favour a more processive action than that of CBHII, i.e. for each encounter with the substrate, CBHI hydrolyzes more bonds than CBHII before dissociation.

There is no detailed structural information yet available for EGI, the *T. reesei* family C endoglucanase but, by analogy with CBHII and E2, it is anticipated that the EGI active site will be an open groove. As expected, the regions corresponding to the CBHI active site loops are deleted in EGI. The active sites of two other endoglucanases are known to be groove-shaped. EGV (family K) from *Humicola insolens* comprises a six-stranded β-barrel core, connected by loops, and three α-helices with a long active site groove that runs the entire length of the molecule (Davies *et al.*, 1993). The three-dimensional structure determined for CelD, a family E endoglucanase from *Clostridium thermocellum*, comprises two domains: an amino-terminal immunoglobulin-like domain packed against a larger catalytic domain. The catalytic domain is described as a "twisted α-barrel" and contains six putative subsites along its active site groove (Juy *et al.*, 1992). Closely related eight-stranded α/β barrel structures have been determined for three family F enzymes: *C. fimi* Cex, *Streptomyces lividans* XynA and *Pseudomonas fluorescens* subsp. *cellulosa* XynA (Derewenda *et al.*, 1994; Harris *et al.*, 1994; White *et al.*, 1994). In all three enzymes, the inferred active site is an open cleft at the C-terminus. The family G xylanases from *T. harzianum*, *B. circulans* and *B. pumilus* all comprise a single domain of two or three β-sheets and one α-helix (Arase *et al.*, 1993; Campbell *et al.*, 1993; Törrönen *et al.*, 1994). The active site cleft is formed by a deep groove between two sheets which spans the whole width of the molecule. Interestingly, a similar structure is described for an *A. aculeatus* endoglucanase from family H (Okada, 1991).

3.3. Cellulose-binding Domains

3.3.1. *Structural Families of Cellulose-binding Domains*

Cellulose-binding domains (CBDs) are the most common ancillary domains in cellulases and xylanases (Gilkes *et al.*, 1991b). Most can be classified into five principal families (I, II, III, IV and VI) according to similarities in primary structure (Table 3). Several CBDs in each of these families have been shown to have affinity for cellulose but others are included on the basis of sequence comparison only. Three unrelated, functional CBDs (Durrant *et al.*, 1991; Py *et al.*, 1991; Ramalingam *et al.*, 1992) are placed in families V, VII and VIII. Numerous other β-1,4-glucanases with affinity for cellulose contain other unrelated non-catalytic sequences, some of which are probably novel types of CBD.

Table 3 Classification of cellulose-binding domains (CBDs) into structurally related families according to amino acid sequence similarities.

Family[a]	Organism	Enzyme	Location[b]	Amino acids[c]	Binding[d]	Accession no.[e]
I	*Agaricus bisporus*	Cel1	C	36		M86356
	Agaricus bisporus	Cel3a	N	36		L24519
	Agaricus bisporus	Cel3b	N	36		L24520
	Fusarium oxysporum	B homologue	N	33		L29377
	Fusarium oxysporum	C homologue 2	C	33		L29379
	Fusarium oxysporum	K homologue	C	37		L29381
	Fusarium oxysporum	Xyn	N	36		L29380
	Humicola grisea	CBHI	C	33		X17258
	Humicola insolens	EII	N	36		X76046
	Humicola insolens	EGV	C	33		Saloheimo *et al.* (1993)
	Neocallimastix patriciarum	XylB	C	33		S71569
	Neurospora crassa	CBHI	C	33		X77778
	Penicillium janthinellum	CBHI	C	33		X59054
	Phanerochaete chrysosporium	CBHI	C	34		M22220
	Phanerochaete chrysosporium	CBHI-1	C	34		Z22528
	Phanerochaete chrysosporium	CBH1-2	C	34		X54411/S40817
	Phanerochaete chrysosporium	CBH1-3	C	34		Covert *et al.* (1992a,b)
	Phanerochaete chrysosporium	CBH1-4	C	34		L22656
	Phanerochaete chrysosporium	CBHII	N	36		Tempelaars *et al.* (1994)
	Porphyra purpurea	PBP[f]	×4	33		U08843
	Trichoderma koningii	CBHI	C	33		X69976
	Trichoderma longibrachiatum	CBHI	C	33		X60652
	Trichoderma reesei	CBHI	C	36	+	P00725
	Trichoderma reesei	CBHII	N	36	+	M16190/M55080
	Trichoderma reesei	EGI	C	33	+	M15665
	Trichoderma reesei	EGII	N	36	+	M19373
	Trichoderma reesei	EGV	C	36	+	Z33381
	Trichoderma reesei	Man	C	34		L25310
	Trichoderma viride	CBHI	C	33		X53931

II	*Butyrivibrio fibrisolvens*	End1	C	95		X17538
	Cellulomonas fimi	CenA	N	106	+	M15823
	Cellulomonas fimi	CenB	C	103	+	M64644
	Cellulomonas fimi	CenD	C	105	+	L02544
	Cellulomonas fimi	CbhB	C	106	+	L25809
	Cellulomonas fimi	CbhA	C	104	+	L29042
	Cellulomonas fimi	Cex	C	106	+	L11080/M15824
	Cellulomonas fimi	XynD	I & C	90	+	X76729
	Cellulomonas flavigena	ORFB	C	106		Al-Tawheed (1988)
	Clostridium cellulovorans	EngD	C	108	+	M37434
	Clostridium longisporum	CelA	C	97	+	L02868
	Dictyostelium discoideum	270-11a	C	98	+	M33861
	Dictyostelium discoideum	270-11b	I	106		M33861
	Microbispora bispora	CelA	C	100		P26414
	Pseudomonas fluorescens	EglA	C	100		X12570
	Pseudomonas fluorescens	CelB	N	102	+	X52615
	Pseudomonas fluorescens	CelC	N	99	+	X61299
	Pseudomonas fluorescens	XynA	N	101	+	X15429
	Pseudomonas fluorescens	XynB/C	N	99	+	X54523
	Pseudomonas fluorescens	XynD	N	102		X58956
	Streptomyces lividans	CelA	N	108		M82807
	Streptomyces lividans	CelB	C	106		U04629
	Streptomyces lividans	ChiC	N	105		D12647
	Streptomyces lividans	XlnB	C	86		M64552/S68767
	Streptomyces plicatus	Chi63	N	102		M82804
	Streptomyces rochei	EglS	C	103		X73953
	Thermomonospora fusca	E1	C	96	+	L20094
	Thermomonospora fusca	E2	C	96	+	M73321
	Thermomonospora fusca	E4	C	104	+	L20093
	Thermomonospora fusca	E5	N	103	+	L01577
	Thermomonospora fusca	XynA	C	86	+	U01242

Table 3—continued

III	*Bacillus lautus*	CelA	C	150	+	M76588
	Bacillus lautus	ORF	C	150		M76588
	Bacillus subtilis DLG	End	C	132		M16185
	Bacillus subtilis BSE616	End1	C	133		D01057
	Bacillus subtilis CK2	Cel	C	133		X67044
	Bacillus subtilis N-24	End2	C	132		M28332
	Bacillus subtilis PAP115	End1	C	132		X04689/Z29076
	Caldocellum saccharolyticum	ManA	I × 2	172		L01257
	Caldocellum saccharolyticum	CelA	I × 2	172/172		L32742
	Caldocellum saccharolyticum	CelB	I	172		X13602
	Caldocellum saccharolyticum	CelC	I × 2	172/172		Bergquist *et al.* (1993)
	Cellulomonas fimi	CenB	I	131	+	M64644
	Clostridium cellulolyticum	CelCCG	I	178		S114528/M87018
	Clostridium cellulovorans	CbpA	N	161	+	M73817
	Clostridium stercorarium	CelZ 1	I	144	+	X55299
	Clostridium stercorarium	CelZ 2	C	133		X55299
	Clostridium thermocellum	Cbh3	I	132		X80993
	Clostridium thermocellum	CelF	I	174		X60545
	Clostridium thermocellum	CelI	C × 2	137/150		L04735
	Clostridium thermocellum	CipA	I	156		L08665
	Clostridium thermocellum	CipB	I	167	+	X68233
	Erwinia carotovora	CelV	C	156		X76000/X79241
IV	*Cellulomonas fimi*	CenC	N × 2	148	+	X57858
	Clostridium cellulolyticum	CelCCE	N	168		M87018
	Streptomyces reticuli	Cel1	N	125		L04735/X65616
	Thermomonospora fusca	E1	N	141		L20094
V	*Erwinia chrysanthemi*	CelZ	C	63	+	Y00540
VI	*Bacillus polymyxa*	XynD	C	90		X57094
	Clostridium stercorarium F9	XylA	C × 2	87/92	+	D13325

	Clostridium thermocellum	XynZ	I	92		M22624
	Limulus sp.	Factor G_α	C × 2	87		Seki *et al.* (1994)
	Microspora bispora	BglA	C	85		L06134
VII	*Clostridium thermocellum*	CelE	I	240	+	M22759
VIII	*Dictyostelium discoideum*	CelA	N	152	+	M33861
IX	*Clostridium thermocellum*	XynX	C × 2	174/189		M67438
	Thermoanaerobacterium saccharolyticum	XynA	C × 2	174/187		M97882
	Thermotoga maritima	XynA	C × 2	170/180		Z46264

[a]Families are numbered as originally proposed by Coutinho *et al.* (1992). Three additional families (VI–VIII) are included to accommodate new sequences.

[b]N, C and I, indicate N-terminal, C-terminal or internal CBDs, respectively; ×2 and ×4, indicate two and four CBDs, respectively.

[c]The number of amino acid residues indicated is based on sequence alignment and is considered tentative.

[d]+, indicates that a functional CBD has been demonstrated experimentally.

[e]GenBank, SWISS-PROT or EMBL database accession numbers are given if available; literature references are cited in other cases.

[f]PBP, polysaccharide binding protein consisting entirely of four type I CBDs.

Family I CBDs are all fungal; they were first identified in cellulases from *T. reesei*. They are 33–36 amino acids long, have highly conserved sequences, and are usually C-terminal. All contain at least four conserved aromatic residues, believed to be important for the interaction with cellulose (Claeyssens and Tomme, 1990; Reinikainen *et al.*, 1992) and four cysteines that form two disulphide bridges (Kraulis *et al.*, 1989).

Family II is currently the largest and includes representatives from *C. fimi*, *P. fluorescens* subsp. *cellulosa*, *S. lividans* and *T. fusca*. Family II CBDs are found in cellulases, xylanases and two chitinases, as well as an arabinofuranosidase (Kellett *et al.*, 1990) and an acetyl esterase (Ferreira *et al.*, 1993). Two family II CBDs have also been identified in a spore germination-specific protein from the slime mould *Dictyostelium discoideum* (Meinke *et al.*, 1991c) and one of these is known to be functional (H. Ennis, personal communication). There are no fungal representatives. Family II CBDs are about 100 residues long; they contain four strictly conserved tryptophan residues and two highly conserved cysteine residues that form a disulphide bridge (Gilkes *et al.*, 1991a; McGinnis and Wilson, 1993; Meinke *et al.*, 1993).

Family III CBDs are prevalent in enzymes from *Bacillus* spp., *Clostridium* spp. and *Caldocellum saccharolyticum*. They contain 130–170 amino acid residues; many are located internally. Family IV comprises a small group of CBDs distantly related to family II (Coutinho *et al.*, 1992). So far, all representatives are from enzymes with catalytic domains belonging to family E. Family VI is a new family of CBDs found predominantly in xylanases. Two copies of this type of CBD are also present in the horseshoe crab, 1,3-β-*D*-glucanase-sensitive coagulation factor G (Seki *et al.*, 1994).

3.3.2. *Functional Characterization of Cellulose-binding Domains*

Only some of the CBDs listed in Table 3 have been characterized biochemically. Affinity and specificity for various insoluble substrates have been demonstrated with full-length enzymes or truncated enzymes produced by partial proteolysis or genetic manipulation. Chimeric proteins containing CBDs have also been produced and characterized. Representatives of all CBD families have been shown to bind to Avicel, phosphoric acid-swollen cellulose or highly crystalline bacterial cellulose. Usually, the physical structure of the cellulosic substrate is not critical but unusual specificities are shown by the CBDs of XynD and CenC from *C. fimi*: the CenC CBD binds to amorphous cellulose but has relatively insignificant affinity for bacterial crystalline cellulose (Coutinho *et al.*, 1992) while XynD CBD has a high affinity for crystalline cellulose but does not bind to phosporic acid-swollen cellulose (Millward-Sadler *et al.*, 1994). Some cellulases show catalytic specificity for "amorphous" or crystalline substrates (Tomme *et al.*, 1988) and CBDs could be involved in targeting (see below). Deletion of the CBDs

from *T. fusca* and *Clostridium stercorarium* xylanases (Irwin *et al.*, 1994; Sakka *et al.*, 1993) drastically reduced their affinities for xylan; other, related CBDs, from both xylanases and cellulases, have no apparent affinity for xylan, indicating that CBDs, even within families, may have different functional roles. Several type II and III CBDs, notably those from *C. fimi* and *Clostridium* sp., bind strongly to α-chitin, a polymer of β-1,4-linked *N*-acetylglucosamine that resembles cellulose (Ong *et al.*, 1993; Tomme *et al.*, 1994). Interestingly, type II CBDs occur in some chitinases (Table 3) (Fujii and Miyashita, 1993; Robbins *et al.*, 1992), but their affinities for chitin and cellulose have not been investigated.

The conditions required to desorb CBDs from cellulose vary considerably. Desorption of some CBDs requires denaturants such as guanidinium hydrochloride, urea or sodium dodecyl sulphate, or organic solvents such as polyethylene glycol or triethylamine; others can be desorbed with water. Even within a given family, the conditions required for desorption appear to vary significantly (Coutinho *et al.*, 1992; Millward-Sadler *et al.*, 1994; Ong *et al.*, 1993; Owolabi *et al.*, 1988; Poole *et al.*, 1992; Py *et al.*, 1991). This variability could reflect inherent differences in binding mechanisms or differences in experimental conditions; in chimeric proteins, the polypeptide that is fused to the CBD can have a substantial influence on adsorption or desorption requirements (Tomme *et al.*, 1994). The mechanism of adsorption to cellulose is still unknown but the conservation of aromatic amino acids in CBDs, especially tryptophan and tyrosine, implies that these residues have crucial roles in binding, as in other protein–carbohydrate interactions (Vyas, 1991). Nitration of tyrosine residues in *T. reesei* CBHI drastically reduces the binding of the enzyme to crystalline cellulose whereas nitration of the core protein lacking the CBD has no effect (Claeyssens and Tomme, 1990). The CBD of CBHI is a wedge-shaped β-sheet structure composed of three antiparallel strands with one hydrophobic and one hydrophilic face (Kraulis *et al.*, 1989). The latter surface contains three tyrosine residues that are exposed to the solvent. Mutation of Tyr 492 at the tip of the wedge, to Ala or Asp, reduces the binding of CBHI for cellulose to the level observed for the core protein. Mutation experiments with two type II CBDs are somewhat more difficult to reconcile. Substitution of the highly conserved N- or C-terminal tryptophan residues (W14 and W68, respectively) in the *C. fimi* CenA CBD by alanine results in a 30- and 50-fold decrease in binding affinity (Din *et al.*, 1994a). High-resolution NMR structural analysis shows that the two corresponding tryptophan residues in the related CBD from *C. fimi* Cex (W17 and W72) are completely exposed to solvent and, like the tyrosine residues in the CBHI CBD, accessible to the substrate (L. Kay, personal communication). Other experiments with the CenA CBD show that the C-terminal region of the CBD is functionally important (Gilkes *et al.*, 1989; Greenwood *et al.*, 1989). In contrast, data for the analogous CBD from *P. fluorescens* subsp. *cellulosa* XynA imply that the N-terminal, but not the

C-terminal, tryptophan residues are important in binding (Poole *et al.*, 1993). Perhaps conserved residues do not have equivalent roles, even in closely related CBDs.

It has been frequently demonstrated that removal of the CBD, genetically or proteolytically, affects enzyme activity on various cellulosic substrates. Removal of the CBD from *C. fimi* CenA reduces the activity of the enzyme on Avicel by 20–50% and on bacterial cellulose by almost 80%, but increases activity on phosphoric acid-swollen cellulose and carboxymethylcellulose (Coutinho *et al.*, 1993; Gilkes *et al.*, 1988). A truncated *Bacillus* endoglucanase, lacking the CBD, has three- to five-fold lower specific activity on amorphous cellulose relative to the wild-type enzyme (Hefford *et al.*, 1992). Removal of the CBDs from *T. reesei* CBHI and CBHII reduces the activity on Avicel by about 85% and 50% respectively, but does not alter the activities towards soluble substrates (Tomme *et al.*, 1988). Proteolytic removal of the *B. succinogenes* endoglucanase CBD decreases the activity of the enzyme for Avicel or regenerated cellulose two-fold, without significantly changing carboxymethylcellulase activity (McGavin and Forsberg, 1989). Similarly, grafting the *T. fusca* E2 CBD onto the catalytic domain of *Prevotella ruminicola* endoglucanase enhances the activity of the enzyme towards amorphous celluloses 7- to 10-fold (Maglione *et al.*, 1992).

Two proposals have been made to explain how CBDs enhance enzyme activity on insoluble substrates: one is that they serve to increase the effective enzyme concentration on the substrate surface; the other, that they play a role in disrupting non-covalent associations, disrupting structure and thereby increasing substrate accessibility (Knowles *et al.*, 1987; Teeri *et al.*, 1992). Either mechanism could involve targeting of enzymes to distinct regions of the substrate by CBDs with particular affinities. Some CBDs clearly differ in their specificities for different physical forms of cellulose, as described above. There is also some evidence that CBDs have specificity for particular faces or corners of cellulose I crystals (Chanzy *et al.*, 1984; Gilkes *et al.*, 1993).

The isolated CBD of *C. fimi* endoglucanase A (CenA) disrupts the structure of ramie fibres and releases small particles of cellulose from both ramie and cotton fibres; it appears to penetrate the fibre at surface discontinuities and slough off cellulose fragments that are non-covalently attached to the underlying substrate; further penetration of the CBD then exfoliates the fibre structure (Din *et al.*, 1991, 1994b). In a similar fashion, the CenA CBD prevents the flocculation of bacterial cellulose microfibrils (Gilkes *et al.*, 1993). However, both these effects appear to involve the disruption of relatively weak interactions between cellulose microfibrils, not disruption of the crystal structure itself, and their relevance to cellulose hydrolysis *in vivo* is difficult to assess. CBDs could also participate in the dissociation of cell wall matrix polysaccharides, such as xyloglucans or xylans from microfibrils (see section 2); this could explain the presence of CBDs

on xylanases. In the absence of a clearly defined role, we cannot assume that all CBDs have the same function, but there is no evidence that any CBDs disrupt the crystalline structure of cellulose, as originally proposed (Teeri *et al.*, 1992). It is now suggested that movement of the CBHI catalytic domain along the cellulose chain could weaken the interaction between adjacent molecules and render the substrate more accessible to other enzymes (Srisodsuk, 1994). It is also proposed that hydrolysis and crystallite disruption are energetically coupled, i.e. that the energy for disruption comes from hydrolysis of the glucosidic bond (Konstantinidis *et al.*, 1993). The CBD is clearly not essential for hydrolysis of crystalline cellulose: for example, the isolated CBHI catalytic domain can still hydrolyze highly crystalline bacterial cellulose (Srisodsuk, 1994), but further experimentation is necessary to define precise roles for these domains.

3.4. Other Domains and Linker Regions

Additional types of ancillary domains are present in many prokaryotic β-1,4-glycanases (Fig. 4). Many cellulases from *Clostridium* spp. have a domain, usually at the C-terminus, containing two very similar regions of 22 amino acid residues. This tandem repeat, or "duplicated segment", allows the enzymes to associate into a complex by binding to a scaffolding protein. Other examples of ancillary domains are the S-layer-like modules implicated in the interaction of bacterial hydrolases with the cell surface (Table 4) and the NodB-like module recently identified in *C. fimi* Xyn D (Millward-Sadler *et al.*, 1994). Functional aspects of these modules are discussed in section 4.

Fn3 modules occur in four *C. fimi* cellulases (Meinke *et al.*, 1993, 1994; H. Shen, unpublished data) (Fig. 4). Although not typical of bacterial or fungal cellulases, these modules occur in a range of other enzymes, usually from soil bacteria, involved in hydrolysis of insoluble polysaccharides (Table 4); a pullulanase from *Thermoanaerobacterium thermosulfurigenes* contains two Fn3 modules and a triplicated S-layer-like segment (Matuschek *et al.*, 1994). Fibronectin is a component of basement membranes in animal cells. It is thought that bacteria acquired Fn3 genes from an animal source after the divergence of prokaryotes and eukaryotes (Bork and Doolittle, 1992). Various animal pathogens, e.g. *Salmonella enteritidis*, produce adhesive appendages called fimbriae that carry fibronectin receptors (Collinson *et al.*, 1993). Fn3 modules could mediate attachment of *C. fimi* cellulases to receptors on the cell surface under particular circumstances; alternatively, they could be involved in the formation of extracellular enzyme complexes. However, there is no evidence for such interactions and the apparent rarity of these repeats in bacterial cellulases indicates that such a mechanism, if operative, is not widespread. Fn3 modules often occur between a catalytic

Table 4 Catalogue of fibronectin type III (Fn3) and S-layer-like modules found in prokaryotic hydrolases and related proteins.

Organism	Enzyme	Copies	Accession no.[a]
Fn3 modules[b]			
Alcaligenes faecalis	PHBD[d]	1	J04223
Alkalophilic eubacterium	AmyA180[e]	3	X53373
Bacillus circulans	ChiA1[f]	2	M57601
Bacillus circulans	ChiD[f]	1	D10594
Cellulomonas fimi	CenB	3	M64644
Cellulomonas fimi	CenD	2	L02544
Cellulomonas fimi	CelE	3	L29042
Cellulomonas fimi	CbhA	3	L25809
Cellulomonas flavigena	EngB	1	Al-Tawheed (1988)
Erwinia chrysanthemi	exo-Gal[g]	1	M31308
Streptomyces lividans	ChiA[f]	1	Watanabe *et al.* (1993)
Streptomyces lividans	ChiB[f]	1	Watanabe *et al.* (1993)
Streptomyces lividans	ChiC	1	D12467
Streptomyces olivaceoviridies	Exo-ChiO1[f]	1	X71080
Streptomyces plicatus	Chi63[f]	1	M82804
Thermoanaerobacter saccharolyticum B6A-RI	Amy[h]	2	L07762
Thermoanaerobacter thermohydrosulfuricum E101-69	Amy[i]	2	M28471/M97665
Thermoanaerobacterium thermosulfurigenes EM1	AmyB[j]	2	M57692

S-layer-like modules[c]			
Acetogenium kivui	S-layer protein	2	M31069
Bacillus sp.	ORFp[k]	2	D28467
Bacillus KSM 635	endoglucanase	3	M27420
Bacillus brevis	S–layer protein	2	M15364/D90050
Bacillus sphaericus	S-layer protein	2	A33856/M28361
Clostridium thermocellum	Xylanase X	3	M67438
Clostridium thermocellum	ORF1p[k]	3	X67506
	ORF2p[k]	3	X67506
	ORF3p[k]	3	X67506
Thermoanaerobacter saccharolyticum B6A-RI	Xylanase	3	M97882
Thermoanaerobacterium thermosulfurigenes EM1	Pullulanase	3	M57692
Thermotoga maritima	Ompα protein[l]	1	X68276
Thermus thermophilus	S-layer protein	1	X57333

[a]GenBank, SWISS-PROT or EMBL database accession numbers are given if available; literature references are cited in other cases.
[b]Sequence alignments are contained in Meinke *et al.* (1991b), Bork and Doolittle (1992) and Hansen (1992).
[c]Sequence alignments are contained in Fujino *et al.* (1993).
[d]Poly-(3-hydroxybutyrate) depolymerase.
[e]Exomaltopentaohydrolase.
[f]Chitinase.
[g]Exo-poly-α-D-galacturonosidase
[h]Amylopullulanase
[i]Bifunctinal α-amylase/pullulanase.
[j]Pullulanase.
[k]Translated open reading frame.
[l]Outer membrane protein.

domain and a carbohydrate-binding domain, so perhaps they function simply as spacer sequences or linkers (see below). Deletion of the Fn3 module between the catalytic domain and the chitin-binding domain in the *Bacillus circulans* WL-12 chitinase A1 drastically reduces the activity of the enzyme on chitin, but the activity on soluble substrates, such as carboxymethylchitin, is not affected; the Fn3 sequence itself does not bind to chitin (Watanabe *et al.*, 1993). In this case, it can be argued that the Fn3 sequence acts as a linker and is required for the proper functioning of both domains on insoluble substrates.

The various domains or modules in multidomain cellulases are typically joined by distinct linker regions (Gilkes *et al.*, 1991b). Most linkers are rich in proline, hydroxyamino acids and often alanine and glycine residues. This composition, and the regular arrangement of proline and hydroxyamino acid residues, probably imparts structural stability through extensive hydrogen bonding while allowing the necessary flexibility (Coggins, 1991; Huber and Bennett, 1983; Jentoft, 1991). Linkers are often glycosylated (Ong *et al.*, 1994; Tomme *et al.*, 1988); their structure and function demand an exposed position in the molecule and glycosylation probably contributes to stability in an aqueous environment and protects against proteolysis (Langsford *et al.*, 1987). The proline- and threonine-rich linkers are reminiscent of the elongated, glycine-, proline- and hydroxyproline-rich extensins of plant cell walls (Cassab and Varner, 1988) which adopt a helical, polyproline II-type conformation, similar to that of collagen (van Holst and Varner, 1984). Glycine-rich linkers occur in a few β-1,4-glycanases such as XynD from *C. fimi* (Millward-Sadler *et al.*, 1994) but theoretical considerations indicate that all-glycine linkers are probably too flexible and unstable to be used as structural elements (Argos, 1990). Long amino acid sequences (309–374 residues) containing hydroxy amino acid-rich regions occur between the two catalytic domains in the XylA, XylB and XylD, bifunctional proteins from *R. flavifaciens* (Flint *et al.*, 1993b) (Fig. 4). These may function as linkers, although other bifunctional β-1,4-glycanases have much shorter linkers (Bergquist *et al.*, 1993); alternatively they could be involved in protein–protein interaction or substrate binding.

It is reasonable to propose that linkers allow flexibility between domains while maintaining a spatial separation appropriate for domain interaction. It is suggested that the flexibility and spacer functions of the *T. reesei* CBHI linker reside in two distinct regions of the polypeptide: an N-proximal glycine-rich region and a C-proximal hydroxyamino acid-rich region, respectively. Both proposals are generally supported by experiments that probe linker function by examining the effects of deletion (Shen *et al.*, 1991; Srisodsuk *et al.*, 1993); however, it is difficult to reconcile these data with the results of another study that shows no effect on adsorption or catalytic activity following deletion of the entire linker from *P. fluorescens* subsp. *cellulosa* XynA (Ferreira *et al.*, 1990). The data for XynA prompted the

curious speculation that bacterial linkers have in fact evolved because their encoding DNA facilitates domain swapping in a manner analogous to that of introns in eukaryotic genes, even though linkers are quite common in eukaryotic proteins.

4. CELLULASE SYSTEMS

4.1. General Aspects

It is convenient to consider enzyme systems for cellulose degradation in isolation although they typically form only part of a spectrum of polysaccharide hydrolases produced by microorganisms to degrade the complex structural components of plant cell walls. However, even the digestion of purified native cellulose under laboratory conditions requires the concerted action of several enzymes. For example, six cellulases have been characterized in *C. fimi* (Meinke *et al.*, 1994; Shen *et al.*, 1994) and the same number in *T. fusca* (Wilson, 1992). Some cellulolytic organisms produce a surprisingly large set of cellulases; for example, one *Clostridium thermocellum* strain has at least 15 cellulase genes (Hazlewood *et al.*, 1988; Millet *et al.*, 1985). The apparent requirement for so many related enzymes is one of the most intriguing aspects of enzymatic cellulose hydrolysis. In addition to hydrolytic enzymes, phosphorolytic enzymes may be involved in cellulose digestion in some bacteria (Sheth and Alexander, 1967) and oxidative enzymes are believed to be important in cellulose degradation in white-rot fungi (Coughlan, 1985). The interaction of enzyme components, even in relatively simple cellulase systems, is still not well understood and continues to be the primary focus of most work in the field.

Some cellulolytic organisms produce cellulase systems that can efficiently and exhaustively digest native cellulose in plant biomass. These "complete" systems, such as those of wood-decaying fungi, *Clostridium* spp. and various rumen bacteria, are evidently designed to exploit plant biomass as a source of carbon and energy. In contrast, many microorganisms have so-called "incomplete" cellulase systems that have presumably evolved for other purposes and have relatively limited hydrolytic capacities. Incomplete systems are found in plant bacterial pathogens, e.g. *E. chrysanthemi* (Chippaux, 1988) and *Pseudomonas solanacearum* (Huang and Schell, 1992; Huang *et al.*, 1989). Others are associated with symbiotic nitrogen-fixing soil bacteria; for example, *Rhizobium leguminosarum* (Mateos *et al.*, 1992) and *Azoarcus* sp. (Reinhold-Hurek *et al.*, 1993) produce cellulases and other hydrolases implicated in localized degradation of the root hair cell wall to permit bacterial infection. In this context, it is intriguing that xylanases (XynD and XynA, respectively) from *C. fimi* and *Cellvibrio mixus* (Haz-

lewood and Gilbert, 1993a; Millward-Sadler *et al.*, 1994) contain amino acid sequences related to ones found in NodB, one of several nodulation factors in *Rhizobium* spp. (Fig. 4). Nodulation factors are involved in the production of chitooligosaccharides that function as signal molecules during root colonization by nitrogen-fixing bacteria; NodB is a chitin deacetylase. XynD does not appear to have chitin deacetylase activity (Millward-Sadler *et al.*, 1994) but it is conceivable that some bacterial xylanases are involved in signal molecule synthesis. As yet, no cellulases with NodB-like sequences have been reported.

In many bacteria, the ecological significance of incomplete systems is obscure; for example, *Bacteroides ruminocola* cannot grow on insoluble cellulose (Williams and Withers, 1985) although it produces at least one endoglucanase that hydrolyzes carboxymethylcellulose and acid-swollen cellulose (Matsushita *et al.*, 1990); likewise, a strain of *Ruminococcus albus* is reported to grow on Avicel but not on cotton (Wood *et al.*, 1982). *B. ruminocola* and *R. albus* may not need complete cellulase systems because they are part of a consortium in the rumen flora that cooperate in the hydrolysis of plant biomass. Such bacteria could be regarded as evolutionary intermediates that have yet to acquire a complete set of cellulase genes (Béguin and Aubert, 1994); conversely, it is possible that some cellulolytic strains have lost essential genes during prolonged laboratory cultivation. The following discussion is primarily concerned with the organization of complete systems, although the distinction between complete and incomplete systems is not always obvious.

Given the taxonomic and ecological diversity of cellulolytic microorganisms, it is not surprising that the ways in which cellulase systems are organized appears equally varied; nevertheless, all systems can be divided into two broad categories: non-complexed and complexed. Non-complexed systems, also called "non-aggregating" systems (Gilbert and Hazlewood, 1993), are characteristic of aerobic fungi and bacteria; they comprise several soluble cellulases and related polysaccharide depolymerases which are secreted into the culture medium. Complexed (or aggregating) systems are generally typical of anaerobic microorganisms, including bacteria and fungi that colonize anaerobic environments in the rumen and hind-gut of herbivores, composting biomass and sewage. In some anaerobic bacteria, notably *Clostridium* spp., the complexed enzymes are contained in distinct high-molecular-weight protein complexes called cellulosomes which are bound, at least initially, to the cell surface; in other anaerobic bacteria the organization of cell-associated enzyme complexes is less well defined. This distinction between cellulase systems of aerobes and anaerobes applies generally, but it is not absolute: some cellulolytic anaerobes, for example, *Bacillus* spp., secrete non-complexed enzymes (Lo *et al.*, 1988), whereas some cellulases in aerobic bacteria may be cell-bound (Schlochtermeier *et al.*, 1992).

4.2. Non-complexed Systems

The soft-rot fungi (e.g. *Trichoderma* spp. (Teeri *et al.*, 1991), *Fusarium* spp. (Wood and McCrae, 1977), *Penicillium* spp. (Wood and McCrae, 1986) and *Talaromyces emersonii* (McHale and Coughlan, 1980)) secrete a combination of endoglucanases and exoglucanases (cellobiohydrolases) into the surrounding medium. The cellulase system of *T. reesei* has been studied extensively and has become a paradigm for this group. It comprises two major cellobiohydrolases, CBHI and CBHII, two major endoglucanases, EGI and EGII (Wood and Garcia-Campayo, 1990) and at least two low-molecular-weight endoglucanases, EGIII and EGV (Saloheimo *et al.*, 1988, 1994). Culture filtrates from these organisms are capable of solubilizing native cellulose. Secreted β-glucosidases relieve product inhibition by hydrolysis of cellobiose and other small soluble cellooligosaccharides (Teeri *et al.*, 1991; Wood and Garcia-Campayo, 1990). White-rot fungi (e.g. *Phanaerochaete chrysosporium* and *Schizophyllum commune*) also appear to use non-complexed systems; these fungi are of particular interest because they are also capable of complete lignin degradation (Broda, 1992). Many other aerobic filamentous fungi, including *Agaricus bisporus* (Raguz *et al.*, 1992), *Humicola* spp. (Hayashida *et al.*, 1988), *Irpex lacteus* (Kanda and Nisizawa, 1988) and *Sclerotium rolfsii* (Lachke and Deshpande, 1988), appear to use similarly organized cellulase systems. *P. chrysosporium* has multiple CBHI-type genes (Sims *et al.*, 1994) and at least one CBHII-type gene (Broda, 1992); it also produces enzymes with endoglucanase activity (Eriksson and Pettersson, 1975). Relatively little information is available on the enzymology of cellulose degradation by brown-rot fungi (e.g. *Poria placenta*). Endoglucanase and β-glucosidase activities have been demonstrated, but there is no evidence for exoglucanases in this group (Eriksson *et al.*, 1990). Non-enzymatic, oxidative processes are implicated in the early stages of cellulose degradation by brown-rot fungi (Kleman-Leyer *et al.*, 1992).

Non-complexed enzyme systems are also produced by aerobic bacteria such as *C. saccharolyticum* (Bergquist *et al.*, 1993), *C. fimi* (Meinke *et al.*, 1993), *P. fluorescens* subsp. *cellulosa* (Hazlewood *et al.*, 1992), *Microbispora bispora* (Yablonsky *et al.*, 1988) and *T. fusca* (Wilson, 1992), but whether these are organized in the same way as the non-complexed systems of fungi remains to be established. Unlike most fungal culture filtrates, cell-free preparations from cellulolytic bacteria sometimes have only weak cellulolytic activity, relative to whole cultures (Su and Paulavicius, 1975); this may be because one or more important hydrolytic enzymes are membrane-bound in bacteria. Bacterial β-glucosidases are often membrane-bound or intracellular (Hägerdal *et al.*, 1978; Yablonsky *et al.*, 1988), so that removal of cells could lead to the accumulation of inhibitory levels of soluble products during cellulose hydrolysis.

Most of the early work on enzymatic cellulose hydrolysis involved

cellulases isolated from the supernatants of fungal cultures because the various components could be prepared with apparent ease. Several independent studies demonstrated that mixtures of isolated components interacted synergistically, i.e. their combined activity on cellulose was greater than the sum of their individual activities. This effect is commonly observed with crystalline substrates but not soluble derivatives such as carboxymethylcellulose. Synergy is discussed here at some length because it is the focus of many studies on cellulase interactions in non-complexed systems; whether or not the same general principles also apply to complexed systems is not yet known. Explanations of synergy all suppose that the substrate concentration for at least one enzyme is rate-limiting and that the other enzyme overcomes this limitation for its partner; the arrangement can be reciprocal.

Preliminary models (Wood, 1968, 1969) sought to describe synergy as the interaction of endoglucanases (so-called C_x enzymes) with C_1, a putative non-catalytic factor responsible for the disruption of hydrogen bonds in cellulose crystals and production of "reactive" substrate (Reese *et al.*, 1950), but later versions abandoned this concept and focused on the interaction of endoglucanases and cellobiohydrolases; several earlier reviews provide background information (Coughlan, 1991; Enari and Niku-Paavola, 1987; Wood and Garcia-Campayo, 1990; Woodward, 1991). A current and widely held model for endo–exo synergy describes the sequential interaction of an endoglucanase with cellobiohydrolases, as shown in Fig. 6: here, the rates of hydrolysis of the exo-acting cellobiohydrolases are limited by the availability of cellulose chain ends; the endoglucanase cannot hydrolyse crystalline cellulose efficiently but cleaves bonds at relatively accessible "amorphous regions" to provide sites for cellobiohydrolase attack.

This general model was developed to explain data for enzymes from *P. chrysosporium* and *Trichoderma koningii* (Eriksson, 1978; Eriksson and Pettersson, 1975; Wood and McCrae, 1978), and later extended to other fungal systems (Henrissat *et al.*, 1985; McHale and Coughlan, 1980; Wood *et al.*, 1980). The endo–exo model is also generally assumed to apply to non-complexed bacterial systems; the synergistic behaviour of *T. fusca* endo- and exoglucanases and the "cross-synergism" of *T. fusca* endoglucanases with *T. reesei* CBHI and CBHII are consistent with this view (Irwin *et al.*, 1993). Although several bacterial exoglucanases have been described, family B bacterial cellobiohydrolases analogous to *T. reesei* CBHII were not reported until recently. It is now evident that *C. fimi* CbhA (Meinke *et al.*, 1994) and *T. fusca* E3 (D. Wilson, personal communication) are bacterial analogues of *T. reesei* CBHII. This suggests that the synergistic interaction of endoglucanases and cellobiohydrolase typified by fungi does indeed operate in bacteria. However, no bacterial CBHI (family C) analogues have yet been reported and it is possible that this type of enzyme is restricted to fungal systems.

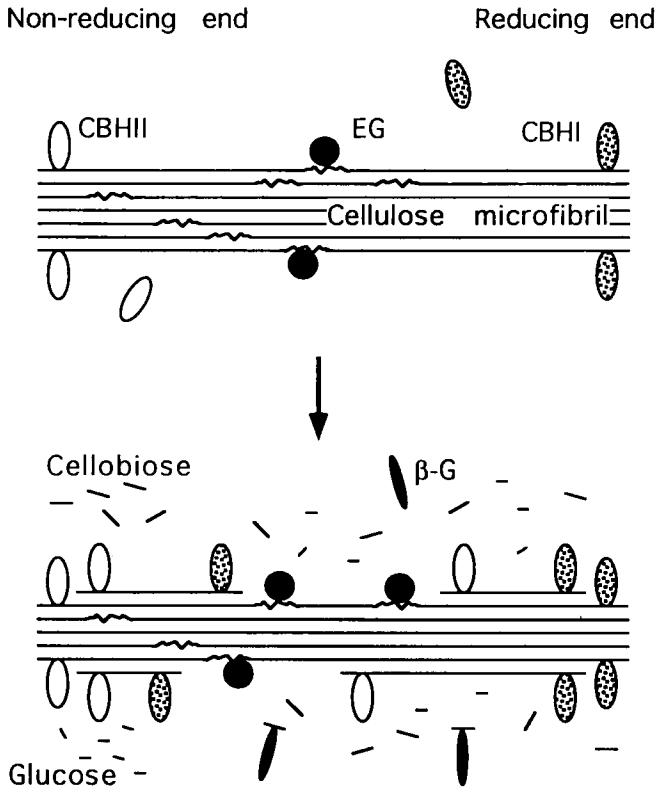


Figure 6 General model of the synergistic interaction of cellobiohydrolases and endoglucanase. The endoglucanase (EG) preferentially attacks the cellulose microfibril at superficial regions where crystal packing is disordered, thus providing additional sites of attack for exo-acting cellobiohydrolases (CBHI and CBHII). Erosion of the surface by cellobiohydrolases exposes new sites for endoglucanase attack. Hydrolysis of cellobiose by β-glucosidase (β-G) relieves product inhibition of endoglucanase and cellobiohydrolases. CBHI and CBHII are shown attacking cellulose chains from the reducing and non-reducing ends respectively, according to the proposed action of *T. reesei* cellobiohydrolases.

Although this general model is still widely accepted, several observations suggest that it is an oversimplification. For example, maximum synergy with *T. reesei* CBHII was obtained at high endoglucanase ratios, as the model predicts, but synergy between endoglucanases and CBHI was maximal when the enzymes were in equimolar proportions (Henrissat *et al.*, 1985). Also, not all endoglucanases appear able to act synergistically with cellobiohydrolases; for example, purified *T. koningii* endoglucanases differed markedly in their ability to synergize with *T. koningii* cellobiohydrolase

(Wood and McCrae, 1978). Similarly, *T. fusca* endoglucanases show varying degrees of synergy with *T. reesei* CBHI or CBHII (Irwin *et al.*, 1993). It is unclear which particular properties allow an endoglucanase to act synergistically with a cellobiohydrolase. Perhaps synergy requires the activities of the two enzymes to be closely coordinated and only certain endoglucanases can form a binary enzyme complex required for concerted action (Tomme *et al.*, 1990; Wood and McCrae, 1978); complex formation could involve protein–protein interactions, or targeting of enzyme pairs, perhaps by CBDs, to particular sites on the cellulose surface. Others suggest that competition for binding sites, involving a mechanism that promotes enzyme turnover, is an important factor (Ryu *et al.*, 1984). However, the finding that *T. reesei* CBHI and EGI can act synergistically even when added sequentially suggests that the simultaneous action of two or more enzymes is not a requirement for all synergistic interactions (Nidetzky *et al.*, 1994).

Although not all endoglucanases synergize with cellobiohydrolases, the ability of some fungal cellobiohydrolases to "cross-synergize" with endoglucanases from other fungi, or with enzymes from bacteria (Gow and Wood, 1988; Irwin *et al.*, 1993; Wood and McCrae, 1979), implies that protein–protein interactions, if involved, are not particularly specific. There is no obvious property (e.g. catalytic domain family, stereospecificity of hydrolysis or general structural and functional organization) common to all endoglucanases that show synergistic interaction with cellobiohydrolases. It has been suggested that only endoglucanases from "complete" cellulase systems (in this case, defined as systems containing a cellobiohydrolase) are effective synergistic partners (Wood *et al.*, 1980). Others have suggested that only endoglucanases with a high affinity for crystalline cellulose are effective (Klyosov, 1990) but *T. fusca* endoglucanases still synergize with *T. reesei* CBHI when their CBDs are deleted (Irwin *et al.*, 1993). Another aspect of endoglucanase activity that may be relevant to synergy is the processivity of attack. It is known that some α-amylases are non-processive and hydrolyze only one α-1,4-glucosidic bond before dissociating from the substrate; others act processively by cleaving multiple bonds, on the same chain or an adjacent chain, before dissociation (Mazur and Nakatani, 1993; Robyt and French, 1970). The general model for endo–exo synergy predicts that non-processive endoglucanase, by creating more ends for attack, would have a greater synergistic interaction with a cellobiohydrolase than a processive endoglucanase. Processivity in β-1,4-glucanases has received relatively little attention, but data for *T. fusca* E2 and E5, two endoglucanases that act synergistically with *T. reesei* CBHI or II, suggest that both are non-processive enzymes (Irwin *et al.*, 1993).

Synergy is also seen between pairs of cellobiohydrolases (Driguez, 1988; Fägerstam and Pettersson, 1980; Henrissat *et al.*, 1985; Nidetzky *et al.*, 1994) and this effect is not explained by the endo–exo model if these enzymes are strictly exo-acting. If exoglucanases are capable of limited endoglucanase

activity, as some data suggest (Ståhlberg *et al.*, 1993a), the exo–exo synergy can still be accommodated by the endo–exo model, but definitive demonstration of exo–exo synergy is problematic. For example, the apparent synergy between conventionally purified *Penicillium pinophilum* CBHI and CBHII on cotton cellulose was no longer evident when CBHII was further purified by ligand-based affinity. However, synergy was restored by a trace amount of a specific endoglucanase, implicating this enzyme in a synergistic ménage à trois (Wood *et al.*, 1989). In contrast, *T. reesei* CBHI and CBHII, purified by similar affinity techniques, did show synergistic behaviour on bacterial cellulose (Driguez, 1988) or filter paper (Irwin *et al.*, 1993). Synergy between pairs of strictly exo-acting cellobiohydrolases could be explained if each enzyme recognizes only one of the two possible stereospecific configurations of cellobiose residues exposed on the cellulose surface; for example, hydrolysis by CBHI could expose a contiguous residue of the opposite type as a substrate for CBHII (Wood and McCrae, 1986). While this explanation is attractive, it currently lacks experimental support. An alternative explanation could involve the preference of CBHI and CBHII for hydrolysis of celluloses Iα and Iβ, respectively (see section 2).

It is clear that the molecular basis of synergy remains ill-defined; however, it is now obvious that data obtained with conventionally purified enzymes should be viewed with extreme caution. Progress towards understanding synergy and related aspects of cellulase action depends on rigorous control of experimental conditions, in particular enzyme purity. The degree of synergy can vary with the enzyme:substrate ratio (Woodward *et al.*, 1988) and the form of cellulose substrate used (Henrissat *et al.*, 1985) so conditions need to be carefully defined and considered when comparing different experiments. Analyses are confounded by changes to the substrate as hydrolysis proceeds and by generation of inhibitory end-products that would normally be consumed *in vivo*. Use of heterogeneous substrates such as Avicel introduces unnecessary complications into analyses; more-defined substrates, such as bacterial cellulose, are preferable unless only empirical data are needed. There are several experimental approaches that could provide new insights into how enzymes interact synergistically. The use of recombinant gene technology to express individual enzymes in a cellulase-free background avoids problems associated with enzyme contamination. Gene manipulation also enables catalytic domains to be produced in isolation in order to analyze the role of ancillary domains. Recombinant gene technology has been applied extensively to bacterial enzymes but less extensively to fungal systems. Gene replacement or deletion (Karhunen *et al.*, 1993; Suominen *et al.*, 1993) is an alternative recombinant technique that provides a way to examine synergistic systems *in vivo*. Detailed analysis of cross-synergism could also be productive: comparison of endoglucanases that cross-synergize with a particular cellobiohydrolase should allow features that are essential for productive interaction to be identified. Three-dimensional

structural characterization of the various enzymes should then allow these features to be defined more precisely.

4.3. Complexed and Cell-associated Systems

4.3.1. *Clostridium spp.*

While there is no compelling evidence for functional multi-enzyme cellulase complexes in aerobic fungi and bacteria, the cellulolytic *Clostridium* spp. produce tightly associated, extracellular cellulase complexes called cellulosomes. In *C. thermocellum*, cellulosome clusters (polycellulosomes) form protuberances on the cell surface and mediate adsorption of the cells to insoluble cellulose; later, the cells desorb leaving a coating of cellulosomes on the residual substrate (Lamed *et al.*, 1983a; Wiegel and Dykstra, 1984). Individual cellulosomes, isolated by elution of substrate with water, are discrete entities that are very resistant to dissociation and highly active on crystalline cellulose. Several strains of *C. thermocellum* have been investigated; all produce cellulosomes but these differ somewhat in size and organization. Generally, individual cellulosomes are complexes of molecular mass 2–6 MDa containing about 50 polypeptide molecules (Mayer *et al.*, 1987). It is estimated that the specific activity of the complexes on crystalline cellulose is about 50-fold higher than the extracellular system produced by *T. reesei* (Johnson *et al.*, 1982). Several important details of the structural and functional organization of the cellulosome have now been elucidated. The discussion that follows attempts to summarize our current understanding with an emphasis on newly acquired data; comprehensive background information is contained in other recent reviews (Bayer *et al.*, 1994; Béguin and Aubert, 1994; Bronnenmeier and Staudenbauer, 1993; Doi, 1994; Felix and Ljungdahl, 1993).

SDS-PAGE analysis of cellulosomes from *C. thermocellum* strain YS showed at least 14 unique polypeptide components ranging in mass from 48 to 210 kDa (Lamed *et al.*, 1983b). Most of the components had β-1,4-glucanase or β-1,4-xylanase activity although the largest, a 210 kDa glycoprotein now known as CipB, had no detectable catalytic activity. Predictably, analyses of various *C. thermocellum* gene banks revealed numerous distinct genes encoding β-1,4-glycanases: for example, genes for fifteen endo-β-1,4-glucanases, two β-1,4-xylanases, one β-1,3(4)-glucanase and two β-glucosidases were detected in DNA from *C. thermocellum* strain NCIB 10682 (Gräbnitz and Staudenbauer, 1988; Hazlewood *et al.*, 1988; Millet *et al.*, 1985; Schimming *et al.*, 1991); a further collection of 12 genes, including two encoding exo-β-1,4-glycanases, was isolated from *C. thermocellum* strain F7 (Bumazkin *et al.*, 1990; Piruzian *et al.*, 1985; Tuka *et al.*, 1990). These enzymes are representative of several different β-1,4-glycanase families

but most carry an additional domain of approximately 65 amino acid residues, usually at the C terminus, containing a pair of well-conserved 22 amino acid repeats (Fig. 4). The function of this duplicated segment was not immediately clear, but it was shown not to be required for either catalytic activity or binding to cellulose.

Electron microscopic studies by Mayer *et al.* provided useful clues to how enzymes are organized within the cellulosome; ultrastructural details were best resolved in so-called "loose" cellulosomes obtained during later stages of cultivation. These contained globular particles linked by threads into ordered chain-like arrays; there were four or more arrays per cellulosome and five to eight particles in each array (Mayer *et al.*, 1987). It was proposed that the particles corresponded to individual endoglucanase molecules, assembled into a linear array on a protein scaffold. It was also suggested that this arrangement of enzymes would enable a coordinated attack on cellulose molecules at the surface of the substrate resulting in cellodextrin release.

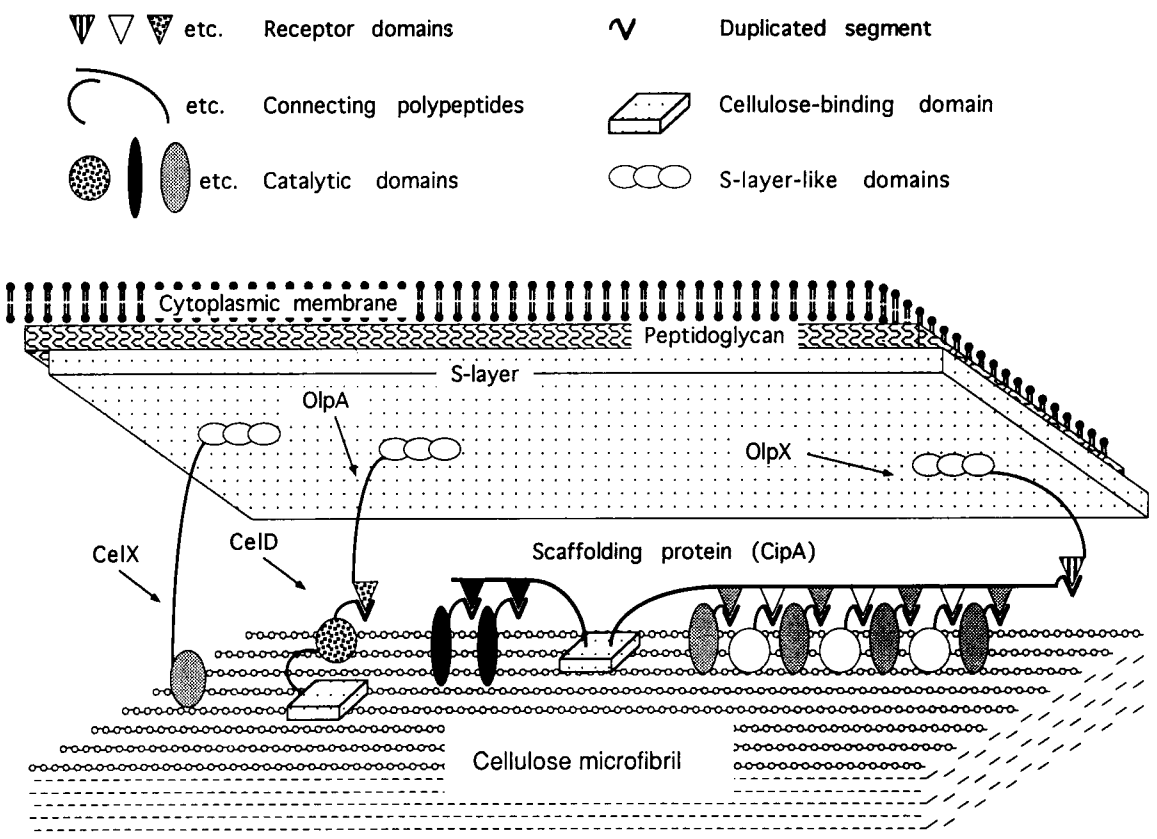
Several independent observations have provided molecular details that confirm the general structural features contained in this model. An enzyme component of the cellulosome from *C. thermocellum* ATCC 27405 called CelS (then called S_S) was shown to have significant activity against Avicel when combined with a non-catalytic protein called S_L, but only very weak activity alone. S_L (now called CipA) also allowed CelS to bind to cellulose. These observations implied that CipA corresponds to the hypothetical scaffolding molecule in Mayer's model and that it promotes cellulose hydrolysis by anchoring enzymes to the cellulose surface (Wu and Demain, 1988; Wu *et al.*, 1988). It was subsequently shown that CipA binds to two other *C. thermocellum* β-1,4-glycanases, CelD and XynZ. It was also shown that binding was mediated by the 22 amino acid duplicated segment at the C-termini of both enzymes (Tokatlidis *et al.*, 1991, 1993).

The structures of CipA and CipB (for cellulosome integrating proteins) are entirely consistent with their role as scaffolding molecules. The deduced CipA amino acid sequence (Gerngross, 1993) shows a type III CBD near its N-terminus and nine reiterated segments of about 166 amino acids; the structural and functional organization of CipB appears to be closely related (Poole *et al.*, 1992). Deletion analysis provides strong evidence that the reiterated segments of CipA correspond to nine receptor domains for the 22 amino acid duplicated segment carried by most *C. thermocellum* cellulases and hemicellulases (Fujino *et al.*, 1992). This conclusion is supported by experiments that demonstrate binding of a fusion protein containing one of the CipA receptor domains (RDCipA) to a second fusion protein containing the duplicated segments of CelD (DSCelD) (Salamitou *et al.*, 1994b). CipA and CipB also have a duplicated segment of their own. Their role is presently unclear, but recent data (Salamitou *et al.*, 1994b) contradict an earlier suggestion (Fujino *et al.*, 1992) that CipA molecules might self-associate by means of DSCipA–RDCipA interactions. Current thinking on how scaffold-

ing proteins mediate attachment of enzyme components to cellulose is summarized by the model shown in Fig. 7. Although the CipA CBD accounts for the affinity of the cellulosome for cellulose, there are other possible substrate interactions: *C. thermocellum* β-1,4-glycanases such as CelE, CelF and XynZ which contain both a duplicated segment and a CBD could also participate.

The mechanism that enables cellulosomes to attach to the cell surface is still under investigation. The nucleotide sequence of a 9.4 kb region of *C. thermocellum* DNA immediately downstream of the gene for CipA contains three open reading frames encoding polypeptides designated ORFp1, ORFp2 and ORFp3. These polypeptides are significant because they all have reiterated segments at their C termini that resemble domains found in certain bacterial cell-surface proteins (so-called S-layer proteins), but ORFp3 is particularly interesting because it also has an N-terminal domain (RDORF3p) related to the receptor domains of CipA. These structural features led to a preliminary model in which ORFp3 mediates the attachment of the cellulosome to the *C. thermocellum* cell surface through the preferential interaction of RDORF3p with DSCipA (Fujino *et al.*, 1993). The model was attractive because it provided a role for DSCipA. Although ORF3p does bind to the cell surface (Salamitou *et al.*, 1994a), the model was later revised because the interaction of RDORF3p with DSCipA could not be demonstrated (Salamitou *et al.*, 1994b). Instead, it is proposed that ORF3p (now renamed OlpA, for outer layer protein) may attach cellulases or hemicellulases containing duplicated segments to the cell surface directly (Fig. 7). Nevertheless, DSCipA does bind to three unidentified polypeptides from *C. thermocellum* cultures (Salamitou *et al.*, 1994b); presumably, these do contain receptor domains specific for DSCipA and could fill the role originally attributed to ORF3p.

Figure 7 Hypothetical organization of the *C. thermocellum* cellulosome. The model shows a scaffolding protein (CipA) containing nine receptor domains that interact with duplicated segments on various β-1,4-glycanases, and a CBD that mediates adsorption to cellulose. CipA is attached to the cell by a hypothetical outer layer protein (OlpX) containing S-layer-like modules and a receptor for the CipA duplicated segment. The regular distribution of enzymes relative to the substrate reflects the proposed simultaneous "multicutting" of individual cellulose molecules to release soluble cellooligosaccharides, according to Mayer *et al.* (1987). Some enzymes, such as CelD, may be attached to the cell through interaction with other outer layer proteins such as OlpA; adsorption of CelD to cellulose is mediated by its own CBD. CelX, a hypothetical enzyme analogous to *Thermoanaerobacterium saccharolyticum* BA-RI xylanase A (Lee *et al.*, 1993) contains a catalytic domain and S-layer-like modules (see Fig. 4), and represents a third possible mode of interaction with the *C. thermocellum* cell surface. Details are contained in the text. (Revised from Fujino *et al.*, 1993).


etc. Receptor domains
Duplicated segment
etc. Connecting polypeptides
Cellulose-binding domain
etc. Catalytic domains
S-layer-like domains
Cytoplasmic membrane
Peptidoglycan
S-layer
OlpA
OlpX
CelX
CelD
Scaffolding protein (CipA)
Cellulose microfibril

The *C. thermocellum* cellulosome has become a paradigm for highly organized bacterial cellulase complexes but cellulosomes are also produced by other cellulolytic *Clostridium* spp. Structural and functional examination of these complexes will provide details about features that are common to all cellulosome systems and the variations in organization that exist between species. *Clostridium cellulolyticum* produces several endoglucanases containing duplicated segments and a large scaffolding protein whose structure and properties are currently under investigation (Bagnara-Tardif *et al.*, 1993). The *Clostridium cellulovorans* cellulosome contains a scaffolding protein, called CbpA (for cellulose-binding protein) (Doi, 1994). Like CipA, it contains a CBD and nine receptor domains (called hydrophobic domains) implicated in enzyme binding; however, it lacks its own duplicated segment, so this domain does not seem to be an essential feature of all scaffolding proteins from *Clostridium* spp. CbpA contains four additional hydrophilic domains whose functions are presently unknown. *C. cellulovorans* EngB, which contains a duplicated segment similar to those found on *C. thermocellum* enzymes, binds to a fusion protein containing one of the receptor domains. Curiously, the same receptor appears able to bind at least one cellulase (Eng D) that does not contain a duplicated segment, suggesting that other types of protein–protein interactions are important in cellulosome organization (Doi, 1994).

An important aspect of the cellulosome model proposed by Mayer *et al.* is that the enzyme complex hydrolyzes crystalline cellulose with remarkable efficiency, not because the enzyme components themselves are unusually active, but because the activities of the individual enzyme are highly coordinated (Mayer *et al.*, 1987). Data showing that the catalytic domains of the cellulases and hemicellulases from *Clostridium* spp. are not unusual but typical of those found in a broad range of cellulolytic bacteria, including species that do not produce cellulosomes, are consistent with this view (Table 1). This coordination can be viewed as a specialized form of synergy in which the spatial organization of the enzymes on the cellulose chain is crucial. The mechanism proposed by Mayer *et al.*, described as a "simultaneous multicutting" of the cellulose chain by adjacent enzymes, involved only endoglucanases; exoglucanases were not implicated because there was no definitive evidence for such enzymes in *Clostridum* spp. at that time (Coughlan and Ljungdahl, 1988). However, there is now strong evidence that the *C. thermocellum* cellulosome contains at least one cellobiohydrolase, called CelS (Wang and Wu, 1993). This enzyme, previously called S_S and first described as an endoglucanase (Fauth *et al.*, 1991), is now recognized as an analogue of a well-characterized *C. thermocellum* cellobiohydrolase called S8 (Morag *et al.*, 1991, 1993). CelS is a member of an emerging family of cellulases, presently called family L (Shen *et al.*, 1994). Related enzymes (CelCCF, P70, Avicelase II and CelB) occur in *C. cellulolyticum*, *C. cellulovorans*, *C. stercorarium* and *Clostridium josui*, respectively, but, like

the various *C. thermocellum* endoglucanases, family L enzymes are not restricted to *Clostridium* spp.: enzymes with related catalytic domains (CelA and CbhB) have also been found in *C. saccharolyticum* and *C. fimi* (Table 1, Fig. 4). CelS and CbhB are major components of their respective cellulolytic systems and this implies that family L enzymes play a key role in cellulose hydrolysis, both in *Clostridium* spp. and in aerobic bacteria. Demonstration of at least one major cellobiohydrolase in the cellulosome suggests that the apparent need for simultaneous multicutting should be re-evaluated. Does the scaffolding protein really coordinate enzyme activities in the way suggested by Mayer *et al.* or simply mediate the adsorption to cellulose of an appropriate combination of exo- and endoglucanases whose synergistic interactions otherwise resemble those of non-complexed systems? In the latter case, the function of the scaffolding protein is reduced to that of a collective CBD which replaces CBDs on individual enzymes and mediates attachment of the complex to the cell. A better understanding of this and related issues requires new techniques that allow analysis of intact cellulosomes, not just its isolated components.

4.3.2. *Other Complexed or Cell-associated Systems*

Complexed or cell-associated cellulase systems are found in several anaerobic bacteria other than *Clostridium* spp. These include *Fibrobacter* (previously, *Bacteroides*) *succinogenes*, *R. albus* and *R. flavefaciens*, the principal bacteria in rumen flora, and other anaerobes such as *Acetivibrio cellulolyticus* and *Bacteroides cellulosolvens* from sewage sludge. Like *Clostridium* spp., these bacteria are typically found to adhere tightly to cellulose during growth (Forsberg *et al.*, 1981; Latham *et al.*, 1978; Minato and Suto, 1978; Morris and Cole, 1987; Stack and Hungate, 1984; Stewart *et al.*, 1990). It is assumed that close contact provides the bacteria with a competitive advantage in mixed cultures; some have suggested that adhesion is essential for cellulose hydrolysis (Kudo *et al.*, 1987). So far, the molecular basis for adhesion is not known, but cell surface proteins are implicated because protease treatment markedly reduces the ability of *F. succinogenes* to bind to cellulose (Gong and Forsberg, 1989; Mitsumori and Minato, 1993). A cellulose-binding protein of molecular mass 120 kDa was isolated from a cell extract of *F. succinogenes* strain S85 (Mitsumori and Minato, 1993). The protein, as yet uncharacterized, appeared to be on the cell surface because it was not detectable in cells treated with proteinase K. A glycosylated cellulose-binding protein of molecular mass 180 kDa from the outer membrane of *F. succinogenes* strain S85 is also implicated in adhesion (Gong and Forsberg, 1993a). At least one *F. succinogenes* cellulase, endoglucanase 2, can bind directly to cellulose (McGavin and Forsberg, 1989). Endoglucanase 2 appears to contain independent catalytic and cellulose-binding domains; however, recognizable CBDs are conspicuously absent from all the *Acetivibrio*,

Bacteroides, *Fibrobacter* and *Ruminococcus* cellulases characterized so far (see Table 3).

In *F. succinogenes*, about half the extracellular carboxymethylcellulase activity was found associated with membrane fragments or vesicles in the medium (Forsberg *et al.*, 1981; Groleau and Forsberg, 1981, 1983) and at least two endoglucanases and a cellobiosidase were cell-associated (Gong and Forsberg, 1993b). *R. albus* carboxymethylcellulase was also predominantly cell-associated but could be released as a high-molecular-mass complex by washing cells with buffer or water. Formation of the enzyme complex, and of spherical vesicles on the capsule surrounding *R. albus* cells, required rumen fluid or 3-phenylproprionic acid, a precursor in capsule synthesis (Stack and Hungate, 1984). Ultrastructural examination of the surfaces of *A. cellulolyticus*, *B. cellulosolvens*, and *R. albus* showed protuberances similar to those seen on cellulolytic *Clostridium* spp. and cell extracts from *A. cellulolyticus* and *B. cellulosolvens* reacted strongly with antibodies against *C. thermocellum* cellulosomes; several non-cellulolytic control strains of bacteria showed neither of these characteristics. Conditions that released most of the cellulase activity from *R. albus* cells also resulted in loss of cell surface protuberances (Lamed *et al.*, 1987, 1991; Stewart *et al.*, 1990). It has been noted that the complexes from many of these anaerobic species contain polypeptides of similar size to the *C. thermocellum* scaffolding protein (Coughlan and Ljungdahl, 1988), but the relationship between the protuberances or high-molecular-weight enzyme complexes on the cell surfaces of rumen bacteria and the cellulosomes of the ecologically distinct *Clostridium* spp. remains to be established. As yet, there is no genetic evidence for cellulosome-like organization in anaerobic bacteria other than *Clostridium* spp.; for example, no genes encoding scaffolding proteins like those in *Clostridium* spp. have been reported in other bacteria. Likewise, although some endoglucanases from rumen bacteria show strong sequence similarity to those from *Clostridium* spp., none has been found to contain a duplicated segment. As in non-complexed systems, some enzymes involved in biomass degradation by rumen microorganisms contain two catalytic domains. These include a xylanase/xylanase (Zhang and Flint, 1992), a xylanase/β-1,3(4)-glucanase from *R. flavefaciens* (Zhang and Flint, 1992) (Fig. 4) and a xylanase/xylanase from *F. succinogenes* (Paradis *et al.*, 1993) but, to date, no enzymes with cellulase activity.

Anaerobic fungi also contribute to biomass digestion in the rumen and hind-gut of herbivores. *Neocallimastix*, *Piromonas* and *Sphaeromonas* spp. produce zoospores, originally thought to be multiflagellated protozoa, that colonize the substrate with invasive hyphae; ultimately, the fungi produce sporangia which release further zoospores (Bauchop, 1989). The penetrative action of the hyphae is thought to contribute to fibre disruption (Heath, 1988). *Neocallimastix* spp. are the most studied representatives of this group; at present, genes for one endoglucanase (Zhou *et al.*, 1994), a multifunctional

β-1,4-glycanase (Xue *et al.*, 1992) and two xylanases (Black *et al.*, 1994; Gilbert *et al.*, 1992) from *Neocallimastix patriciarum* have been described. A 750–1000 kDa complex with activity against crystalline cellulose was isolated from *Neocallimastix frontalis* culture filtrate (Wilson and Wood, 1992a, b). The complex contained multiple polypeptide components and at least three β-1,4-glucanase activities; its composition varied under different growth conditions. These data indicate that the cellulase systems of *Neocallimastix* spp. and related anaerobic fungi are also of the complexed type. It is not known how these complexes are organized but they may be associated with the fungal cell wall because treatment of *N. frontalis* culture filtrate with chitinase preferentially inactivates a component involved in crystalline cellulose hydrolysis (Wilson and Wood, 1992b).

Interestingly, cell surface protuberances have also been reported in *Cellulomonas* sp. and *Thermomonospora curvata*. Their appearance on *Cellulomonas* sp. cells was correlated with cellulase induction; also, cell extracts, like those from the rumen bacteria, reacted with antibodies against *C. thermocellum* cellulosomes. Similarly, the protuberances were seen on *T. curvata* cells during growth on cellulose or xylan, but not on pectin or starch. Although these data provide only circumstantial evidence for enzyme complexes in these species, they do suggest that the distinction between the cellulase systems of anaerobic bacteria and the non-complexed systems of the aerobic species may not be absolute. Biochemical studies on the cellulase systems of aerobic bacteria are normally focused on non-complexed enzymes, but the possibility that complexed or cell-associated enzymes are important in some species, or under particular growth conditions, cannot be dismissed entirely. The role of Fn3 modules in the *C. fimi* cellulases has not been elucidated and it is conceivable that these could be involved in enzyme complex formation (see Section 3.4).

5. GENETICS OF CELLULASES AND RELATED HYDROLASES

5.1. General Aspects

Recent reviews have covered the genetics and physiology of cellulolytic microorganisms in general (Béguin, 1990; Béguin and Aubert, 1994); others have covered groups of related organisms, namely the clostridia (Bronnenmeier and Staudenbauer, 1993; Hazlewood and Gilbert, 1993b), the actinomycetes (Wilson, 1992) and the ruminococci (White *et al.*, 1993). Others have considered specific organisms such as *P. fluorescens* subsp. *cellulosa* (Hazlewood *et al.*, 1992) and *T. reesei* (Fowler *et al.*, 1993; Kubicek *et al.*, 1993a, b; Penttilä *et al.*, 1993; Teeri *et al.*, 1991). Such widespread interest reflects the potential applications of cellulases and related enzymes. Successful application of a cellulase system or of some of its components will require

economical, large-scale production of the enzymes. An understanding of the genetics and physiology of the organism producing the system will allow manipulation of the organism to maximize production of either the entire system or of selected components.

The natural substrates for cellulolytic microorganisms are plant cell wall materials, which contain both cellulose and hemicelluloses. Organisms that may be exposed to these polymers only transiently, such as those in soil and water, may induce the hydrolytic enzymes as needed. Others, such as those in the rumen, which are exposed constantly to plant cell wall material, may produce the enzymes constitutively. Induction of hydrolytic enzymes in response to cellulose or hemicellulose requires hydrolysis of the insoluble polymers to soluble products which can be transported into the cell to effect induction. Therefore, some of the enzymes in an inducible system may be synthesized constitutively, perhaps at low levels, to effect formation of the inducer. The cellulase- and xylanase-hydrolyzing systems examined to date are mostly quite complex, comprising numerous enzymes. The regulation of the systems may be relatively simple or complex, depending on the organization of the genes encoding the enzymes and on the nature of the controlling elements. The genes may be controlled quite independently, or they may be controlled in groups in independent operons or in regulons. They may be subject to global systems of regulation, such as catabolite repression.

Complete understanding of the hydrolytic systems of an organism can be obtained only by identifying and characterizing all of the components of a system, and by determining the organization and regulation of the genes encoding them. Systems for genetic analysis *in vivo* are still lacking for many of the organisms. Physiological analyses are difficult because of the complexities of both the natural substrates and the hydrolytic systems. Determination of total cellulase or xylanase activity is virtual meaningless because it gives no indication of the enzymes being produced under particular conditions. This requires determination of the levels of each enzyme in the system, but biochemical resolution of all the components of a given system is usually both difficult and time-consuming. In spite of these difficulties, which are largely surmountable by manipulation *in vitro* of the genes encoding the proteins in a system, it is clear that there are two general mechanisms of control of the synthesis of cellulases and related enzymes, i.e. induction in the presence of the polysaccharides and repression by carbon sources metabolized more readily than the polymers (Béguin, 1990). Details of the mechanisms of regulation, however, are relatively few.

5.2. Genetic Organization of Cellulase and Xylanase Systems

Cloning of cellulase, xylanase and related genes from a microorganism gives an indication of the genetic organization. If each gene is present on a distinct

fragment in a restriction endonuclease digest of genomic DNA, the genes generally are not closely linked on the genome, and therefore not organized into operons. They may be part of a regulon, however. If more than one of the genes is present on a DNA fragment, sequencing of the fragment will show how closely linked the genes are and if they may be part of an operon. In many instances, the presence of related genes on a particular DNA fragment was revealed by DNA sequencing. In most of the microorganisms examined to date, the genes encoding cellulases, xylanases and related enzymes are scattered on the genomes, with little or no linkage between individual genes. In others, some genes are linked, but most are scattered, individual genes. In a few organisms, there is extensive linkage between the genes. The linkages are outlined below.

In *Bacillus* sp. N-4, the genes for two cellulases, *celA* and *celB*, are virtually contiguous on the genome. The predicted amino acid sequences of the encoded proteins are very similar, suggesting that the genes arose by duplication (Fukimori *et al.*, 1987). In *Bacillus polymyxa, xynD*, encoding a family F xylanase, is 155 base pairs upstream of, and in the same orientation as, *gluB*, which encodes a lichenase (Gosalbes *et al.*, 1991). The genes known to encode cellulases and xylanases are scattered on the genome of *C. thermocellum*; however, *bglA*, encoding a β-glucosidase, and *licB*, encoding a β-1,3(4)-glucanase, are close together but convergent (Schimming *et al.*, 1992). Four genes, at least one of which encodes a structural protein of the cellulosome, are closely linked in the same orientation and in the order *cipA*-ORF1-ORF2-ORF3 on the genome of *C. thermocellum*. The polypeptide encoded by ORF3, now called OlpA, contains S-layer-like modules and a cohesin domain; CipA, or scaffoldin, is the major structural component of the cellulosome (Fujino *et al.*, 1993). In *C. cellulolyticum*, on the other hand, five genes are clustered in the same orientation in the order *cipCCA-celCCF-celCCC-celCCG-celCCE*. The spaces between the genes are about 100 base pairs. CipA is a scaffoldin; the polypeptides encoded by the other four genes are cellulases (Bagnara-Tardif *et al.*, 1992; Belaich *et al.*, 1993). As in *C. thermocellum*, most of the genes known to encode cellulases and xylanases are scattered on the genome of *P. fluorescens* subsp. *cellulosa*. However, *xynB* and *xynC*, encoding a xylanase and an arabinofuranosidase, respectively, are in the same orientation and only 148 base pairs apart (Kellett *et al.*, 1990). The genes *celA* and *xynA*, encoding an endoglucanase and a xylanase, respectively, are also closely linked in the same orientation (Gilbert *et al.*, 1990). In *B. fibrisolvens* GS113, three genes are clustered in the same orientation in the order ORF1-*xylB*-ORF3, the genes being separated by 15 and 33 base pairs, respectively. *xylB* encodes a bifunctional protein with xylosidase and arabinofuranosidase activities. Although incomplete on the DNA fragment sequenced, the short distances between the genes suggest that ORF1 and ORF2 encode proteins whose functions are related to those of *xylB* (Utt *et al.*, 1991). Three xylanase genes are clustered in a 3.8 kb region

of the genome of *Bacteroides ovatus* (Whitehead and Hespell, 1990). In *C. fimi*, the only linkage detected to date is between *cbhA* and *cenD*, encoding a cellobiohydrolase and an endoglucanase, respectively, which are 129 base pairs apart and in the same orientation (Meinke *et al.*, 1993, 1994). In the fungus *P. chrysosporium*, two genes that are similar to *cbh1* of *T. reesei* are within 750 base pairs of each other (Covert *et al.*, 1992b).

The most extensive linkage of polysaccharidase-encoding genes described to date is in the anaerobic, thermophilic bacterium *C. saccharolyticum*. There is a cluster of 12 contiguous genes, all in the same orientation, at least nine of which encode proteins involved in xylan utilization. The order of the genes is *orf1-xynG-xynH-orf2-xynF-xynE-xynD-xynA-xynC-orf3/4-xynB* (Bergquist *et al.*, 1993; Lüthi *et al.*, 1990). *xynE* and *xynA* encode xylanases, *xynD* and *xynB* encode xylosidases, *xynC* encodes an acetylxylan esterase, *xynF* encodes a protein with xylanase and arabinofuranosidase activity, and *xynG* and *xynH* may be involved in the transport of hydrolysis products. A second cluster of four contiguous genes, all in the same orientation, encodes enzymes involved in cellulose and mannan hydrolysis. The order of the genes is *celA-manA-celB-celC* (Lüthi *et al.*, 1991). The proteins encoded by the second cluster of genes will be discussed later. It is possible that there are similar clusters of hydrolytic genes of related function in *Cellvibrio mixtus*, in which, within a 94.1 kb segment of the genome, there are groups of enzymes for the hydrolysis of starch, esculin, cellobiose, chitin, carboxymethylcellulose and polygalacturonic acid, in that order (Wynne and Pemberton, 1986).

A cluster of genes in the same orientation may or may not be in an operon. If each gene is separated from the next by only a few base pairs, they probably comprise an operon. If the intervening sequences are longer, the absence of putative promoters and transcription terminators in those sequences is suggestive of an operon. In many of the clusters described above, the genes are far enough apart for transcript analysis to be required before the genes can be designated as an operon.

5.3. Multifunctional Proteins

Enzymes can also be coordinately regulated by fusing the genes encoding them, resulting in the production of multifunctional proteins. In *C. saccharolyticum*, all of the proteins encoded by the *celA-manA-celB-celC* gene cluster are multidomain enzymes, each of which contains two independently functioning catalytic domains (Gibbs *et al.*, 1992; Saul *et al.*, 1989, 1990). The catalytic domains are at the N- and C-termini of the proteins; between the catalytic domains are family III CBDs, two in CelA, ManA and CelC, one in CelB; the domains are connected by linkers rich in proline and threonine. In CelA, the catalytic domains are cellulases of families E2 and

L (Bergquist *et al.*, 1993; Shen *et al.*, 1994); in ManA, they are a β-mannanase and an endoglucanase (Gibbs *et al.*, 1992); in CelB, they are a family of F xylanase and a family A1 cellulase (Saul *et al.*, 1990); in CelC, they are a family E2 cellulase and a β-mannanase related to the mannanase in ManA (Bergquist *et al.*, 1993). The *celA* gene, however, has two frameshifts in the sequence encoding the C-terminal mannanase domain, so that *C. saccharolyticum* does not produce the intact protein (Bergquist *et al.*, 1993). In *R. flavefaciens* 17, *xynA* and *xynD* encode multidomain, bifunctional enzymes. XylA has an N-terminal family G xylanase connected to a C-terminal family F xylanase by a sequence of 374 amino acids very rich in glutamine and asparagine; the domains can function independently, the family G domain producing xylooligosaccharides, the family F domain producing xylose (Zhang and Flint, 1992). XylD has an N-terminal family G xylanase connected to a C-terminal β-1,3(4)-glucanase by a sequence of 309 amino acids, which includes a sequence of 30 amino acids very rich in threonine and serine, of unknown function (Flint *et al.*, 1993a). The polypeptide encoded by *xynC* of *F. succinogenes* has an N-terminal xylanase followed by a second xylanase domain and a C-terminal domain of unknown function. The first xylanase domain is active on xylan; the second is active on arabinoxylan and cellulose (Zhu *et al.*, 1993). In the rumen fungus *N. patriciarum*, *xynA* encodes a protein with two family G xylanases separated by a linker; the xylanase sequences are 92% identical, suggestive of gene duplication. Connected to the C-terminus of the second xylanase domain by a linker is a duplicated sequence of 40 amino acids (Gilbert *et al.*, 1992).

The activities of such multifunctional proteins may be modified by proteolysis because linker sequences are very sensitive to proteases. Such proteins may well be a by-product of evolution by transfer and shuffling of discrete coding sequences. Further analysis is required to understand their roles in the regulation and activity of the hydrolytic systems to which they belong.

5.4. Transcription and its Regulation

Cloned genes or fragments thereof are used as probes to detect transcripts of the genes and to determine the sites of initiation and termination of transcription. Analysis of the DNA sequences upstream of the initiation site usually reveals putative promoter sequences on the basis of similarity to promoters identified previously in the same or related organisms. It is advisable to analyse transcripts produced in the organism from which the gene was obtained, even if the gene is transcribed from a promoter on the cloned DNA fragment in a heterologous host because different transcription start sites can be used in the homologous and heterologous hosts. With the *celA* gene of *C. thermocellum*, for example, the major transcripts in *C.*

thermocellum and *E. coli* are initiated at sites 77 base pairs apart, preceded by a *Bacillus subtilis* σ^{28}-like promoter in *C. thermocellum* and by an *E. coli* σ^{70}-like promoter in *E. coli*. A minor transcript is initiated in *C. thermocellum* at the *E. coli* σ^{70}-like promoter (Béguin *et al.*, 1986). It is not clear if *C. thermocellum* uses this promoter preferentially under some conditions. The transcription initiation sites for *celD* of *R. flavefaciens* are different in the normal host and in *E. coli* (White *et al.*, 1993), as are the initiation sites for *celA* of *R. albus* AR67 (Vercoe and Gregg, 1991).

The transcripts are monocistronic for virtually all of the cellulase and xylanase genes analysed to date, with the mRNA detected corresponding in length to little more than the coding sequence of the gene. This is to be expected, with so few of the genes linked to related genes. In those organisms for which several genes have been analysed, expression of the genes is not always strictly coordinated, which may reflect the need in some organisms for constitutive synthesis of enzymes required to release inducing products from the polymers. Sometimes the transcripts have ragged 5′-ends, as if initiation occurs at any one of a number of closely spaced sites. It has been suggested, however, that this may be an artefact, a consequence of nibbling of the ends of DNA–RNA hybrids by S1 nuclease (Mishra *et al.*, 1991).

In *C. thermocellum*, genes encoding cellulases are expressed constitutively, but subject to catabolite repression. When cells are grown on cellobiose, the genes analysed to date are derepressed in the order *celA*, then *celD* and *celF*, and lastly *celC* as cellobiose becomes limiting. Transcripts of *celA*, *celD* and *celF* are present in the late exponential and early stationary phases; those of *celC* almost exclusively in the early stationary phase. CelA, D and F but not CelC are components of the cellulosome. Transcripts of *celF* are initiated at a unique site; those of *celD* at different sites separated by 170 base pairs in late exponential and early stationary phases (Mishra *et al.*, 1991).

In *C. cellulolyticum*, probes from *celCCC* and *celCCG* both hybridize to transcripts of approximately 5 and 6 kb (Bagnara-Tardif *et al.*, 1992). Transcripts of this length can encode CelCCC, CelCCG and a third polypeptide. Stable hairpin structures are possible between *cipCCA* and *celCCF*, and between *celCCG* and *celCCE*, leading to the suggestion that there is probably an operon, *celCCF-celCCC-celCCG*, which is transcribed into a tricistronic message (Belaich *et al.*, 1993).

The endoglucanase gene *engB* of *C. cellulovorans* is transcribed from a single transcription initiation site, which is preceded by sequences similar to promoter sequences in other Gram-positive bacteria. There is a stem-loop structure downstream of *engB* which may be a transcription termination signal (Foong, *et al.*, 1991). The transcript of 1.6 kb is monocistronic. The level of transcript is 2- to 3-fold greater in cells grown on cellulose than in cells grown on cellobiose, suggesting that *engB* is transcribed constitutively at a relatively low level and induced in the presence of cellulose (Attwood *et al.*, 1994).

The genes *celA*, *B*, *C* and *D* are unlinked in *R. flavefaciens* FD-1. The transcripts of the genes appear to be monocistronic. *CelA* and *C* are expressed constitutively, whereas *celB* and *D* are induced in the presence of cellulose (Doerner *et al.*, 1992). Transcription of the *celE* gene in this organism may also be induced in the presence of cellulose (Wang *et al.*, 1993b). Transcription of *xynA* and *B* in *R. flavefaciens* growing on cellobiose is induced by the addition of xylan (Flint *et al.*, 1991). The transcript of *egl1* in *R. albus* F-40 is monocistronic. There are putative -10 and -35 sequences upstream of the coding sequence; deletion of the -35 sequence results in a carboxymethylcellulase-negative phenotype in *E. coli* (Ohmiya *et al.*, 1989). Transcription of the *celA* gene of *R. albus* 8 is initiated at different sites 370 base pairs apart in cultures with cellobiose or cellulose as carbon source. The transcripts are also of different sizes: about 0.6 and ~3.0 kb on cellobiose, and about 2.7 kb on cellulose (White *et al.*, 1993).

There are three monocistronic transcripts of *celE*, encoding endoglucanase E_5, in *T. fusca* XY, all with identical 3′ ends but different, closely spaced 5′ ends. The mRNA level is always proportional to the level of E_5 in cells grown on different carbon sources. The relative levels of mRNA on glucose, cellobiose and cellulose are 0.015, 0.11 and 1.0, respectively, brought about by a combination of glucose repression and induction in the presence of cellulose (Lin and Wilson, 1988b). *T. fusca* XY cells grown on cellulose, but not on other carbon sources, contain a protein which binds to a target sequence of approximately 21 base pairs, within which there is a 14 base pair inverted repeat, ~45 base pairs downstream of the putative *celE* promoters (Lin and Wilson, 1988a). The identical inverted repeat is present in the same position near the *celB* and *D* genes of *T. fusca* XY (Lao *et al.*, 1991). The identical 14 base pair inverted repeat is present in somewhat similar positions near *celA* of *S. lividans* JI66 and *casA* of *Streptomyces* sp. KSM-9, both of which encode cellulases (Fernández-Abalos *et al.*, 1992). Three copies of the sequence, two identical to the *T. fusca* sequence, one differing by a single base, are upstream of *celA* in *Streptomyces halstedii* JM8; one is upstream of the putative -35 sequence, one at the putative-10 sequence, one between the -10 sequence and the translation initiation codon (Fernández-Abalos *et al.*, 1992). A somewhat similar sequence is in a similar position near *cenC* of *C. fimi* and a β-1,3-glucanase gene of *Oerskovia xanthineolytica* (Fernández-Abalos *et al.*, 1992). All of the organisms in which the sequences have been identified are related. The exact function of the binding protein in *T. fusca* XY is not known. Its presence only in induced cells suggests that it could block the action of a repressor (Wilson, 1992).

The transcripts of *cenA*, *B* and *C*, and of *cex*, are monocistronic in *C. fimi*. Transcription of the genes is not coordinately regulated. Glycerol-grown cells contain low levels of *cenA* and *cenB* transcripts only; glucose-grown cells contain very low levels of *cenB* transcripts only; carboxymethylcellulose-grown cells contain high levels of transcripts of all four genes. Only CenB

is produced constitutively, but it is induced by carboxymethylcellulose (Greenberg *et al.*, 1987a, b; Moser *et al.*, 1989). *C. fimi* uses different promoters for constitutive and induced transcription of *cenB* (Greenberg *et al.*, 1987a). A sequence of 50 base pairs with 64% sequence identity, and which includes the putative promoters, is present immediately upstream of the transcription initiation sites of *cenA*, *cenB* and *cex* (Greenberg *et al.*, 1987a). This sequence is not present near *cenC*, but only *cenC* has near it the 14 base pair inverted repeat identified in *T. fusca*, suggesting that *cenC* is regulated quite differently.

When induced, *T. reesei* produces very large amounts of CBHI, CBHII, EGI and EGII in the proportions 6:1.5:1:1, respectively. The genes encoding the proteins are present on different chromosomes in single copies, so their promoters must be very efficient (Teeri *et al.*, 1991). The genes are coordinately regulated because the transcripts of the genes are always present in the same proportions as the proteins they encode; the levels of the transcripts vary with the growth conditions (Fowler *et al.*, 1993). The genes are induced fully by the addition of 2 mM sophorose to cultures growing on sorbitol. When sophorose is added to cultures on glucose, transcription does not increase (Penttilä *et al.*, 1993). There is some increase in transcription when glucose becomes limiting in the absence of cellulose (Teeri *et al.*, 1991). There appear to be no common regulatory sequences upstream of the genes, but upstream of *cbh1* are three repeats of a 6-base sequence which may be involved in glucose repression of the gene (Penttilä *et al.*, 1993). Transcription of *cbh1* in *T. reesei* is induced by cellulose or sophorose. Transcripts appear 14 h after the addition of cellulose, but only 4 h after the addition of sophorose. If cultures are induced with cellulose for 21 h, then glucose added for 1 h, transcripts cannot be detected. Polyclonal antiserum to the major cellulases blocks transcription of *cbh1* if added before cellulose, but not if added 10 h after cellulose. Antiserum to individual cellulases does not block induction (El-Gogary *et al.*, 1989). Either all of the major cellulases of *T. reesei* are required to effect induction by cellulose, or induction is dependent on a minor component of the system. Two minor endoglucanases, at least, in addition to CBHI, CBHII, EGI and EGII, are produced by *T. reesei* (Håkannson *et al.*, 1978; Saloheimo *et al.*, 1994; Ward *et al.*, 1993).

Transcripts of *cel1* in *Agaricus bisporus* are six times more abundant in cellulose-grown than in glucose-grown cultures. Putative glucose- and cAMP-responsive sequences lie upstream of *cel1* (Yagüe *et al.*, 1994). *P. chrysosporium* produces a number of cellulases with sequences and functions related to those of cellulases from *T. reesei*. It produces several CBHI isozymes (Covert *et al.*, 1992a; Sims *et al.*, 1988, 1994) and a single CBHII (Tempelaars *et al.*, 1994). Six *cbhI* genes were cloned and sequenced. The gene sequences belong to a family with two sub-families: *cbhI.1* and *cbhI.2* form one subfamily, *cbhI.3–cbhI.6* form the other (Covert *et al.*, 1992a). *CbhI.1*, *cbhI.2* and *cbhI.3* are clustered within a 25 kb region. The relative

transcript levels for *cbhI.1*, *cbhI.2*, *cbhI.3*, *cbhI.4*, *cbhI.5* and *cbhI.6* in cultures grown on cellulose are 0.00002, 0.00002, 0.02, 1.0, 0.04 and 0.1, respectively (Covert *et al.*, 1992b; Vanden Wymelenberg *et al.*, 1993). *CbhI.1*, *cbhI.2* and *cbhII* are not transcribed when glucose is the carbon source. All are transcribed with cellulose as the carbon source but only *cbhII* is transcribed with oat spelt xylan as the carbon source (Tempelaars *et al.*, 1994).

There must be promoters upstream of the genes for which transcripts have been analysed, but none has been identified unequivocally by footprinting with the homologous RNA polymerase. Sequence comparisons with known promoters in other organisms, especially related organisms, will often reveal putative promoters. Related, appropriately positioned sequences upstream of different cellulase genes in the same organism can also define putative promoters in the absence of similarity to promoters in other organisms. Although putative promoters are located upstream of most of the genes discussed above, a consensus sequence for a cellulase or xylanase promoter has not emerged. Transcript analysis has shown that, as might be expected, the patterns of regulation vary in different organisms, with uncoordinated regulation in some, and coordinated regulation in others.

5.5 Inducers of Cellulase and Xylanase Synthesis

Measurement of enzyme levels in cultures growing on different carbon sources will give insights into the general mechanisms regulating enzyme synthesis, such as catabolite repression or related processes, and induction. Unless individual enzymes are quantified, however, perhaps with specific substrates or with specific antibodies, measurements of total activity against substrates such as carboxymethylcellulose, cellulose, xylan and cellodextrins, give little information on the regulation of all the genes comprising the system. Transcript analysis is more revealing if specific probes are available. The same criticism can be made of attempts to identify inducers and the enzymes which produce them from the polysaccharides: as yet unidentified enzymes may be responsible. The enzymes involved will be produced constitutively, perhaps at low levels which rise upon induction. It is possible that different genes in a system respond to different inducers. Cellobiose is a logical candidate for an inducer; it is an end product of the hydrolysis of cellulose by cellobiohydrolases and of cellodextrins by endoglucanases and cellodextrinases, and cellulolytic organisms generally transport it. However, membrane-associated and intracellular retaining β-glucosidases could convert cellobiose to products such as sophorose by transglycosylation. Sophorose appears to function as an inducer in some organisms. It is also possible that soluble oligosaccharides could be transported to serve as inducers. These comments apply also to induction by xylan. The logical place to look for the

true inducer(s) is inside the cell. It may not be transported from the exterior.

Extracellular cellobiose induces the components of the cellulosome in *C. thermocellum* (Bhat *et al.*, 1993). *F. succinogenes* produces xylanase activity constitutively (Forsberg *et al.*, 1981); Egl1 is repressed by cellobiose, but it produces Egl2 and Egl3 constitutively (McGavin *et al.*, 1990); it has a cellodextrinase in the periplasm which converts soluble cellodextrins to cellobiose and glucose (Huang and Forsberg, 1987, 1988) which makes it unlikely that cellodextrins are transported. In contrast, culturing of *P. fluorescens* subsp. *cellulosa* on cellobiose, glucose or xylose represses production of endoglucanase and xylanase activities, but *celC*, encoding an endoglucanase, is not repressed during growth on cellobiose. Surprisingly both endoglucanase and xylanase activity are induced by growth on Avicel, filter paper, carboxymethylcellulose or xylan (Hazlewood *et al.*, 1992). However, cross-contamination of the substrates must be ruled out before any conclusions can be drawn from the last observation. Avicel can be contaminated with xylan, for example.

In *T. fusca* XY, endoglucanase activity is inducible by cellulose, cellodextrins or cellobiose, and cellulases are involved in inducer generation from cellulose. Mutants which synthesize cellulase constitutively are still subject to growth rate repression (Lin and Wilson, 1987). In *C. fimi* NRRL B-402, some endoglucanases are synthesized constitutively, others are catabolite repressed (Kolios *et al.*, 1991). In a *C. fimi* strain isolated from biogas digester slurry, both xylanase and endoglucanase activity are induced by carboxymethylcellulose or xylan (Khanna and Gauri, 1993). In *B. subtilis*, xylanase activity is produced constitutively during exponential growth. As with the analyses of transcription, these sample studies of enzyme levels indicate the complexity and the diversity of the cellulolytic and xylanolytic systems and their regulation in different bacteria.

Sophorose is an especially effective inducer of cellulases in *T. reesei*. It is transported into the cytoplasm but is hydrolysed slowly by β-glucosidase, and so might be transglycosylated to an inducer. In this context, it is interesting that the extracellular β-glucosidase Bgl1 is required by *T. reesei* for rapid induction by Avicel or lactose (Fowler *et al.*, 1993), and that multiple copies of the *bgl* gene enhance induction by cellulose and sophorose, even though sophorose is a relatively poor substrate for Bgl (Kubicek, 1993). Low concentrations of sophorose appear in the medium during growth of *T. reesei* on cellulose (Kubicek *et al.*, 1993a). It should be borne in mind, however, that it is the intracellular concentration of sophorose which is critical if it is a true inducer. Sophorose does induce a sophorose permease (Kubicek *et al.*, 1993b). However, limiting cellobiose is an effective inducer if β-glucosidase activity is inhibited (Kubicek *et al.*, 1993a). *T. reesei* has a constitutive cellobiose permease, of high affinity but slow rate; a mutant in which the permease level is reduced 12-fold is not induced by cellulose or

sophorose, but it is induced by lactose (Kubicek *et al.*, 1993b). The mycelium of *T. reesei* does not contain constitutive cellulases, but the conidia do carry surface-associated cellobiohydrolases, which could produce cellobiose for induction (Kubicek, 1993). The ratios of the major cellulases are always constant in the mycelium, but not in the conidia. Although deletion or replacement of individual genes show that neither Cbh1, Cbh2, Egl1 nor Egl2 is required for their induction by lactose (Fowler and Brown, 1992; Suominen *et al.*, 1993), their requirement for induction by cellulose is not known. Clearly, cellulase induction in *T. reesei* is far from solved.

6. MICROBIAL CELLULASES IN BIOTECHNOLOGY

Although animals cannot use cellulose or hemicelluloses directly, many have evolved symbiotic relationships with cellulolytic microorganisms to tap this vast supply of carbon and energy. Ruminants and other herbivores are familiar examples, but there are many others: shipworms, wood-boring bivalves of the family Teredinidae, harbour vast populations of cellulolytic bacteria in specialized glands (Waterbury *et al.*, 1983) and numerous insects are associated with symbiotic cellulolytic fungi (Cooke, 1977). Symbiosis reaches a sophisticated level among the Attini, a tribe of leaf-cutting ants that tend luxuriant monocultures of saprophytic fungi in subterranean jungle gardens to satisfy all their dietary needs. Humans also exploit the ability of microorganisms to hydrolyse plant biomass, through agriculture: the cultivation of edible mushrooms and the farming of ruminant livestock can be considered as early examples of the application of cellulases to biotechnology. The use of cellulases and hemicellulases to improve the nutritional value of forage crops or cereal-based pig and poultry feeds (Gilbert and Hazlewood, 1991; Henk and Linden, 1992) are relatively conservative extensions of these traditional practices; attempts to use transgenic animal technology to facilitate the digestion of plant material by non-ruminants represent a more radical approach to dietary manipulation (Hall *et al.*, 1993).

Cellulases could be used to produce edible sugars from biomass for human consumption but the product could not compete with existing sources such as sugar cane or corn syrup. However, interest in the use of cellulases to produce fermentable sugars from cellulosic wastes was stimulated in the United States in the late 1970s by the need to overcome dependence on foreign oil imports (Stout, 1982). Agricultural and forest residues are available on a large enough scale to be considered as a source of sugars for the production of alcohol-based fuels. Urban wastes are an additional source of feedstock: it is estimated that 40% of municipal solid waste is cellulose (Walter, 1981). However, the biological conversion of cellulosics is currently hampered by the costs of substrate pre-treatment and enzyme production

(Saddler, 1993). Mechanical, chemical, biological or thermal pre-treatment improves the accessibility of wood wastes to cellulases by removing lignin and hemicelluloses and by partially disrupting fibre structure. Pre-treatment with high-pressure steam is a promising technique although not yet generally applicable to softwoods. Hydrolysis to fermentable sugars requires large quantities of enzymes because cellulases have low turnover numbers and are very sensitive to product inhibition. Enzyme recycling could help to reduce production costs. Product inhibition is alleviated by β-glucosidases or avoided in "simultaneous saccharification and fermentation" schemes where substrate, enzymes and ethanol-tolerant yeasts that ferment cellobiose are mixed in a single reaction vessel. Because most plant biomass includes a significant hemicellulose fraction, efficient bioconversion schemes are likely to incorporate processes for fermentation of pentose sugars. Genetic approaches to the problems now facing biomass conversion include attempts to engineer recombinant bacteria with genes for both hydrolysis and fermentation (Wood and Ingram, 1992). White-rot fungi (e.g. *P. chrysosporium*) and actinomycete bacteria (e.g. *Streptomyces* spp.) have considerable potential in this and other applications because they can degrade both polysaccharides and lignin (Broda, 1992; Jeffries, 1990). Ethanol derived from starch hydrolysates is presently used to supplement gasoline fuels but it is unlikely that production could be sustained on a global scale. Removal of economic barriers to lignocellulose bioconversion is a major challenge to engineers, biochemists and molecular biologists while reserves of biomass are available at current levels and costs.

Because of the current problems associated with extensive hydrolysis, applications that involve only limited hydrolysis of cellulose or hemicellulose have more immediate potential. Xylanases are now used to aid lignin removal during the manufacture of pulps for paper making; the process is environmentally significant because it reduces the need for chlorine bleaching (Wong and Saddler, 1992). Other promising applications in this area include the use of cellulases to remove "fines" or modify pulp fibre surface properties and to facilitate the de-inking of recycled paper (Ciba Geigy Corporation, 1994; Jeffries *et al.*, 1992). Enzyme preparations for these purposes are available commercially (Ciba Geigy Corporation, 1994). Several suppliers also produce cellulase preparations for textile treatment to reduce fuzz or piling or to enhance the softness, lustre and colour brightening of fabrics made from cotton, flax or ramie fibres. Preparations with alkaline pH optima, e.g. from *Humicola* or *Bacillus* spp., are suitable as detergent additives to assist dirt removal and improve fabric appearance. Similarly, cellulases could replace the use of pumice in the manufacture of stone-washed denim (Lange, 1993).

Other potential applications involve non-hydrolytic properties of cellulases. Isolated CBDs modify the surface of cellulosic fibres (Din *et al.*, 1991) and could find applications in the textile, pulp and paper industries. CBDs can be fused to heterologous proteins as affinity tags for immobilization or

purification purposes; appropriate protease cleavage sites can be introduced to facilitate subsequent tag removal. Cellulose matrices are attractive because they are cheap and available in a range of configurations (Kilburn *et al.*, 1993). The scaffolding proteins of cellulosomes could be modified to allow assembly and coordination of multiple enzymatic and non-enzymatic components for complex biochemical processes (Bayer *et al.*, 1994). *T. reesei* CBHI coupled to silica is enantioselective and can be used to resolve racemic mixtures of various drugs (Ståhlberg *et al.*, 1993b). The linkers that join the various modules of multidomain cellulases are potentially useful tools for the genetic engineer: for example, the interdomain linker from *T. reesei* CBHI was used to join the variable domains of a single chain antibody produced in *E. coli* (Takkinen *et al.*, 1991).

The production of fungal cellulases has been considerably improved by strain selection but the genetic engineering of cellulase systems offers the flexibility needed to tailor systems to specific applications. By this means, it will be possible to delete unwanted enzymes or overproduce others and to engineer the properties of individual enzymes. The *T. reesei cbh1* promoter has been used for high-level production of CBHI itself, other *T. reesei* cellulases, and a selection of heterologous proteins including *Phlebia radita* lignin peroxidase and calf chymosin (Paloheimo *et al.*, 1993). Similarly, the *cbhI.1* promoter of *P. chrysosporium* has been isolated and attempts to develop appropriate transformation systems for gene manipulation and high-level expression are in progress (Broda, 1992).

7. CONCLUSION

We can confidently predict that molecular biology, high-resolution X-ray crystallography, NMR spectroscopy and other biochemical techniques will continue to provide us with important information on enzyme regulation, on the structure of individual cellulases and hemicellulases, and on their mechanisms of action on soluble substrates. Understanding how these enzymes cooperate to hydrolyse insoluble substrates, particularly crystalline celluloses, is a further challenge. There is no doubt that cellulases effect important changes to their substrate before releasing soluble products and the key to understanding cellulase action appears to rest in the examination of these events. Progress in this area is currently limited by the availability of appropriate analytical tools although new techniques, such as atomic force microscopy (Hanley *et al.*, 1992), are promising. Recent advances in our understanding of cellulose structures and the increasing availability of defined cellulosic substrates should also facilitate research. It is clear that the properties of cellulases are profoundly altered by the presence of trace enzyme contaminants. It is not unreasonable, given the confusion caused by

the use of impure enzymes (Wood and Garcia-Campayo, 1990), to propose that future studies *in vitro* be restricted to enzymes from recombinant sources. It is still far from clear why the individual cellulolytic bacteria and fungi require so many related cellulases with specificities that overlap, but perhaps this is because we underestimate the complexity of the substrates and of the task these microorganisms face.

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Calcium and Bacteria

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1. INTRODUCTION

Life evolved in an environment containing many different cations, including Ca^{2+}. In excess these cations are frequently toxic and among those cations present in the early oceans Ca^{2+} presented a particular problem. Many calcium salts are relatively insoluble, including those of phosphate and a

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variety of simple organic acids. Thus from an early period in evolution there must have been some means of removing Ca^{2+} from the cell in order to prevent precipitation of cellular constituents. The removal of Ca^{2+} requires energy in one form or another to pump the Ca^{2+} against the concentration gradient. The Ca^{2+} within the cell could be bound into a harmless form by Ca^{2+} chelating materials, but in doing so the free Ca^{2+} concentration within the cell is decreased. A net Ca^{2+} concentration gradient across the cell membrane is formed which by present-day values would be of the order of 10^{-3} to 10^{-4} M. This gradient has to be resisted by the impermeability of the cell membrane and maintained by continuous exclusion of Ca^{2+} from the cell. The ability to control Ca^{2+} content or at least intracellular free Ca^{2+} concentration may thus be a fundamental attribute of all cells, including the prokaryotes.

Because the cations were present during early evolution, they would also have participated in the random selection processes that eventually yielded the structural and functional components of cells. The attributes of particular cations suited them to particular roles. Unlike Mg^{2+}, Ca^{2+} does not often feature as a co-factor of present-day enzymatically active proteins, but, with some notable exceptions, Ca^{2+} appears more frequently as a constituent of the structural components of the cell. As the extensive investigations of eukaryotic cells have shown, Ca^{2+} also became important in signal transduction, as a signal transmitter in membrane depolarization events, as an intracellular second messenger and as an effector of actino-myosin contraction. The common evolutionary origins of the prokaryotes and eukaryotes and the many examples of evolutionary conservation of structure and function that have been shown to exist between them support the concept (Norris *et al.*, 1991) that such evolutionary conservation should extend to the role of Ca^{2+}. Many of the functions that Ca^{2+} has been shown to perform in eukaryotes may therefore be expected to be present in prokaryotes. It is clear from the literature that the choice of analytical techniques and the approaches made in many investigations of Ca^{2+} in prokaryotes involve the implicit assumption of such evolutionary conservation.

Despite the recognition of the importance of Ca^{2+} in eukaryotic cells, there have been relatively few systematic studies of the role of Ca^{2+} in bacteria. Much of what little is known has been acquired serendipitously as the result of research investigations that were not directed at Ca^{2+}. Often the involvement of Ca^{2+} has come as a surprise and is sometimes reported as such (cf. Vyas *et al.*, 1987). Consequently the current picture of the distribution of Ca^{2+} in prokaryotes and its functions in the prokaryotic cell is incomplete and often superficial. Recently, however, there has been an increased interest in the role of Ca^{2+} in the prokaryote. A number of investigations have reported data that add substantially to the view that Ca^{2+} does indeed contribute to the structure and regulatory functions of the prokaryotic cell.

This review describes the recent advances and hypotheses concerning the role of Ca^{2+} in prokaryotes. In order to assist in providing some element of structure and a thematic approach, the review is divided into three sections that concern the cell wall and membrane, the cytoplasm and the prokaryotic nucleoid. Other recent reviews and articles in the same subject area include those by Smith (1988), Norris (1989a, b), Norris *et al.* (1991), and Onek and Smith (1992).

2. THE CELL WALL AND CELL MEMBRANE

A large fraction of the Ca^{2+} associated with the prokaryotic cell is found in the region of the cell wall and cell membrane. Chang *et al.* (1987) using X-ray microprobe analysis found that 10-nm-wide regions which included the cell envelope contained an average of 17 times more Ca^{2+} than corresponding cytoplasmic regions of exponential phase cells of *Escherichia coli* (32.6 versus 1.5 mM kg^{-1}). Part of this Ca^{2+} is bound on the outer surface of the cell. The outer lipopolysaccharide layer of *Salmonella typhimurium* has been shown to contain high-affinity binding sites for Ca^{2+}. It is also thought that Ca^{2+} is required for the stabilization of the lipopolysaccharide layers of Gram-negative bacteria (Schindler and Osborn, 1979). Labelling studies with ^{45}Ca have also shown that a large fraction of the Ca^{2+} associated with prokaryotic cells is bound externally and may be removed by washing procedures (Gangola and Rosen, 1987) or treatment with Ca^{2+} chelating agents such as ethyleneglycol-bis-(β-aminoethylether)-N,N,N′,N′,-tetraacetic acid (EGTA) (Smith *et al.*, 1987a). For example, about half the ^{45}Ca associated with long-labelled cultures of the cyanobacterium *Nostoc* PCC 6720 was removed by brief treatment with growth medium containing 2 mM EGTA. This EGTA-removable fraction persisted in cultures that were boiled or sonicated to break open the cells, but the EGTA-resistant fraction, which is presumed to represent the internal Ca^{2+}, was removed by such treatment (Smith *et al.*, 1987a).

2.1. Ca^{2+} and the Cell Wall

In addition to occurring in the lipopolysaccharide layer, Ca^{2+} is also involved in the regular outer surface layers, known as S-layers, that occur in most bacteria. X-ray diffraction and microscopic studies have shown that these S-layers are composed of one or more monolayers. Each monolayer is formed from one species of acidic, bilobed protein that is organized in a regular crystalline array within the mono-protein layer. The interaction of these bilobed proteins in the crystalline array involves the binding of divalent cations. Studies on the so-called A-layer of *Aeromonas salmonicida*—which

is equivalent to an S-layer—showed that the structure is altered by growth under Ca^{2+} limiting conditions or by treatment with Ca^{2+} chelating compounds such as ethylenediamine tetraacetic acid (EDTA) or EGTA (Garduno *et al.*, 1992). After divalent cation depletion, the ordering of the protein subunits assumed regular interactions that are distinct from those found in the A-layer of cells grown in Ca^{2+} replete culture conditions. The integrity of the A-layer was decreased in Ca^{2+} depleted conditions as shown by the release into the growth medium of tetrameric complexes rather than the monomeric form of the constituent A protein.

The structural role of Ca^{2+} in the cell wall and cell membranes of prokaryotes is perhaps reflected in the requirement of 1 mM or 0.1 mM Ca^{2+}, respectively, for the optimal growth of an *E. coli* L form, NC7, in medium containing either NaCl or KCl as the osmotic stabilizer (Onada and Oshima, 1988). In KCl-supplemented cultures the requirement for Ca^{2+} was shown to be temperature-sensitive. L forms arise spontaneously in bacterial cultures and are frequently isolated from subcultures of pathogenic bacteria. They are characterized by the lack of a complete cell wall with resultant fragility and strict growth requirements. The lack of a complete cell wall in the L form suggests that Ca^{2+} may have a role in the maintenance of the structure and integrity of either the remaining cell wall or the exposed cell membrane.

The importance of Ca^{2+} in the structure of the bacterial cell wall and the detrimental effects of Ca^{2+} depletion on its integrity are readily discerned in cultures of prokaryotes grown in Ca^{2+} deficient media, though the addition of a Ca^{2+} chelating agent such as EGTA to the growth medium may be required to deplete sufficiently the Ca^{2+} content of the culture. The relatively large cells of filamentous cyanobacteria such as *Anabaena* and *Nostoc* species assist the microscopic observation of the effects of Ca^{2+} depletion. In cultures of *Nostoc* PCC 6720, grown for two generation times in medium containing 2 mM EGTA, the cell wall appeared less compact and in some cells was deformed. More obvious was the rapid degeneration of the filaments, which break down to single cells as an apparent consequence of the further loss of cell wall integrity. Most of the single cells appeared to be arrested in the process of cell division because they contained an incomplete latitudinal invagination of the cell wall (R. J. Smith, unpublished observations).

2.2. Ca^{2+} and the Cell Membrane

There is evidence that Ca^{2+} influences phospholipid metabolism and membrane structure; several individual reports link lipid metabolism and Ca^{2+}. Some bacterial phospholipases, for example, have been found, like their eukaryotic counterparts, to require Ca^{2+}. Concentrations of Ca^{2+} exceeding 1 nM are required to maintain the activity of phospholipase A1

which resides on the outer membrane of *E. coli* (Elsbach, 1985) and, *in vitro*, Ca^{2+} has been shown to inhibit phosphatidylglycerol synthesis (Demant, 1982). These reports lend credence to the hypotheses of Murthy and co-workers, who have supplied substantial evidence in a series of reports for the role of Ca^{2+} and a calmodulin-like protein in the regulation of phospholipid metabolism in Mycobacteria. The work describes the effects of glucose supplementation to glycerol-grown cultures of *Mycobacterium phlei*. Addition of glucose (optimum concentration, 5% (w/v)) was found to increase growth and the phospholipid content of the cells. The increase in general and individual phospholipids, including phosphatidylglycerol, phosphatidylinositol and phosphatidylethanolamine, is positively correlated with an increase in the cellular content of the calmodulin-like protein (Reddy *et al.*, 1992). Supplementation of glucose to 7.5% (w/v) does not promote the maximum accumulation of phospholipids nor, notably, the increase in the calmodulin-like protein. Further evidence comes from the incorporation of ^{32}Pi into total phospholipids during the glucose supplementation. The incorporation was inhibited by the addition of known antagonists of eukaryotic calmodulins (trifluoperazine or phenothiazine) to the culture (59% inhibition was observed at a dose of 40 μM) or by the addition of EGTA (35% inhibition at a dose of 2 mM) (Reddy *et al.*, 1992).

The relevance of this hypothesis is brought into focus by observations of the interaction of Ca^{2+} and charged lipids and their effects on the structure and function of bacterial membranes. Ca^{2+} promotes *in vitro* the lateral segregation of acidic phospholipids within a membrane into domains (Cullis and de Kruijff, 1979). The formation of such domains of phospholipids in *S. typhimurium* membranes has been ascribed to a Ca^{2+}–phospholipid interaction (Metcalf *et al.*, 1986). Membrane fusing and the blebbing of the erythrocyte envelope has also been attributed to Ca^{2+}, which has been proposed to play a major role in the formation of non-bilinear phospholipid configurations (Cullis and de Kruijff, 1979; Zachowski, 1987). More recently, evidence has been presented which indicates that the formation of such non-bilayer membrane structures in *E. coli* may be involved in the translocation of proteins across membranes (Killian *et al.*, 1990). Consequently, it would appear that such fundamental physiological processes as protein export and cell division may involve an effect of Ca^{2+} on membrane structure. However, the interaction of charged lipids and Ca^{2+} may affect not only the structure of the membrane itself, but also the structure and function of proteins embedded in it (Shapiro, 1993).

2.3. Protein and Lipid Domains

The concept of fluidity in biological membranes (Singer and Nicholson, 1972) infers the ability of the lipids composing the membrane and of the proteins

embedded within it, to move within the linear dimension of the membrane. A natural consequence of such fluidity is the ability of the membrane to differentiate so that specific areas of the membrane may possess distinct structure and functions. The asymmetrical distribution of proteins and enzyme activities in the compartmentalized eukaryotic cell has long been accepted. Only more recently has it been understood that this asymmetry does not depend solely on cell organelles, but also occurs through the sequestering of specific proteins to different domains of plasma membranes. The prokaryote, although devoid of cell organelles and considered small enough that diffusion serves to distribute metabolites, also possesses such asymmetry (Shapiro, 1993). The most extensively documented examples of the assembly of differentiated protein complex structures within domains of bacterial membranes are the cell division apparatus (Bi and Lutkenhaus, 1991) and the chemosensory apparatus (Alley *et al.*, 1992; Maddock and Shapiro, 1993).

Data obtained from the use of nuclear magnetic resonance (NMR), electron spin resonance (ESR), freeze fracture and fluorescence studies provide substantial evidence for the occurrence of other less obvious domains in cell membranes (Tocanne *et al.*, 1989). In bacteria the technique of photodimerization of neighbouring phospholipids has shown that a heterogeneous distribution exists in the membranes of *Micrococcus luteus* (De Bony *et al.*, 1989), while fluorescence reactivation after photobleaching has revealed the existence of large charged lipid domains in *S. typhimurium* membranes (Metcalf *et al.*, 1986). Autoradiography was used to demonstrate the existence of large numbers of smaller domains in the cell membrane of *E. coli* (Green and Schaechter, 1972). The asymmetrical heterogeneity of the lipid composition of the membranes of bacteria has also been demonstrated by the analysis of minicells. These arise from an abnormal cell division at the cell pole of a bacterial cell and thus present an opportunity to study the lipid and protein contents of the membrane derived from this discrete portion of the cell. The lipid composition of the membranes of *Bacillus subtilis* and *E. coli* minicells have been shown to be different from that of normal cell membranes for both the phospholipid and the fatty acid ratios (Goodell *et al.*, 1974; Shohayeb and Chopra, 1985). Enzymatic studies of minicell membranes (Dvorak *et al.*, 1970; Frazer and Curtis, 1975; Buchanan, 1981) and the asymmetrical distribution of penicillin-binding proteins in whole cell membranes (Leidenix *et al.*, 1989; Bayer *et al.*, 1990) provide evidence for the presence of protein domains within bacterial membranes.

The available evidence thus supports the notion that not only may proteins associate to form specific domains within the membrane, but also the constitution of the membrane in terms of the diversity of the lipid content may vary from area to area. These domains, for example, may be particularly rich in charged lipids. Such domains may in turn affect the activity of proteins embedded within the membrane. They may, for example, promote the

association or dissociation of membrane-bound proteins to either form or disaggregate protein complexes and thus influence the activity of individual proteins or protein complexes. The work of Cullis and de Kruijff (1979) and Zachowski (1987) indicates that Ca^{2+} has major effects on the diversity of membrane structure. Ca^{2+} may therefore influence the activity of proteins within the membrane as a consequence of these protein/protein and protein/lipid interactions through an alteration in the composition of the lipids surrounding them. The fluidity of the membrane also implies that domains are not permanent structures, but may alter with time either as an aspect of the cell cycle or in response to external influences.

On the basis of such conjectures, Norris (1989a, b, 1992) has proposed a mechanism for the regulation of DNA replication and cell division in prokaryotes that brings together the effects of Ca^{2+} on membrane structure and the proposed Ca^{2+}/calmodulin-mediated regulation of phospholipid synthesis. It is suggested that a sudden increase in cytoplasmic Ca^{2+} may trigger a major translocation of phospholipids from the inner to the outer monolayer of the bilayered cell membrane. A steady accumulation of phospholipids during the cell cycle would, through the eventual formation of a critical packing density of charged lipids, promote the translocation. This would result in the sudden equalization of the packing density between the two layers of the membrane. Since the translocation may be expected to disrupt momentarily the integrity of the membrane, depolarization of the membrane and a substantial influx of Ca^{2+} might occur. The loss of charged lipids from the inner monolayer of the membrane may be presumed to decrease substantially the ability of the inner layer of the membrane to associate with Ca^{2+}. Thus bound Ca^{2+} within the cell, including that entering due to the translocation, would necessarily be redistributed to cytoplasmic chelators. It is suggested that this could account for the redistribution of Ca^{2+} from the cell wall/membrane region to the cytoplasm of the dividing *E. coli* cell that was observed by Chang *et al.* (1987).

Because Ca^{2+} is also thought to be bound by the nucleoid regions, the replication of the genome affects Ca^{2+} metabolism in terms of the balance between influx, efflux and the intracellular chelation of Ca^{2+}. Norris (1992) has further suggested that the cessation of Ca^{2+} binding to newly made DNA, which presumably occurs on the termination of replication, may also contribute to an overshoot on Ca^{2+} influx leading to an increase in the cellular Ca^{2+} content. This increase could plausibly promote the formation of charged acid lipid domains and thus increase the packing density to the critical value required to trigger translocation of the charged lipids between the monolayers of the membrane. Thus the termination of replication could trigger the sequence of events that lead to cell division and the next round of replication. However, if Ca^{2+} were the only arbiter of the proposed sequence of events, spurious Ca^{2+} influx might lead to premature cell division. To counter this possibility it was suggested that the *E. coli* cell only becomes receptive to

the translocation phenomenon (and therefore the effects of Ca^{2+} on translocation) at the appropriate time. Prior to the publication of the observations of Murthy and associates, Norris (1989a) suggested that the process which controlled susceptibility to charged lipid translocations might be the accumulation of the charged lipids in the inner monolayer of the cell membrane. This accumulation, it was suggested, would occur gradually during the cell cycle, such that, by the time the cell had terminated DNA replication and was ready to commence cell division, the packing density of charged lipids in the inner monolayer would be reaching the critical level. The observations of Murthy and associates indicate that just such an accumulation of charged lipids occurs in the membranes of *M. phlei* and *M. smegmatis* and thus add credence to the hypothesis (Reddy *et al.*, 1992).

Norris has taken the potential role of lipid domains in cell division a step further in a second hypothesis, the "membrane tectonics model" (Norris, 1989b) which attempts to explain the manner and means by which the site of bacterial cell division is positioned. The model is based on the formation of membrane domains. In brief the hypothesis suggests that at least three domains exist in the cell membrane: the polar domains at the ends of the cell, the chromosomal domains that form in the centre of the cell and migrate apart during nucleoid segregation, and the septal domain that forms by nucleation between the separating chromosomal domains and acts as the precursor for septum formation and consequent cell division. These domains call not only for the formation of phospholipid domains, but also the formation of protein island domains. The consequences of charged phospholipid accumulation and the influence of charged phospholipids on membrane-bound proteins are thus extended in theory to both the regulation and the positioning of cell division. Given the known attachment of the bacterial chromosome to the inner monolayer of the cell membrane (Rosenburg and Cavalieri, 1968; Abe *et al.*, 1977; Green and Schaechter, 1972), and in particular the association with the cell membrane of the hemimethylated origin of replication, OriC, in *E. coli* (Ogden *et al.*, 1988; Campbell and Kleckner, 1990), it is reasonable to suggest that the initiation of DNA replication is also controlled by a mechanism that involves Ca^{2+}-mediated effects on the membrane. Notably, proteins involved in the initiation of DNA replication show a sensitivity to phospholipid composition (Yung and Kornberg, 1988) and, as described below, may be influenced by Ca^{2+} and calmodulin.

The hypotheses described by Norris are supported by, and offer an explanation for, other observations. For the present purpose, they serve to demonstrate how Ca^{2+} may influence the structure and function of the membrane and thereby play a role in the regulation of the spatial organization of the cell and the metabolic and physiological functions it performs. However, the effects of Ca^{2+} on the cell membrane of the prokaryotic cell are not limited to DNA replication and cell division, but might include the

transport of proteins (De Vrije *et al.*, 1985; Lill *et al.*, 1990), ions (George *et al.*, 1989; Martinac *et al.*, 1990) and sugars (Seto-Young *et al.*, 1985; Letellier *et al.*, 1977), respiration (Esfahani *et al.*, 1977; Dancey and Shapiro, 1977) and cell wall synthesis (Beadling and Rothfield, 1978; Lee *et al.*, 1980).

Support for the idea that Ca^{2+} may influence membrane-associated processes comes from the observed effects of Ca^{2+} on two electron transport complexes in prokaryotes. The sulphate-reducing bacterium *Desulphovibrio gigas* couples the reduction of sulphite to sulphide from hydrogen using the enzyme desulphoviridin which is supplied via an electron transfer chain that includes cytochrome c_3 and either ferredoxin or flavodoxin. Attempts to reconstitute the chain *in vitro* using purified protein extracts have not proven successful so that studies of the process have generally used methyl viologen as an artificial electron carrier between the hydrogenase and the terminal reductase, desulphoviridin. Preparations of the chain manufactured from dialysed crude extracts of *D. gigas* have a small residual activity *in vitro*. Chen *et al.* (1991) found that this residual activity was substantially enhanced by the addition of Ca^{2+} or Mg^{2+}. Using this enhancement of the crude preparation as an assay system, a coupling factor protein of molecular mass 65 kDa was isolated from *D. gigas*. The protein was required for the enhancement and was capable of stimulating the activity of either the crude system *in vitro* or one prepared from the purified constituent proteins. In the presence of the coupling factor, either 40 μM Ca^{2+} or 40 μM Mg^{2+} promoted a 10-fold enhancement of the activity of the purified protein preparation. Analysis of the coupling factor indicated that it binds four Ca^{2+} per molecule and contains amino acid sequences that have putative structural and functional homology with the (EF) hand motif of eukaryotic Ca^{2+} binding proteins. The site of action of the coupling factor protein is not known, but the authors speculated that it may stabilize the complex of proteins formed between the components of the electron transport chain.

There is also substantial evidence for the involvement of Ca^{2+} in cyanobacterial photosynthetic electron transport. Despite the similarity of the O_2-evolving photosystem II (PSII) activities found in the cyanobacteria to those of higher plants, the Ca^{2+} requirements in cyanobacterial photosystems may be different (Debus, 1992). A Ca^{2+} mediated activation of O_2 evolution was first found in extracts of *Phormidium luridum* (Piccioni and Mauzerall, 1978). An eight-fold increase in O_2 evolution on addition of both Mg^{2+} and Ca^{2+} was ascribed to a four-fold increase in the number of active photosynthetic units due to Ca^{2+} and a doubling of turnover rate due to Mg^{2+}. Evans and associates have made an extensive study of the requirement for Ca^{2+} to sustain the activity *in vitro* of PSII extracts of the unicellular cyanobacterium, *Synechococcus* sp. PCC 7942 (formerly known as *Anacystis nidulans*). The maintenance of activity was shown to be strongly dependent

on the presence of Ca^{2+} in the extraction buffers used to prepare the *in vitro* system (England and Evans, 1983) and on the Ca^{2+} content of the medium in which the culture was grown (Roberts and Evans, 1988). The activity of preparations *in vitro* was inhibited when Ca^{2+} was removed, but could be restored by the addition of Ca^{2+} and Na^{+} (England and Evans, 1983). The requirement for Ca^{2+} has been ascribed to electron transport between component Z and phytochrome P_{680} on the water splitting side of the PSII complex (Evans *et al.*, 1983; Satoh and Katoh, 1985). This requirement for Ca^{2+} in the PSII complex of *Synechococcus* sp. has been confirmed by Becker and Brand (1985), who also found that it could be satisfied by Na^{+}. More recently the Ca^{2+} dependent activity has been shown to be inhibited by an antibody raised against guinea pig calmodulin. Two proteins have been identified in thylakoid membrane extracts of *Synechococcus* sp. that bind Ca^{2+} in the assay system devised by Maruyama *et al.* (1984). One corresponded to a protein of about 60 kDa and another to a protein of 18 kDa. The 60 kDa protein was also detected in Western blots of thylakoid extracts by the guinea pig calmodulin antibody and was thus ascribed a function in the Ca^{2+} dependency of the PSII preparations. McColl and Evans (1990) suggested that this protein may represent an aggregate of the D1 protein, which is a 32 kDa protein of the cyanobacterial O_2 evolving complex of the PSII particle (Barbock, 1987) and akin to the Ca^{2+} binding protein of the chloroplast (Koike and Inoue, 1985). This suggestion was confirmed by the finding that PSII preparations prepared from a *Synechococcus* sp. mutant in which the gene encoding the major D1 protein (*psb*A1) was deleted, cannot be reactivated *in vitro* after Ca^{2+} depletion (H. Evans, personal communication). The 18 kDa protein was suggested as being analogous to the light harvesting proteins of the chloroplast which also bind Ca^{2+} (Webber and Gray, 1989).

2.4. Extracellular Proteins

A greater proportion of secreted proteins appear to bind Ca^{2+} than do cytoplasmic proteins. In a survey of protein sequence databases using search matrices based on the EF hand motif, Kretsinger *et al.* (1991) identified several putative Ca^{2+} binding proteins including five from prokaryotic sources. Two of these are secreted proteins, namely the haemolysin A from *E. coli* which was known to bind Ca^{2+} and require it for attachment to the erythrocyte membrane (Ludwig *et al.*, 1988), and cellulase A of *Clostridium thermocellum*. Other recently reported examples are: (a) the extracellular Ca^{2+}-requiring proteases from the marine bacterium JT0127 (Inagaki *et al.*, 1991) and the two *Xanthomonas* species, *X. maltophillia* (Debette, 1991) and *X. campestris* (Dow *et al.*, 1990); and (b) two *B. subtilis* enzymes, a

levansucrase (Chambert and Petit-Glatron, 1990) which requires either Ca^{2+} or Fe^{2+} for refolding, and a pectate lyase B (Nasser *et al.*, 1990) which is activated by Ca^{2+} and inactivated in the presence of excess EDTA. Since it is expected that, like *E. coli* (Gangola and Rosen, 1987), all bacteria maintain an intracellular free Ca^{2+} concentration of the order of 1 μM or less, one aspect of the Ca^{2+} requirement of secreted proteins is that they are unlikely to fold into an active form in the cytoplasm. The Ca^{2+} activation of such proteins may therefore be a mechanism that has evolved as a means of avoiding premature activation prior to secretion. One type of extracellular protein that requires particular mention is the adenylate cyclase toxins that are secreted by *Bacillus anthracis* and *Bordetella pertussis* (Gordon *et al.*, 1989). Although these two novel toxins differ in size, both enter eukaryotic target cells and are activated by the cellular calmodulin to catalyse the synthesis of cAMP from ATP. Apart from the adenylate cyclase of *Anabaena* (Bianchini *et al.*, 1990), the toxins are currently the only known prokaryotic enzymes to be activated by calmodulin and the only ones known to be activated by a eukaryotic calmodulin.

3. THE CYTOPLASM

Our knowledge and understanding of the metabolism of Ca^{2+} and the molecular biology of Ca^{2+} mediated signal transduction and cellular regulation is almost entirely derived from the extensive investigations of animal cells, which have been prompted and supported by the medical implications that include carcinogenesis and the immune response. This work has shown that in eukaryotic cells, including those of higher plants, the role of Ca^{2+} as a second messenger is involved in a variety of diverse processes. These include such disparate functions as cell growth, division, elongation and mobility, glycogen metabolism, cyclic nucleotide metabolism, protoplasmic streaming and cell organelle mobility, microtubule assembly, gene expression—including both transcription and translation—and the activation of a limited number of individual enzymes. The extent of the eukaryotic work is such that an investigation of Ca^{2+} in prokaryotes must rely heavily on it as a source, not only of analytical techniques, but also of the concepts used in the interpretation of data. A prime example is the investigations in prokaryotes of calmodulin-like proteins in which both the criteria for the identification of the putative prokaryotic calmodulin and the interpretation of the potential role that such a protein might play rest entirely on the eukaryotic role model. Another example involves the many investigations of prokaryotic processes that have made extensive use of inhibitors of calcium channels and Ca^{2+}/calmodulin activity. Almost exclusively, it has been assumed that the action of the inhibitor in the prokaryote is the same as in

eukaryotic cells. Only in a very few cases has this assumption been directly tested.

3.1. The Eukaryotic Role Model

The maintenance of a low intracellular free Ca^{2+} concentration (i[Ca^{2+}]) is required not only to protect the cell from toxic effects of Ca^{2+}, but also to permit the use of Ca^{2+} as a second messenger. Any increase in the free i[Ca^{2+}] due to the propagation of a signal must be negated in order that the next signal may occur. Calcium enters the eukaryotic cell through voltage-gated channels. In order to maintain the steady state of the free i[Ca^{2+}], Ca^{2+} is excluded from the eukaryotic cell by a Ca^{2+}dependent ATPase. The buffering of the cytoplasmic free i[Ca^{2+}] is further assisted by other Ca^{2+}dependent ATPases that move the Ca^{2+} into cellular organelles. In muscle cells, which make extensive use of Ca^{2+} fluxes to produce contraction, a specialized compartment is employed, called the sarcoplasmic reticulum, which contains the Ca^{2+} binding protein calsequestrin. However, all animal cells are now thought to contain Ca^{2+} sequestering compartments. Nerve cells contain a second Ca^{2+} transport system, a Ca^{2+}/Na^{+} antiporter, by which Ca^{2+} is excluded in exchange for an influx of Na^{+}. This antiporter has a relatively low affinity for Ca^{2+} and so only operates efficiently when the internal free i[Ca^{2+}] substantially exceeds the normal concentration. This can presumably occur in nerve cells when frequent action potentials depolarize the membrane and permit Ca^{2+} influx through the voltage-gated Ca^{2+} channels.

An increase in the local free i[Ca^{2+}] in a cell may occur either by an influx of external Ca^{2+} through the Ca^{2+} channels in the outer membrane of the cell or by release from a Ca^{2+} sequestering compartment through similar channels. In eukaryotes, the release of sequestered Ca^{2+} involves a cascade of molecular events known as the inositol triphosphate pathway (Fig. 1). Receptor proteins are present within the cell membrane which have domains projecting from both the outer and inner surface of the membrane. These diverse receptors possess specific sites in the outer domain to which external activator molecules, such as peptide hormones, may bind. The inner domain of the receptor protein is bound to one member of a family of membrane-associated G (GTP binding) proteins. When activated by the binding of an external activator the receptor protein releases the associated G protein which is now able to bind GTP. The released G protein activates in turn a membrane-associated phosphoinositide specific phospholipase which hydrolyses a minor membrane phospholipid called phosphoinositol 4,5,-bisphosphate to release an inositol triphosphate (IP_3) and the membrane lipid diacylglycerol (DAG). IP_3 diffuses through the cytoplasm to act on gated Ca^{2+} channels and release Ca^{2+} from the calcium sequestering

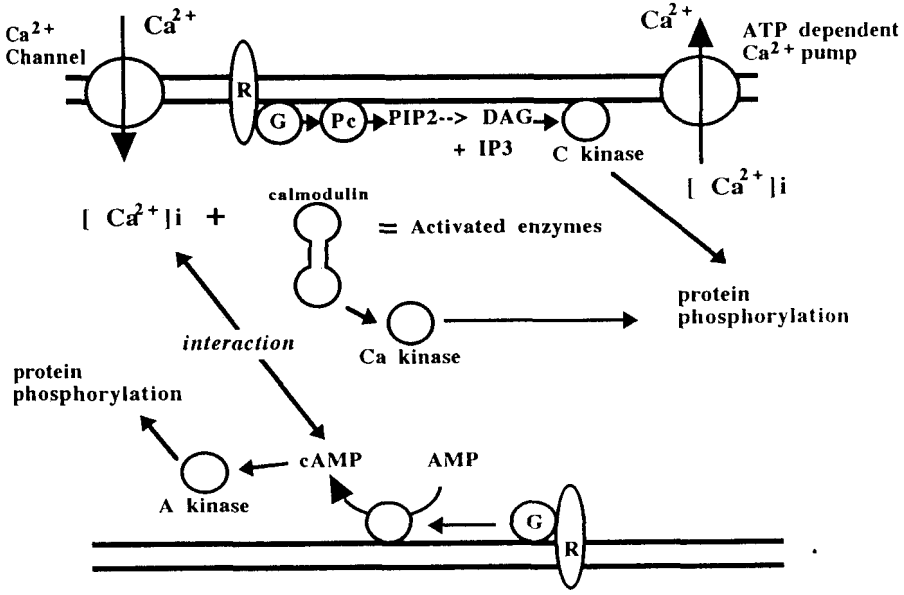


Figure 1 A representation of the Ca^{2+}/calmodulin and cAMP response pathways in eukaryotic cells. R, receptor protein; G, G-protein; Pc, phospholipase C; PIP2, phosphoinositide 4,5,-bisphosphate; IP3, inositol triphosphate; DAG, diacyl glycerol. The protein kinases are labelled C and A. The response pathways are affected by Ca^{2+} channel inhibitors (e.g. verapamil) or activators (e.g. BayK 8644), calmodulin inhibitors (e.g. trifluoperazine) and phorbol esters which mimic the effect of DAG and activate the C kinase.

compartment(s), thus increasing the local free i[Ca^{2+}]. DAG activates a membrane-associated protein kinase C and so directly influences the phosphorylation of proteins.

The released Ca^{2+} acts on Ca^{2+} binding proteins. The known Ca^{2+} binding proteins belong to a related family of proteins called the EF hand family after the conserved, Ca^{2+} binding domain which they all possess. These proteins detect the Ca^{2+} signal and initiate a regulatory event. One such signal transducing protein is calmodulin, which has diverse effects within the cell. On binding Ca^{2+}, the conformation of calmodulin changes revealing a hydrophobic site on the elongated protein that is capable of binding to a receptor site possessed by certain enzymes and membrane transport proteins. Calmodulin activates a limited number of enzymes; currently about 20 different enzymes in animal cells are known or suspected of being activated directly by calmodulin (Moncreif *et al.*, 1990).

Ca^{2+}/calmodulin also activates protein kinases and in particular the Ca-kinase, which is a multifunctional kinase with a broad substrate specificity. The increase in Ca^{2+}/calmodulin activation caused by an increase in

free i[Ca^{2+}] is necessarily transient because the removal of Ca^{2+} from the cytoplasm to maintain the steady state concentration is rapid. Protein phosphorylation establishes a more prolonged response to the Ca^{2+} mediated signal as well as a further amplification and diversification of it. Two-dimensional PAGE has revealed literally hundreds of phosphorylated proteins in eukaryotic cells, far more than can be attributed to currently known protein kinases (Cozzone, 1993). Proteins activated (or deactivated) by phosphorylation may perform many tasks and set in motion complex events involving, for example, cell growth, division or differentiation. Protein phosphatases are also present in the cell and in removing phosphate groups from proteins permit deactivation of the Ca^{2+}/calmodulin stimulated event.

Ca^{2+} mediated regulation is thus complex, but it is also interactive with other regulatory mechanisms, such as cyclic nucleotide mediated regulation. Via receptor proteins and G proteins, which are similar to those involved with the IP_3 pathway, external signals activate membrane-associated adenylate cyclase and increase the cytoplasmic concentration of cAMP. The cAMP directly activates specific enzymes and is able to effect protein phosphorylation by activation of protein kinase A. In association with Ca^{2+}, calmodulin regulates some adenylate cyclases and cAMP phosphodiesterases while kinase A phosphorylates and thereby influences the Ca^{2+} channels and Ca^{2+}/ATP pumps. Kinase Ca and kinase A frequently phosphorylate different sites on the same enzyme. By these interactions, Ca^{2+}/calmodulin and cAMP interact, not only directly upon each other's concentrations, but also indirectly in their respective regulatory effects. A prime example of this interaction is the regulation of phosphorylase kinase and its role in glycogen degradation. Many eukaryotic cells also contain cGMP, and although the general role of this regulator is not well understood, it also probably contributes to the complex regulatory interaction. The multiplicity of the receptor proteins and protein kinases (and also the methylases, acetylases, uridylases and adenylases) provides for a panoply of external signals to be interpreted in multiple different ways by different cell types.

3.2. Ca^{2+} Channels

The presence of voltage-gated, mechanosensitive Ca^{2+} channels in the membranes of eukaryotic cells is well established. Their importance in nerve cell depolarization has ensured considerable research into their structure and function. There is, however, little direct evidence for similar channels in bacterial membranes even though other bacterial ion channels have been detected and investigated. For example, the presence of the ubiquitous proton translocating ATPase in bacterial membranes is well established

(Monticello *et al.*, 1992). Several bacterial toxins are also known that form channels. Colicin E1 forms a voltage dependent channel in the inner membrane of target bacteria and permits a K^+ flux (Merrill and Cramer, 1990). The F1 antigen of *Yersinia pestis* (Rodriguez *et al.*, 1992) and the mosquitocidal δ-endotoxin of *Bacillus thuringiensis* (Slatin, 1992) form channels in the cell membranes of their respective eukaryotic target cells. More directly, the recent development of methods such as the preparation of bacterial membranes from giant protoplasts (Saimi *et al.*, 1992) has permitted the use of patch clamp techniques to obtain data on channels present in both Gram-negative and Gram-positive bacteria. The presence of voltage gated (Delcour *et al.*, 1989) and mechanosensitive (Martinac *et al.*, 1990) cation selective channels has been demonstrated and something of their conductance and regulation is now being revealed. However, the use of these techniques to investigate Ca^{2+} channel activity has not been reported.

The most direct evidence for the existence of Ca^{2+} channels in bacteria is found in a brief report by Matsushita *et al.* (1989) who have shown that right-side-out membrane vesicles prepared from *B. subtilis*, strain W23, accumulated ^{45}Ca when a membrane potential was generated by K^+ efflux in the presence of valinomycin. The voltage dependent influx of Ca^{2+} was partially inhibited by standard concentrations of known inhibitors of eukaryotic Ca^{2+} channels such as ω-conotoxin (10 μM; 78% inhibition), nitrendipine (10 μM; 77% inhibition) and verapamil (50 μM; 52% inhibition) and completely inhibited by La^{3+} (0.25 mM $LaCl_3$). Protein fractions containing polypeptides of 32,35 and 42 kDa were purified by chromatography and used to reconstitute Ca^{2+} channels in planar phospholipid bilayers. Using patch clamp analysis, the reconstituted channel was shown to provide a typical single channel current. The conductance of the channel was estimated as 25 pS for Ba^{2+} as the charge carrier and the mean open time of the channel was found to be 20 ms. The current through the reconstituted channel was inhibited by ω-conotoxin and $LaCl_3$ and increased by BAY K 8644, a known enhancer of Ca^{2+} channel conductance in eukaryotic membranes. This brief report gave support not only to the view that chemotaxis in *B. subtilis* involves Ca^{2+} channels, but also that bacterial membranes contain Ca^{2+} channels which appear similar to those found in eukaryotes.

Further evidence for the existence of Ca^{2+} channels in prokaryotes comes from studies with ^{45}Ca. However inadequate the technique, the flux of ^{45}Ca into whole cells (Gangola and Rosen, 1987; Smith *et al.*, 1987a) and from everted cell membrane vesicles (cf. Brey and Rosen, 1979) clearly demonstrates the effect. However, the most substantial body of evidence for the widespread existence of Ca^{2+} channels in bacteria, equivalent to those found in eukaryotes, comes from the use of Ca^{2+} channel inhibitors (Table 1). The investigations of a variety of putative Ca^{2+} dependent effects in

Table 1 Effects of Ca^{2+} channel and calmodulin inhibitors in prokaryotes.

Inhibitor	Effect	Action	Reference
Verapamil	Chemotaxis, *B. subtilis*	−	Matsushita *et al.* (1989)
	Protein phosphorylation, *S. coelicolor*	+	Stowe *et al.* (1992)
	N_2 fixation, *Nostoc* 6720	+/−	Onek and Smith (1992)
	Ariel mycelium, *S. alboniger*	−	Natsume and Marumo (1992)
ω-conotoxin	Chemotaxis, *B. subtilis*	−	Matsushita *et al.* (1989)[a]
	Chemotaxis, *E. coli*	−	Tisa *et al.* (1993)[a]
Dimethylbenzamil	Competence, *S. pneumoniae*	−	Trombe *et al.* (1992)
BayK 8644	Chemotaxis, *B. subtilis*	−	Matsushita *et al.* (1989)[a]
Trifluoperazine	N_2 fixation, *Nostoc* 6720	+/−	Onek and Smith (1992)
	Ariel mycelium, *S. alboniger*	−	Natsume and Marumo (1992)
	Phospholipid synthesis, *M. phlei*	−	Reddy *et al.* (1992)
	Growth, *M. tuberculosis*	−	Ratnakar and Murthy (1992)
Chlorpromazine	Protein phosphorylation, *S. coelicolor*	+	Stowe *et al.* (1992)
	Ariel mycelium, *S. alboniger*	−	Natsume and Marumo (1992)
Nitrendipine	Chemotaxis, *B. subtilis*	−	Matsushita *et al.* (1989)[a]
Compound 48/80	N_2 flixation, *Nostoc* 6720	+/−	Onek and Smith (1992)
	Cell division, *E. coli*	−	Chen *et al.* (1991)

[a]Denotes that the effect of the inhibitor on Ca^{2+} was confirmed. For a list of inhibitors of putative prokaryotic calmodulins, see Onek and Smith (1992).

bacteria and particularly the Ca^{2+} dependence of prokaryotic motility have employed an array of inhibitory agents that are known to antagonize Ca^{2+} channel activity in eukaryotic cells. The use of such data as evidence for the existence of bacterial Ca^{2+} channels assumes that the observed physiological effects of the inhibiting agent are the result of the inhibition of a Ca^{2+} channel. Only in a very few instances has the effect of a Ca^{2+} channel inhibitor on Ca^{2+} influx into a bacterium been directly tested (cf. Matsushita *et al.*, 1989; Tisa and Alder, 1992). Even in eukaryotes the effects of Ca^{2+} channel inhibitors are known to vary in different cells. However, such is the diversity of both the Ca^{2+} channel inhibitors that have been used and of the strains of bacteria and physiological processes to which they have been applied, that there can be little doubt that the data provide substantial evidence for the existence of Ca^{2+} channels in bacteria. Furthermore, such data suggest that the bacterial channels must bear a strong structural and functional resemblance to those present in eukaryotic cells, because many agents such as ω-conotoxin are thought to have specific interactions with the channels.

However, this concept of evolutionary conservation of Ca^{2+} channel structure and function between bacteria and eukaryotes can appear strained. For example, the effects of abscisic acid on the cyanobacterium *Nostoc* 6720 (Smith *et al.*, 1987b, Marsalek *et al.*, 1991) are attributed, by comparison with the effects of the Ca^{2+} ionophore, calimycin (Compound 23187) (Smith *et al.*, 1987a), to an increase in Ca^{2+} influx. The idea of a higher plant hormone having effects on a prokaryote becomes more acceptable in the light of abscisic acid effects on Ca^{2+} dependent depolarization of nerve cells (Huddart *et al.*, 1986), the promotion of a Ca^{2+} flux in stomatal guard cells (Hetherington and Quatrano, 1991) and the presence of abscisic acid in animal brain tissue (La Page-Degivry *et al.*, 1986). The mode of action of abscisic acid on Ca^{2+} channels may be to affect the physical qualities of the lipid environment of the channel rather than a direct effect on the channel itself. The Ca^{2+} channels of diverse species could be expected to react to this quality and no direct interaction of the hormone with the channel would be required to explain the apparently conserved action of abscisic acid across such a wide evolutionary divide. In addition, the evolutionary origin of abscisic acid may be somewhat older than hitherto expected because recent evidence indicates that it is present in cyanobacteria (Hirsch *et al.*, 1989) and is synthesized in response to stress conditions (Marsalek *et al.*, 1992). Less readily explained, except perhaps in terms of the possible effects of degradation products, are the observations of Abeliovitch and Gan (1983) who found that the neurotransmitter, acetylcholine, lowered the threshold Ca^{2+} concentration in the growth medium that was required for trichome tip movement and increased the number of responsive trichomes when added to cultures of the cyanobacterium *Spirulina platensis*.

3.3. Ca^{2+} Efflux

Our current knowledge of the mechanisms of Ca^{2+} efflux from bacterial cells owes much to the work of Rosen and associates which dominates the literature (Rosen, 1982, 1987). Ca^{2+} efflux was first reported in *E. coli* by Silver and Kralovic (1969) whose observations of the temperature dependence of the efflux evoked the suggestion of an energy-dependent process. It has been demonstrated that the efflux in *E. coli* is driven by the proton motive force (Tsujibo and Rosen, 1983) and, from detailed studies of everted membrane vesicles, is catalysed by a Ca^{2+}/H^+ antiporter (Rosen and McClees, 1974; Brey *et al.*, 1978; Brey and Rosen, 1979). A second system for Ca^{2+} efflux has also been identified (Ambudkar *et al.*, 1984) which involves a calcium phosphate symporter/H^+ antiporter system. The requirement for a proton motive force for efficient extrusion of Ca^{2+} from *E. coli* has been confirmed by the use of an energy-depleted mutant which lacks the proton translocating ATPase. When supplied with energy sources such as lactate or succinate, the mutant produces a proton motive force and rapid Ca^{2+} efflux, but little or no substrate level phosphorylation. Other bacteria that have been shown to use Ca^{2+}/H^+ antiporters are *Azotobacter vinelandii* (Bhattacharyya and Barnes, 1976), *Bacillus megaterium* (Golub and Bronner, 1974), *B. subtilis* (Matsushita *et al.*, 1986), *Chromatium vinosum* (Davidson and Knaff, 1981), *Mycobacterium phlei* (Kumar *et al.*, 1976) and *Rhodobacter capsulata* (Jasper and Silver, 1978). Two species are known, *Halobacterium halobium* (Belliveau and Lanyi, 1978) and *Bacillus spA-007* (Ando *et al.*, 1981), that possess Ca^{2+}/Na^+ antiporters.

A gene believed to encode the Ca^{2+}/H^+ antiporter has recently been cloned on the basis of its ability to restore Na^+ resistance and Na^+/H^+ antiporter activity to a mutant lacking both of the known Na^+/H^+ antiporter activities (Ivey *et al.*, 1993). The gene, designated *cha*A, when expressed from a multicopy plasmid, apparently provides sufficient Na^+/H^+ antiporter activity to complement the mutant's deficiencies. Analysis of the product encoded by the gene was undertaken by assay in everted membrane vesicles prepared from an *E. coli* strain expressing *cha*A. The Ca^{2+}/H^+ antiporter activity promoted in response to energizing the system with the addition of $NADH^+$ exceeded, in both the initial and sustained rates of Ca^{2+} uptake into the vesicles, that which could be attained in vesicles prepared from wild-type cells. The *cha*A gene maps to a different region of the *E. coli* chromosome than the *cal*A, *cal*B *cal*C and *cal*D loci that were characterized in studies of the Ca^{2+}-sensitive mutants of *E. coli* isolated by Brey and Rosen (1979). However, the phenotypic characteristics that a *cha*A mutation confers correspond to those observed in studies of the *cal* loci (Brey *et al.*, 1978; Brey and Rosen, 1979). One interpretation is that the *cha*A gene, which appears to encode the structural component of a Ca^{2+}/H^+ antiporter, might be regulated by a product of the *cal* loci. Alternatively, there might be a

multiplicity of Ca^{2+} antiporter proteins in the *E. coli* cell, including those whose primary role is in Mg^{2+} transport, but which are also able to use Ca^{2+}. The deduced amino acid sequence of the *cha*A gene product is that of an extremely hydrophobic protein of molecular mass 39 kDa, which contains 11 membrane-spanning regions. ChaA also contains a region, in one of the predicted hydrophilic loops that bridge between the membrane-spanning segments, which possesses a close sequence similarity to the eukaryotic protein calsequestrin.

Eukaryotic cells use both secondary transport systems, of which the Ca^{2+}/H^+ and Ca^{2+}/Na^+ antiporters are examples, and primary ATP dependent pumps. Bacteria appear to favour the former, secondary systems, because only two groups have been found so far to use Mg-ATP-dependent Ca^{2+} efflux. Three species of *Streptococcus*, *S. faecalis* (Kobayashi *et al.*, 1987), *S. sanguis* (Houng *et al.*, 1986) and *S. lactis* (Ambudkar *et al.*, 1986) and one Gram-negative soil bacterium *Flavobacterium odoratum* (Gambel *et al.*, 1992), have been reported to yield everted membrane vesicles exhibiting ATP-dependent and vanadate-sensitive Ca^{2+} uptake. The streptococcal Ca^{2+}-ATPase has been solubilized and reconstituted into proteoliposomes. The apparent K_m values for ATP and Ca^{2+} were determined for this preparation as 100 μM and 0.5 μM, respectively (Gambel *et al.*, 1992). One other vanadate-sensitive Ca^{2+}-ATPase has been found in the cyanobacterium *Anabaena variabilis* (Lockau and Pfeffer, 1983). In *F. odoratum* the Ca^{2+}/ATPase activity is a high proportion of the total ATPase activity that can be measured using a soluble ATP hydrolysis assay without the need to reconstitute the protein into liposomes or everted membrane vesicles. A partially purified fraction provided estimates of the apparent K_m values for ATP and Ca^{2+} of 130 μM and 2 μM, respectively, and the activity was 50% inhibited by 1.6 μM vanadate. These values are similar to those observed with preparations of the eukaryotic sarcoplasmic reticulum Ca^{2+}/ATPase. The *F. odoratum* ATPase is also phosphorylated in the presence of ATP, probably on the cytoplasmic side of the membrane-bound protein, in a reaction that is inhibited by vanadate (Gambel *et al.*, 1992). A gene, *pacl*, encoding a Ca^{2+}/ATPase has been isolated from the cyanobacterium *Synechococcus* sp. PCC 7942 (Berkelman *et al.*, 1994). The gene encodes a product similar to other bacterial P-type ATPases, e.g. the K^+/ATPase (Walderhaus *et al.*, 1987) and the Cd^{2+} and Mg^{2+}/ATPase (Nucifora *et al.*, 1989; Snavely *et al.*, 1991a, b). These in turn show sequence homology with eukaryotic ATPases (Gambel *et al.*, 1992).

3.4. Ca^{2+} Metabolism

The total Ca^{2+} content of *E. coli* has been shown to vary with the external Ca^{2+} concentration. Estimates based on the ^{45}Ca labelling of *E. coli* in the

presence of external Ca^{2+} concentrations ranging from 0.08 to 10.08 mM showed that the total Ca^{2+} content of the cell increased from 0.013 and 1.39 mM, in proportion to the external Ca^{2+} concentration (Gangola and Rosen, 1987). Considered as a concentration, the total Ca^{2+} present in the cell is therefore maintained below that of the external Ca^{2+} concentration. These values may be compared with the independent assessment of 0.65 mM which was based on X-ray microprobe analysis of the cytoplasmic regions of *E. coli* cells (Chang *et al.*, 1987). This analysis made a point of avoiding the Ca^{2+} rich cell wall and membrane region of the *E. coli* cells. Consequently, both estimates confirm that the cytoplasmic content of Ca^{2+} in *E. coli* is within the millimolar range.

In contrast, estimates of the free intracellular Ca^{2+} concentration ($i[Ca^{2+}]$) which were derived from the fura-2 fluorescence of loaded *E. coli* cells are of the order of 10^{-7} M. These estimates were obtained by Gangola and Rosen (1987) using *E. coli* cells treated to make them permeable to fura-2 esters which, once absorbed, are hydrolysed to release the fura-2. The fura-2, being unable to cross the cell membrane, is thus trapped in the cell and fluoresces in the presence of free Ca^{2+}. The technique demonstrated that, in energy-replete cells, the free $i[Ca^{2+}]$ did not vary significantly even when the external Ca^{2+} concentration and consequent total cellular Ca^{2+} was increased by a factor of 100 (Fig. 2). *E. coli* has therefore been shown to maintain a free $i[Ca^{2+}]$ comparable to that of eukaryotic cells. Furthermore, since the total cellular Ca^{2+} increased with the external calcium concentration and is large compared with the free $i[Ca^{2+}]$, the cell clearly contains a large excess of Ca^{2+} buffering capacity. This is also evident in the cellular Ca^{2+} concentrations that were found in energy depleted cells which, being unable to maintain a proton gradient, must also be unable to extrude Ca^{2+} by use of the Ca^{2+}/H^{+} antiporter. In such cells, total cellular Ca^{2+} appears approximately equal to the external Ca^{2+} concentration, but the free $i[Ca^{2+}]$ does not increase above 0.7 μM. Analysis of the data provided by Gangola and Rosen (1987) shows that, in energy depleted cells, the free $i[Ca^{2+}]$ increases with the total cellular Ca^{2+} up to a Ca^{2+} content equivalent to 0.5 mM (expressing the total Ca^{2+} as a concentration). Thereafter the additional total Ca^{2+} accumulated by the cell appears to be fully chelated so that a further increase in the free $i[Ca^{2+}]$ is effectively prevented (Fig. 2). This implies that the cell contains a variety of Ca^{2+} chelating compounds with differing dissociation constants such that, in *E. coli* at least, the increase in free $i[Ca^{2+}]$ is not permitted to exceed about seven times the steady state concentration maintained in energy-replete cells, even though the total Ca^{2+} content of the cell rises by 100-fold.

Stated in other terms, the energy-replete *E. coli* cell contains about 10^6 bound Ca^{2+} ions and 100 free Ca^{2+} ions. In energy-depleted cells the bound Ca^{2+} increases to about 1.5×10^7, but the free intracellular Ca^{2+} increases to only 700 ions. However, estimates of total cellular Ca^{2+} based on ^{45}Ca

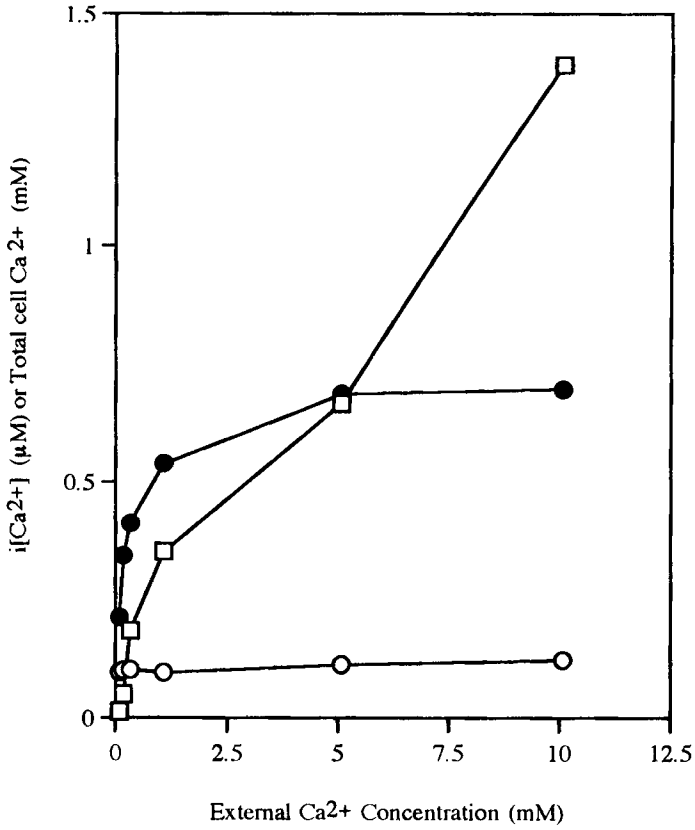


Figure 2 The free Ca^{2+} concentration in *E. coli* cells as a function of the external concentration. ○, i[Ca^{2+}] energy-replete cells; □, total cell Ca^{2+} (mM) energy-replete cells; ●, i[Ca^{2+}] energy-depleted cells. Note that the total cell Ca^{2+} of energy-depleted cells is not shown, but is approximately equal to the external concentration. In energy-depleted cells the free Ca^{2+} concentration is limited to 0.7 μM even though the total cell Ca^{2+} reaches an equivalent concentration of 10 mM. (Figure drawn from the data of Table 1 in Gangola and Rosen, 1987.)

labelling in energy-depleted cells may be unreliable. In the experiments described by Gangola and Rosen (1987), energy-depleted cells were placed in a buffer containing ^{45}Ca for 1 h. Growth and Ca^{2+} efflux from such cells are inhibited so that Ca^{2+} may enter the cell, but not leave. In these conditions the equilibration of the internal and external Ca^{2+} is unlikely. However, the estimates of the cellular Ca^{2+} content are based on the assumption that the external and internal specific radioactivities of Ca^{2+} are equal.

Another apparent anomaly is the finding that the total cellular Ca^{2+} in

energy-depleted cells increased in direct proportion to the external Ca^{2+} concentration and, when expressed in terms of a free Ca^{2+} concentration, was approximately equal to the external Ca^{2+} concentration. Since the free i[Ca^{2+}] is maintained at a low value (0.07 μM) by chelation of the excess Ca^{2+}, the concentration gradient for Ca^{2+} across the membrane does not decline appreciably. Ca^{2+} should continue to enter the cell until the Ca^{2+} chelation capacity of the cytoplasm is saturated and the free i[Ca^{2+}] equals that of the external free calcium concentration. However, this did not occur during the 1 h incubation allowed by the technique. In energy-depleted cells incubated in an external Ca^{2+} concentration of 10.08 mM, the Ca^{2+} influx during the 1 h incubation was equivalent to an average rate of 2.5×10^5 Ca^{2+} ions min^{-1} or an increase in the nominal total cellular Ca^{2+} concentration of 0.16 mM min^{-1}. Although the influx rate will vary in direct proportion to the external Ca^{2+} concentration, it is difficult to accept that the equivalence of the external Ca^{2+} concentration and the nominal total cellular Ca^{2+} in energy-depleted cells is a chance result of the conditions used. Unfortunately, data describing the rate of accumulation of ^{45}Ca by energy-depleted *E. coli* cells with time were not reported, but anything other than a linear relationship would provide evidence for the more satisfactory hypothesis that Ca^{2+} influx is regulated in accordance with both free i[Ca^{2+}] and the external Ca^{2+} concentration. The latter would be necessary to account for the increase in total cellular Ca^{2+} in proportion to external Ca^{2+} concentration in both energy-replete and -depleted cells.

Knight *et al.* (1989) used the photoprotein, aequorin, which emits blue light on binding Ca^{2+}, to measure the free Ca^{2+} concentration in *E. coli* cells. To avoid the difficulties inherent in inserting the protein into bacterial cells, they constructed a recombinant *E. coli* that expressed the aequorin gene. They have shown that a variety of conditions, including an increase in external Ca^{2+} concentration, promote a brief increase in the free i[Ca^{2+}]. The elevated i[Ca^{2+}] returns to the steady state concentration over a period of less than 30 s, demonstrating that *E. coli* senses and responds rapidly to the increase in external Ca^{2+} concentration. This response is not dissimilar to that observed by Gangola and Rosen (1987) on addition of 10 mM glucose to energy-depleted, Ca^{2+}-loaded *E. coli* cells. One interpretation of this rapid response would be that the rate of influx is decreased by closure of voltage-gated calcium channels and/or the rate of efflux is increased to remove the excess Ca^{2+} that has entered before closure of the channels was effected. However, an interpretation based on the results obtained by Gangola and Rosen (1987), in particular the observed increase in total cellular Ca^{2+} in proportion to the external Ca^{2+} concentration, suggests that the response is not so simple. The rapid return of the free i[Ca^{2+}] to the steady-state concentration observed by Knight *et al.* (1989) is not perhaps initially a function of Ca^{2+} exclusion from the cell, but instead that of the chelation of free Ca^{2+} ions.

The time required to re-establish the steady state may thus reflect the time required to chelate the Ca^{2+} that has entered the cell. However, Gangola and Rosen (1987) found that the addition of glucose to energy-depleted, Ca^{2+}-loaded cells enabled them to return the total cellular Ca^{2+} and the free i[Ca^{2+}] to values typical of energy replete cells within 10 min. This argues that, as suggested by Gangola and Rosen (1987), the capacity for Ca^{2+} efflux is large and that the free i[Ca^{2+}] and total cellular Ca^{2+} pools are in rapid equilibrium. Their other assertion, that a restricted entry of Ca^{2+} into the cell contributes to the low cytoplasmic Ca^{2+} concentration, requires further analysis. It may well be true, but the combination of influx, chelation and efflux presents a complex process in which the kinetics of these three components are not easily distinguished. The eukaryotic calmodulin is activated by a Ca^{2+} concentration of 1.0 μM (Norris, 1992). An understanding of the fluxes affecting free i[Ca^{2+}] in bacteria is an important component in determining the effective activation of calcium binding proteins, such as calmodulin, that may act in Ca^{2+}-mediated regulation. Assuming that a similar free i[Ca^{2+}] concentration is required to activate a prokaryotic calmodulin, this concentration is well above that found even in energy-depleted *E. coli* cells. Consequently, activation may require local transient concentrations within the cell or perhaps concentrations that normally occur, but once in a cell generation as suggested by Norris (1992) and Chang *et al.* (1987).

3.5. Protein Phosphorylation

Although the concepts and basic mechanisms of metabolic and genetic regulation were originally defined in bacterial systems, our knowledge of integrated cellular regulation, and regulation in response to environmental factors in bacteria, has lagged far behind our understanding of eukaryotic systems. A key factor in this discrepancy was the recognition of protein phosphorylation as a prime regulatory event in eukaryotic cells. Although the reversible phosphorylation of proteins was shown to be a major process in all eukaryotes (Edelman *et al.*, 1987) there existed no parallel investigation of the phenomenon in bacteria (Cozzone, 1993). It was not until the late 1970s that work with *Salmonella typhimurium* (Wang and Koshland, 1981) and *E. coli* (Mania and Cozzone, 1979; Enami and Ishihama, 1984) provided conclusive evidence for the presence of phosphorylated proteins in bacteria. To date, increasing awareness and research has revealed protein phosphorylation events in over 50 different species of eubacteria, archebacteria and cyanobacteria (Cozzone, 1993). The application of molecular, genetic and biochemical techniques in the last 5 years has shown that protein phosphorylation events are components of regulatory pathways concerned with motility, cellular development and virulence, homeostasis and carbon

and nitrogen source assimilation (Saier, 1993a, b; Titgemeyer, 1993; Parkinson 1993). Three main regulatory mechanisms based on protein phosphorylation have been found in bacteria. Two of these mechanisms involve the phosphorylation of histidinyl residues in sensor proteins that, like their eukaryotic counterparts, are membrane bound.

The so-called two-component, sensor kinase/response protein mechanism is represented by the prime examples of osmoregulation, the regulation of nitrogen assimilation and the more complex mechanisms regulating chemotactic and phototactic responses (Saier, 1993a; Stock *et al.*, 1993). In simple general terms these mechanisms consist of a sensor which is a histidine protein kinase (HPK) and is capable of self-phosphorylation using ATP as the phosphate donor. The sensor kinase interacts with a response regulator protein. The histidinyl phosphate of the HPK may be transferred to an aspartic acid residue within a conserved domain of the response regulator protein. This event serves to activate the response regulator. For example, osmoregulation involves the sensor protein EnvZ and the response protein OmpR. The permeability of the outer membrane of Gram-negative bacteria such as *E. coli* is thought to depend primarily on a small number of channel-forming proteins known as porins. In *E. coli* K12 the two main porins are encoded by the *ompF* and *ompC* genes. The *ompF* gene encodes a protein that forms a larger pore than does the OmpC protein. Changes in the ratio of the two porins reflect changes in the osmotic potential of the culture medium. Porin gene expression is controlled by the inner-membrane spanning EnvZ sensor kinase protein. The periplasmic domain of the protein is thought to act as an environmental sensor and so controls the phosphorylation of the histidinyl residue in the cytoplasmic domain. In turn this regulates the phosphorylation of the response regulator OmpR which then regulates the expression of the *ompF* and *ompC* genes (Stock *et al.*, 1993).

The phosphoenol pyruvate-dependent phosphotransferase system (PTS) forms a second distinct protein phosphorylating mechanism in bacteria which is involved in the import and phosphorylation of sugars and the regulation of carbohydrate catabolism (Saier, 1993b). All the proteins involved (HPr and enzymes I, II and III) are phosphorylated on histidinyl residues by donation of a phosphate group from phosphoenolpyruvate. The system exerts regulation by both protein phosphorylation and allosteric mechanisms. For example, the expression of the β-glucoside operon of *E. coli* and the sucrose regulon of *B. subtilis* are thought to be controlled by transcriptional antiterminators which are phosphorylated or dephosphorylated by the membrane-bound enzyme II protein. The equilibrium between phosphorylated and dephosphorylated antiterminator is controlled by the extracellular availability of the appropriate carbon source. In a similar manner, some catabolic enzymes and carbohydrate permeases are controlled by phosphorylation involving transfer of the phosphate group from HPr by a mechanism in which the presence of an extracellular carbohydrate controls

the phosphorylation state of HPr and consequently by equilibrium that of the target enzymes. The PTS system interacts with other regulatory processes. The phosphorylation state of enzyme III for example is believed to regulate allosterically adenylate cyclase as well as other catabolic enzymes and carbohydrate permeases (Saier, 1993b). However, in contrast, Bianchini *et al.* (1990) reported that the adenylate cyclase activity of a cyanobacterium *Anabaena* sp. is activated by an endogenous calmodulin-like protein.

The third type of protein phosphorylation activity found in bacteria corresponds to the classical serine/threonine protein kinases and the tyrosine kinases found in eukaryotes (Cozzone, 1993). The serine/threonine kinases use nucleoside triphosphates, usually ATP, as phosphate donors and, unlike the histidinyl kinases of the two-component sensor/response and the PTS mechanisms, do not autophosphorylate. The bacterial serine/threonine kinases that have been investigated appear to differ from their eukaryotic counterparts in that they are activated primarily by cellular metabolites rather than by second messengers such as cAMP or Ca^{2+}.

3.6. Ca^{2+} and Protein Phosphorylation

Several studies have examined the possibility that a calmodulin-like protein might be involved in the regulation of protein phosphorylation in bacteria. The principal approach has been one in which either authentic calmodulin (Londesborough, 1986) or Ca^{2+}, EGTA, calmodulin inhibitors and anti-calmodulin antibodies (Stowe *et al.*, 1992) have been added separately *in vitro* to assays of protein phosphorylation in whole cell extracts. Both stimulation and inhibition of protein phosphorylation were observed which, coupled with the identification of calmodulin-like activities in bacteria (Onek and Smith, 1992), support the concept of Ca^{2+} mediated regulation of protein phosphorylation in bacteria. However, the concentrations of additives required to achieve the observed effects were generally higher than those required in eukaryotic systems or present within the bacterial cell (Stowe *et al.*, 1992), which questions whether the observed effects represent physiological events. A direct stimulation of a prokaryotic protein kinase by calmodulin has not been reported. However, two reports indicate that protein kinase C-like activities exist in bacteria. An activity has been described in a pathogenic strain (MAR001) of *E. coli* which is Ca^{2+} and phospholipid dependent and is stimulated by diacylglycerol (DAG) and phorbal esters (Norris *et al.*, 1991). Evidence based on cross-reaction with different monoclonal antibodies and phosphatidylserine fixation indicates that a similar activity is present in *B. subtilis* (Seror quoted in Cozzone, 1993; Orr quoted in Norris *et al.*, 1991; Sandler *et al.*, 1989). These observations must raise the question of whether there exists in bacteria a mechanism akin to the IP3–DAG pathway, but as yet there are insufficient data to yield an answer.

The deduced amino acid sequences of the cytoplasmic and membrane-bound forms of the isocitrate dehydrogenase kinase/phosphatase, which is the best known example of a bacterial serine/threonine kinase, together with those of various bacterial histidinyl kinases suggest that the bacterial kinases differ significantly from their eukaryotic counterparts (Cozzone, 1993). In contrast, two serine/threonine kinases of *M. xanthus* encoded by the genes *pkn1* (Munoz-Dorado *et al.*, 1991) and *pkn2* (Inouye, quoted by Cozzone, 1993) possess significant sequence homology to the eukaryotic enzymes. Tyrosine kinases play important roles in eukaryotes, but, excepting a few specific examples, the functions served by both the serine/threonine and the tyrosine kinases found in bacteria are unknown (Cozzone, 1993). They do not appear to be activated by cyclic nucleotides because several reports agree that the addition *in vitro* of cAMP or cGMP to cell extracts obtained from a variety of bacteria does not affect the patterns of protein phosphorylation observed on subsequent SDS-PAGE analysis of the extracts. These observations are unlikely to be due to an artefact of the assay *in vitro* because no changes in the patterns of phosphorylation have been observed *in vivo* where protein extracts of ^{32}P-orthophosphate labelled, wild type and adenylate cyclase mutants of *S. typhimurium* and *E. coli* have been compared (Cozzone, 1993). These observations have been used to suggest that in bacteria the phosphorylation of proteins as a regulatory device is confined to the control of metabolic processes and is controlled directly by metabolites (Cozzone, 1993). However, exceptions exist. For example, of two different protein kinases found in *Legionella micdadei*, one is cyclic nucleotide independent, but the other is activated by cAMP and cGMP (Saha *et al.*, 1988).

Several prokaryotic processes have been reported as capable of responding to treatments or agents that are known or expected to influence cellular Ca^{2+} (Smith, 1988; Norris *et al.*, 1991; Onek and Smith, 1992). In most instances the mechanism of the putative Ca^{2+} mediated regulation is not known. However, recent investigations have begun to identify the mechanisms by which Ca^{2+} affects the regulation of prokaryotic chemotaxis and phototaxis.

3.7. Bacterial Motility

The abilities of motile species of bacteria to be attracted or repelled by chemicals or different qualities or intensities of light are known respectively as chemotaxis and phototaxis. Flagellated bacteria swim in a "random walk" consisting of periods of smooth unidirectional swimming interspersed with tumbling, which changes the direction of the subsequent smooth swim. Tumbling is caused by a reversal of the direction of rotation of the flagellum. Attraction or repulsion therefore consists of the activation of the tumbling

response such that the sum of the smooth swimming is either towards or away from the attractant or repellent. Gliding bacteria and cyanobacteria are able to move on a solid surface. Although there are several hypotheses, the basis of this form of motility is not well understood. Ca^{2+} has been shown to be required for both these forms of motility.

The work of Castenholz and associates (Castenholz, 1967, 1973; Halfen and Castenholz, 1971) established that Ca^{2+} is an essential factor for phototactic motility in the trichomes of filamentous cyanobacteria. However, stimulation of motility in *Spirulina subsala* cultures was found to require Na^{+} as well as Ca^{2+}, leading Abeliovitch and Gan (1983) to suggest that the site of action of Ca^{2+} was external. In contrast, Ruthenium Red and lanthanum, agents assumed to block Ca^{2+} channels, inhibited motility in *Phormidium uncinatum* (Haeder, 1982). Murvanidze *et al.*, (1982) observed a wave of membrane depolarization that is transmitted along filaments of *P. uncinatum* on switching from light to dark. This depolarization, and the photophobic reversals in trichome movement that accompany it, was found to be Ca^{2+} dependent.

Myxobacteria are gliding organisms that move on a solid surface both as single cells and as aggregates of cells. Cellular aggregates form fruiting bodies consisting of several thousand cells that raise aerial structures to release microcysts. Both the induction of motile ability, the developmental process resulting in cell aggregation and motility itself have been reported to be Ca^{2+} dependent (Womack *et al.*, 1989). Concentrations of 0.5 (minimum) to 1 mM Ca^{2+} were required to induce motility in liquid cultures of *Stigmatella aurantiaca*, but not in cultures of *Myxococcus xanthus*. However, both species required a lower (0.1 to 0.3 mM) concentration of Ca^{2+} to support gliding motility when induced cultures were subsequently placed on agarose. Inhibitors of protein synthesis prevented the induction of motile ability in *S. aurantiaca* by Ca^{2+}. Both motility and development are now known to employ a series of proteins encoded by the *frzA-G* genes, which closely resemble the corresponding genes and proteins that regulate motility in enteric bacteria (Zusman and McBride, 1991). Motility in *M. xanthus* is induced by starvation conditions and the motility and cell aggregation form part of a sporulation process. As described below, Ca^{2+} is also involved in the cellular differentiation that leads to the formation of the mycospores. A Ca^{2+} binding protein, known as protein S, is produced in quantity and used to coat the mycospores (Inouye *et al.*, 1983).

Ca^{2+} has also been found to be involved in the tactic responses of flagellated bacteria; Ca^{2+} is not required for the induction of motility or for swimming *per se*, but appears to act on the sensor/response regulator mechanisms that control smooth swimming versus tumbling. *Halobacterium halobium* possesses both chemotactic and phototactic responses; the latter is sensed by retinal-containing photoreceptors (Hildebrand and Dencher, 1975; Schmiz and Hildebrand, 1979). Externally applied Ca^{2+} and cGMP

have been found to affect the frequency of tumbling, the observed phases of increasing and decreasing responsiveness to light stimuli and the length of the refractory period (Schmiz and Hildebrand, 1987). Furthermore, a decrease in the external Ca^{2+} concentration of *H. halobium* cultures has been shown to result in an increase in the methylation of membrane proteins and a decrease in the response to added attractants (Omirbekova *et al.*, 1985; Schmiz and Hildebrand, 1987). The molecular mechanism regulating *H. halobium* motility is not known, but appears to resemble closely the mechanism employed by the eubacteria. In *E. coli* the CheB protein controls the methylation and thus the sensitivity of the secondary sensor proteins (Stock *et al.*, 1993). The finding that Ca^{2+} affects the methylation of membrane proteins in *M. xanthus* as well as affecting the length of the refractory period implies that the methylated proteins are receptor proteins. Plausibly, the effect of Ca^{2+} may be to act on the halobacterial equivalent of CheB or its activation by phosphorylation.

Chemotaxis in *B. subtilis* has been extensively reported to be regulated by Ca^{2+}. Inhibitors of Ca^{2+} channels, including ω-conotoxin, which has been shown to inhibit effectively the voltage-dependent uptake of Ca^{2+} into membrane vesicles prepared from *B. subtilis*, also inhibit the organisms' chemotaxis towards the attractant L-alanine (Matsushita *et al.*, 1989; Kusaka and Matsushita, 1987). Early work on *E. coli* chemotaxis appeared to indicate that Ca^{2+} had no effect on motility in this bacterium since neither the addition of the Ca^{2+} chelator EGTA nor the addition of Ca^{2+} to the culture medium—both of which are now known not to affect the free i[Ca^{2+}] of *E. coli* cells (Gangola and Rosen, 1987)—had any observable effect on either the rate or direction of chemotaxis. In a paper that will surely stand as a milestone in the investigation of Ca^{2+}-mediated regulation in bacteria, Tisa and Alder (1992) have elegantly and decisively shown that this interpretation was incorrect. Using the caged calcium compounds, Nitr-5 or DM-nitrophen, they have shown that the free i[Ca^{2+}] concentration of the cell influences the frequency of tumbling versus smooth swimming behaviour of *E. coli* K12.

The caged Ca^{2+} compounds are photosensitive Ca^{2+} chelators which release the chelated Ca^{2+} by photocleavage when they are exposed to light of an appropriate wavelength. After loading with Ca^{2+}, the compounds are introduced into the bacterial cell by electroporation. On exposure to light of 340 nm, the compounds are cleaved within the cell and release Ca^{2+}. When *E. coli* cells that had been loaded with Nitr-5/Ca^{2+} or DM-nitrophen/Ca^{2+} were exposed to such light, their swimming behaviour changed from the normal smooth/tumbly behaviour to a predominantly tumbling behaviour. Replacing the Ca^{2+}, by loading the chelators with either Mg^{2+} or Ba^{2+}, had no effect. Further confirmation that the effect was due to Ca^{2+} was obtained by the use of diazo-2. This compound increases its affinity for Ca^{2+} on exposure to light of 340 nm wavelength and would therefore be expected to

decrease transiently the free i[Ca^{2+}] in treated cells. Such treatment of *E. coli* K12 inhibited tumbling and promoted smooth swimming.

Using a series of *E. coli* mutants, the tumbling induced by the caged Ca^{2+} was shown to require the presence of the CheA, CheW and CheY proteins. Based on the current understanding of the molecular pathways involved in the regulation of chemotaxis in eubacteria, the authors suggested that Ca^{2+} may stimulate the autophosphorylation of CheA or the transfer of the phosphate group from CheA to CheY. The authors also briefly reported on the introduction of Fura-2 into *E. coli* cells by electroporation to measure the free i[Ca^{2+}] and the use of the technique to confirm that Ca^{2+}-mediated effects on the frequency of tumbling activity are natural phenomena and not artificially contrived by the use of the caged Ca^{2+} compounds. Applying the technique to *E. coli* cultures, they observed that an attractant produced a transient decrease in the free i[Ca^{2+}], whilst a repellent produced a transient increase in the free i[Ca^{2+}].

In a separate report, Tisa *et al.* (1993) have shown that, as in the case of *B. subtilis*, the Ca^{2+} channel blocker ω-conotoxin inhibits chemotaxis by promoting smooth swimming and preventing tumbling. The effects of Ca^{2+} channel inhibitors on tumbling versus smooth swimming in *E. coli* indicate that Ca^{2+} flux through the channels is responsible for the transient changes in the free i[Ca^{2+}] that accompanies the effects. By extension, the results also indicate that the observed effects of Ca^{2+} on motility in other diverse prokaryotic species occur in a similar manner. This indication is reinforced by observations such as the Ca^{2+}-mediated depolarization of *P. uncinatum* and the effects of Ca^{2+} on the frequency of tumbling in *H. halobium*. Thus the role of Ca^{2+} in prokaryotic motility appears to have been conserved across the prokaryotic kingdom. It is thought that signal transduction in PTS-mediated chemotaxis employs the CheY and CheB pathway (Titgemeyer, 1993). Consequently, the chemotactic aspects of this regulatory pathway may also be influenced by Ca^{2+}.

The work of Tisa and Alder (1992) has shown that a two-component, sensor kinase/response protein mechanism in bacteria is influenced by the free i[Ca^{2+}]. A study of a recombinant protein known as Taz1 suggests that Ca^{2+} may affect other two-component mechanisms. Taz1 is a hybrid molecule that was engineered to contain the N-terminal receptor domain of the Tar protein, which binds aspartate, and the C-terminal, signalling domain of the EnvZ protein, which is involved in the phosphorylation of the OmpR protein and the consequent regulation of the expression of the porin genes in *E. coli*. The two domains are connected by the transmembrane spanning domain of Taz. Expressed in *E. coli*, Taz1 regulates the expression of the porin gene OmpC in response to the addition of aspartate to the growth medium, indicating the successful splicing of the two functional domains (Utsumi *et al.*, 1989).

In an *in vitro* system containing Taz1-enriched membrane fractions, the

activation of Taz1 phosphorylation was found to be stimulated by Ca^{2+} concentrations ranging from 0.06 to 2.6 mM (Rampersaud *et al.*, 1991). $CaCl_2$ (5 mM) also enhanced the phosphorylation of intact, genomically encoded membrane-bound EnvZ and also that of a cytoplasmic fragment of EnvZ that lacks the N-terminal receptor and transmembrane domains. These results indicate that the probable site of action of Ca^{2+} stimulation of the autophosphorylation reaction of EnvZ lies within the cytoplasmic signalling domain of the protein. The concentrations of Ca^{2+} used to observe the effect *in vitro* are considerably higher than those which have been shown to occur within the *E. coli* cell (Gangola and Rosen, 1987). Whether this effect of Ca^{2+} on EnvZ phosphorylation reflects a regulatory function *in vivo* is unknown. Even so, the finding that Ca^{2+} affects the principal phosphorylation event of two of the best known, two-component regulatory mechanisms in bacteria must be considered significant.

Another sensory protein of *E. coli* is the periplasmic D-galactose binding protein, which interacts with the membrane-bound Trg chemotactic signal transducer protein. During refinement of X-ray crystallographic studies on the D-galactose binding protein, Vyas *et al.* (1987) discovered the unexpected presence of Ca^{2+} within the protein crystal. Further analysis revealed an amino acid sequence that closely resembles the EF hand motif of eukaryotic Ca^{2+} binding proteins. The Ca^{2+} binding site is in the C-terminal domain of the ellipsoidal D-galactose binding protein. Like other secreted proteins, the D-galactose binding protein contains two domains with a deep cleft between them containing the D-galactose binding site. The site of interaction with Trg is on the N-terminal domain at the opposite end of the molecule from the Ca^{2+} binding site.

Many secreted proteins appear to bind Ca^{2+}, and the Ca^{2+} site in the D-galactose binding protein may merely serve a function in protein folding and activation. Other sensory proteins such as the arabinose and sulphate binding proteins, which are also required for transport but, unlike the D-galactose binding protein, are not known to serve a function in chemotactic responses, were not found to contain Ca^{2+} during X-ray crystallographic studies by the same authors. While it does not appear likely that the binding of Ca^{2+} by the D-galactose binding protein serves any regulatory function, the presence of a protein sequence motif akin to that of the EF hand adds to the accumulating evidence that prokaryotes possess such sequences and that the evolutionary origin of the motif may be considerably older than hitherto suspected.

3.8. Diazotrophy

Diazotrophy in bacteria (Wilson, 1971; Norris and Jensen, 1957) and cyanobacteria (Gallon, 1992; Rodriguez *et al.*, 1990; Smith *et al.*, 1987a)

has been shown to require Ca^{2+}. One requirement for Ca^{2+} is in the mechanism(s) that protects the labile nitrogenase complex from O_2 (Gallon, 1992). The addition of calmodulin inhibitors (Onek and Smith, 1992) is among a range of treatments (Smith *et al.*, 1987a, b) that inhibit nitrogenase activity in aerobic cultures of *Nostoc* PCC 6720 and suggest that Ca^{2+} binding proteins are involved in the protection of the nitrogenase complex from O_2. However, treatments that enhance cellular Ca^{2+}, such as increasing the Ca^{2+} concentration in the growth medium above 10^{-5} M, or the addition of the Ca^{2+} ionophore calimycin in the presence of Ca^{2+}, decrease the rate of nitrogen fixation. Furthermore, dose–response curves for antagonists of Ca^{2+} binding such as lanthanum and the calmodulin inhibitors have shown that, at low concentrations, the antagonists enhance nitrogen fixation (Smith *et al.*, 1987a; Onek and Smith, 1992). These observations have prompted the suggestion that a second mechanism exists that is distinct from the O_2 protection mechanism and normally depresses nitrogen fixation below the maximum rate that is attainable by the culture (Onek and Smith, 1992). The mechanism by which Ca^{2+} might inhibit nitrogen fixation is not known, but the reported effect of Ca^{2+} on the comparable process of sulphite reduction in *D. gigas* (Chen *et al.*, 1991) offers a plausible precedent.

3.9. Bacterial Calmodulins and EF Hand Proteins

Eukaryotes possess a family of EF-hand, Ca^{2+} binding proteins. The EF hand is the name given to a sequence of nine amino acids that forms a loop preceded by a β-turn and so forms a structure in which one Ca^{2+} ion is surrounded by six ligands, provided by the oxygen atoms of the amino acids (Kretsinger, 1987). Although related by evolutionary descent, the family is functionally diverse. These functions include exocytosis, contractile functions, Ca^{2+} chelation/buffering, the activation of enzymes and nuclear spindle formation. There is good evidence that calmodulin, the enzyme-activating member of the family, interacts with at least 12 and possibly as many as 20 different enzymes or structural proteins. Except for plant NAD kinase, this evidence relates to mammalian proteins. Although the presence of calmodulin in plants implies that there may be calmodulin-activated plant enzymes that have yet to be discovered and/or fully investigated, it has been suggested that the greater conservation of calmodulin sequence structure in animals may be the result of a wider use of calmodulin activation in animals than in plants. As might be expected from their functional diversity, the known calmodulin activated proteins do not exhibit structural or amino acid sequence homology. However, several of them share cationic, amphipathic α helices which have been proposed as the sites at which calmodulin binds in activating the enzymes (Moncreif *et al.*, 1990).

Eukaryotic calmodulin is a small (15–22 kDa) globular and monomeric

protein that is heat stable. The ability of calmodulin to activate target proteins is most frequently assayed using bovine brain cAMP phosphodiesterase (PDE) or NAD kinases (Klee, 1988). These activations are Ca^{2+} dependent and are inhibited by a variety of compounds that bind to calmodulin, such as phenothiazines (Weiss *et al.*, 1985) and naphthalene sulphonamide derivatives (Hidaka *et al.*, 1979). Animal calmodulins and to a slightly lesser extent plant calmodulins are highly conserved but, despite this conservation, there are immunological differences (Jablonsky *et al.*, 1991). For instance, polyclonal antibody preparations raised against spinach calmodulin do not show a significant cross-reaction with vertebrate or protozoan calmodulin, even though the two proteins differ by only 13 amino acid residues (Lukas *et al.*, 1984). This effect probably arises from the suppression of an immune reaction to most of the epitopes that are presented by calmodulin, since they are regarded as self-antigens by the challenged animal's immune system. Unfortunately, this complicates the use of anti-calmodulin antibodies as a tool in the identification of a protein as a calmodulin.

All of these characteristics have been used as assays to provide evidence for the presence of calmodulin in eukaryotic cells and subsequently for the same purpose in investigations of bacteria. However, the adoption of these techniques carries with it the implicit assumption that bacterial calmodulin or calmodulin-like proteins will have sufficient structural or functional homology with the eukaryotic calmodulin. While the assumption may thus involve a bias towards the identification of homologous bacterial proteins, there is some meagre evidence that bacterial calmodulins and eukaryotic calmodulins will activate, albeit less efficiently, each other's target proteins (Bianchini *et al.*, 1990; Gordon *et al.*, 1989; Onek and Smith 1992).

To date, 14 independent investigations have reported the discovery of Ca^{2+}-binding, calmodulin-like proteins in bacteria. A recent review of these investigations may be found in Onek and Smith (1992). Two studies (Iwasa *et al.*, 1981; Harmon *et al.*, 1985) presented evidence for the existence of an *E. coli* calmodulin-like activity. Iwasa and associates described a heat-stable activity that activated PDE, an ATPase and myosin light chain kinase in the presence of Ca^{2+}. Harmon *et al.* (1985) reported the presence of three high-affinity, heat-stable, Ca^{2+} binding proteins (60, 47 and 33 kDa) that were identified by column chromatography. The proteins preferentially bound Ca^{2+} when suspended in buffers containing micromolar concentrations of Ca^{2+} and other ions present at physiological concentrations. Following chromatography, crude fractions containing the proteins were found not to be able to activate NAD kinase, but a total cell crude extract was shown to contain an antigen which was recognized by a commercially available anti-animal calmodulin antibody. Fry *et al.* (1986, 1991) described the purification and properties of a calmodulin-like intracellular protein of *B. subtilis* (23–25 kDa). The protein was present in the soluble fraction of cell-free extracts derived from sporulating cultures. It was found to be heat

stable and to stimulate PDE in a Ca^{2+}-dependent and trifluoperazine-inhibited reaction. The protein also displaced authentic bovine calmodulin from its own antibody.

Another heat-stable, Ca^{2+} binding protein, which has been isolated from dormant spores of *Bacillus cereus* (Shyu and Foegeding, 1991), bound Ca^{2+} in the Western blot technique of Maruyama *et al.* (1984). It is not known whether this protein possesses the ability to activate calmodulin-activated proteins. The amino acid compositions of both *Bacillus* proteins have been determined. Neither composition profile resembles that of eukaryotic calmodulin nor do they resemble each other. These findings imply, at least for the calmodulin-like protein of *B. subtilis* that, while parts of the protein, such as the Ca^{2+} binding and protein interaction sites, may resemble the structure of eukaryotic calmodulin, there is no overall conservation of sequence structure.

M. xanthus is known to produce protein S, which has been shown to be the most abundant protein produced during differentiation of the fruiting bodies, following the aggregation of cells that is induced by starvation on a solid surface. The reported rate of synthesis of protein S increased to reach 15% of total protein synthesis and accumulated in the cells until sporulation, when most of it was assembled on the outer surface of the myxospores, with a smaller amount found inside (Inouye *et al.*, 1983). Protein S is encoded by the *tps* gene. A second gene, *ops*, is present nearby that encodes a protein which shows close homology to protein S. The protein encoded by *ops* is expressed only late in differentiation and accumulates inside the spores. Protein S contains four homologous domains akin to those found in calmodulin and γ-crystallins. Domains 1 and 3 show homology with the EF hand motif, with domains 2 and 4 possessing less homology. However, it does not have the helix–loop–helix conformation found in calmodulin, being composed primarily of β-pleated sheets. Protein S has been shown to bind two Ca^{2+} ions at sites with dissociation constants of 27 and 76 μM. Site-directed mutation studies showed that loss of the ability to bind Ca^{2+} at either or both sides did not reduce the synthesis of the protein, but reduced and, in the case of the double mutant, eliminated binding of the mutant protein to the myxospores (Tientze *et al.*, 1988).

Another calmodulin-like protein has been identified immunologically in *M. smegmatis* (Reddy *et al.*, 1992) and *M. bovis* (Falah *et al.*, 1988). The intracellular levels of the calmodulin-like protein were found to increase in glucose-supplemented cultures and were correlated with the increase in total lipid and phospholipids (Reddy *et al.*, 1992). A partially purified extract, from which an inhibitor was removed by ammonium sulphate precipitation, was able to stimulate the activity of PDE (Falah *et al.*, 1988).

A calmodulin-like protein in *B. pertussis* (Nagai *et al.*, 1990) was purified 3200-fold compared with crude cell supernatants. This putative calmodulin was found to be a small (11 kDa), acidic (pI = 4.25) protein. The purified

preparation activated the *B. pertussis* adenylate cyclase toxin, a mammalian adenylate cyclase prepared from rabbit brain and PDE in a Ca^{2+}-dependent manner. The extract provided half the specific activity of authentic bovine brain calmodulin, was heat stable (80°C for 10 min), inactivated by a protease and inhibited by compound W-7. Similar calmodulin-like proteins were isolated from *Bordetella parapertussis* and *B. bronchiseptica*. The *Bordetella* adenylate cyclase toxin is an extracellular protein that is normally activated by the calmodulin of the recipient eukaryotic target cell. As such it is probably not a normal substrate for the *Bordetella* CAM-like protein, and the role of the calmodulin-like protein in *Bordetella* species is consequently no better defined than that of other prokaryotic calmodulin-like proteins.

Leadlay and associates described the first prokaryotic protein that was shown to possess an EF hand motif (Leadlay *et al.*, 1984; Swan *et al.*, 1987). The 20 kDa Ca^{2+} binding protein, which was isolated from *Saccharopolyspora erythraea*, possesses four helix–loop–helix EF hand-containing domains, but one of these Ca^{2+} binding sites is probably defunct. The protein does not activate PDE and the originally perceived similarities to calmodulin have given way to an even closer homology with a group of invertebrate sarcoplasmic Ca^{2+} binding proteins (SCP). These SCPs bind two or three Ca^{2+} ions per protein because one of the four EF hand-containing domains does not conform to the sequence structure and is unable to bind Ca^{2+}. A recent report revealed that the *S. erythraea* protein, now called calerythrin, possesses only three Cd^{2+} binding sites and consequently three Ca^{2+} binding sites (Bylsma *et al.*, 1992). Although calerythrin appears to lack a site for interaction with other proteins, preliminary experiments suggest that it undergoes marked structural changes upon binding Ca^{2+}. Like the SCP, calerythrin may serve as an intracellular Ca^{2+} buffer, but this function does not provide a simple explanation for the structural changes.

An approach that does not assume comparability between eukaryotic and prokaryotic Ca^{2+} binding proteins has been adopted by Holland and associates (Laoudj *et al.*, 1994). The Ca^{2+} channel inhibitors verapamil and diltiazem have been shown to inhibit the growth of an *E. coli acr* mutant (strain N43) (Nakamura and Suganuma, 1972), which is hypersensitive to many drugs. In further work, Laoudj *et al.* (1994) isolated mutants of the strain which are resistant to inhibition of growth by diltiazem, but which are hypersensitive to the addition of EGTA to the culture medium. When grown to an $OD_{600\ nm}$ of 0.5 in a modified version of M9 medium lacking Ca^{2+}, the addition of 100 mM EGTA did not inhibit growth of the parental strain N43 (*acr*), but caused an immediate inhibition of growth of the diltiazem resistant mutant, known as N43 verA1, followed by a gradual recovery of the growth rate. Notably, in cultures grown to an $OD_{600\ nm}$ of 0.5 and diluted to an $OD_{600\ nm}$ of 0.1 prior to addition of EGTA, both the parental and mutant strain suffered long-term inhibition of growth. The verA1 mutant

strain was inhibited more effectively by this treatment than was the parental strain.

Using one- and two-dimensional PAGE analysis of ^{35}S-methionine-labelled protein extracts of both the parental and mutant strain, the effect of EGTA addition was investigated. The synthesis of at least 25 polypeptides was enhanced and that of at least a further 11 polypeptides was decreased by the treatment. Neither heat-shocked nor detergent-treated cultures showed the same response, indicating that the observed effects are not a part of a general stress response. BAPTA, a more specific Ca^{2+} chelator, elicited a similar pattern of polypeptide induction and repression. The same response was observed in the parental strain.

The majority of the induced proteins are low molecular mass, heat stable, acidic proteins. Five of the induced proteins also cross-react with an antibody raised against calerythrin, the Ca^{2+} binding protein isolated from *S. erythraea*. This suggests that these five proteins and possibly others of the induced group are Ca^{2+} binding proteins. On the basis of these results, it was suggested that the genes encoding the proteins constitute a regulon, the expression of which is controlled by the free i[Ca^{2+}]. Some of the EGTA-induced proteins, particularly a 17 kDa polypeptide, were found to be expressed at a higher level in the verA1 mutant than in strain N43. Such changes in specific protein content may account for the resistance to diltiazem treatment and provide a means to assess whether changes in free i[Ca^{2+}] are related to the induction or repression of specific proteins. The physiological roles of these proteins are not known, but their identification will permit the isolation of genes and the construction of recombinant *E. coli* and so facilitate an analysis of protein function.

Four reports describe calmodulin-like proteins in cyanobacteria. Kerson *et al.* (1984) described stimulation of the uptake of inorganic phosphate by *Oscillatoria limnetica* and deposition of phosphate in polyphosphate granules, a process that was inhibited by fluphenazine (a known inhibitor of eukaryotic calmodulin). The presence of antigens, in crude protein extracts of *O. limnetica*, which cross-reacted with an antibody raised against a commercially available animal calmodulin, reinforced the suggestion that a cyanobacterial calmodulin-like protein was involved in the uptake of organic phosphate. Pettersson and Bergman (1989) also relied on immunological identity as a means of detecting a putative calmodulin-like protein in three strains of *Anabaena*. Western blot analysis of crude protein extracts using a polyclonal, anti-spinach calmodulin antibody demonstrated the presence of a 17 kDa polypeptide. Localization of this antigen using the labelled antibody in electron microscopy studies showed that the protein was distributed throughout the cytoplasmic regions of both the vegetative cells and heterocysts of *Anabaena variabilis*. Pettersson and Bergman (1989) went on to provide the first demonstration that cyanobacteria contained a heat-stable material that was capable of activating a eukaryotic calmodulin-

dependent enzyme, the cucumber NAD kinase, in a Ca^{2+}-dependent and chlorpromazine-sensitive reaction.

Bianchini *et al.* (1990) investigated *Anabaena* sp. and identified three polypeptides that were heat stable and activated PDE. The partially purified extracts activated, in the presence of Ca^{2+}, an adenylate cyclase isolated from *Anabaena* sp. This finding constituted the first and only demonstration of a Ca^{2+}-dependent activation of an endogenous, intracellular, prokaryotic enzyme. As part of an investigation of the Ca^{2+} requirement of PSII activity in *Synechococcus* PCC 7942, McColl and Evans (1990) reported the presence of two proteins (60 and 18 kDa) that bound Ca^{2+} in the Western blot procedure of Maruyama *et al.* (1984). The 60 kDa protein was also detected in Western blots by an antibody raised against guinea pig calmodulin. The antibody inhibited *in vitro* the Ca^{2+}-dependent reactivation of Ca^{2+}-depleted PSII preparations, suggesting that the 60 kDa protein is involved in the reactivation. Onek *et al.* (1994) described in *Nostoc* PCC 6720 three proteins (33, 21 and 17 kDa) that bind Ca^{2+}. The 21 kDa protein was shown to cross-react weakly with two different polyclonal antibody preparations raised against spinach calmodulin, but not with a commercial antibody preparation raised against bovine brain calmodulin. Similarly a polyclonal antibody raised against the purified 21 kDa protein weakly cross-reacted with spinach calmodulin, but not with bovine brain calmodulin. The purified 21 kDa protein activated commercially obtained PDE and a pea NAD kinase preparation in the presence of Ca^{2+} in a reaction that was inhibited by trifluoperazine. The 21 kDa protein also exhibited a Ca^{2+}-dependent shift in mobility during SDS-PAGE; this property is characteristic of eukaryotic calmodulin and other Ca^{2+}-binding proteins (Garrigos *et al.*, 1991; Jablonsky *et al.*, 1991).

4. THE PROKARYOTIC NUCLEOID

Electron microprobe analysis of DNA extracted from *Pseudomonas tabacii* has shown K^+/phosphate and Ca^{2+}/phosphate ratios of 31/100 and 17/100, respectively (Sigee and El-Masry, 1987). The extraction technique employed involves the use of RNAse and phenol treatments plus ethanol precipitation; even so, it is believed to preserve the essential structure of the nucleoplasm (Materman and Van Gool, 1978). Additional data on the relative proportions of Ca^{2+}, K^+ and phosphate in different samples lend support to the proposal that, whereas K^+ primarily forms an electrostatic association within the hydration shell of the treated DNA, divalent cations including Ca^{2+} are chelated within the nucleoid (Daune, 1979). Additional evidence obtained by autoradiography of bound ^{45}Ca and X-ray microprobe analysis of nucleoid material extruded from lysed bacteria supports the interpretation that Ca^{2+}

is bound with the *P. tabacii* chromosome and nucleoid regions (Sigee *et al.*, 1985). Consequently, the nucleoid may act as a Ca^{2+} chelating buffer in bacteria. If the concept is transferred to *E. coli* and a Ca^{2+}/phosphate ratio of 0.17 is assumed, one genome equivalent could bind 7.8×10^5 Ca^{2+} ions. This would constitute an appreciable fraction of the total cellular Ca^{2+}.

The Ca^{2+} bound to the nucleoid may have a role as a bridging ion between the negatively charged DNA and the surrounding matrix of acidic proteins (Williams, 1975). The formation of DNA, divalent cation and protein complexes has been proposed as a general feature of DNA–protein binding (Bere and Helene, 1979; Helene and Lancelot, 1982). The addition of EGTA and EDTA to cultures of *E. coli* has been shown to promote premature cell division (Nanninga *et al.*, 1983) and segregation of the nucleoids (N. Nanninga, quoted in Norris *et al.*, 1991). A comparable effect of Ca^{2+} on nucleoid structure has been observed in *Y. pestis* where the conversion of the compact nucleoid to axial filaments has been reported to depend on the Ca^{2+} concentration in the growth medium (Perry and Brubaker, 1987; Hall *et al.*, 1974). A role for Ca^{2+} in the nucleoid structure would be consistent with these observations and has been used to support the hypothesis that Ca^{2+} is involved in the regulation of replication and cell division in prokaryotes (Norris *et al.*, 1991).

4.1. Associated Proteins

Three of the five prokaryotic proteins identified by Kretsinger *et al.*(1991) as containing putative Ca^{2+}-binding sites comparable to the EF hand motif are associated with nucleoid functions. Two are concerned with gene expression: the EFTS translation elongation factor which is required for the ribosome translocation step and the transcriptional termination factor Rho. The third is the replication initiation factor for the plasmid R6K. Two other proteins have been identified that are thought to be involved in the initiation of DNA replication in *E. coli*. Guzman *et al.* (1991) described an 11 kDa protein which contains no isoleucine and is expressed under stringent control. An open reading-frame (ORF) encoding a protein with these characteristics has been sequenced; the protein contains four putative Ca^{2+}-binding sites. The second protein, DnaK, is one of the heat shock proteins of *E. coli* that is also involved in the initiation of DNA replication of both the genome and some plasmids (Sakakibara, 1988; Wickner, 1990). The autophosphorylation activity of DnaK *in vitro* is increased 10-fold by the addition of Ca^{2+} (Cegielska and Georgopoulos, 1989). Like other heat shock proteins, DnaK shows evolutionary conservation and possesses a high degree of homology with the eukaryotic counterpart known as Hsp70. Notably, this homology includes the conserved 21 amino acid sequence that is known to form the binding

site of calmodulin (Stevenson and Calderwood, 1990). The independent identification of putative Ca^{2+}-binding sites in three different proteins that are all involved in the stringent regulation of the initiation of DNA replication events contributes to the hypotheses of Norris, Holland and associates (Norris, 1989a,b, 1992; Norris *et al.*, 1991).

Other proteins that are involved in chromosome and cell division events in eukaryotes have been identified in bacteria (Holland *et al.*, 1990). Microfilaments containing tubulin have been implicated in chromosome segregation at mitosis, and myosin appears to be involved in nuclear segregation and cell division in lower eukaryotes (De Lozanne and Spudich, 1987; Watts *et al.*, 1985). Several reports have described polypeptides present in mycoplasmas and *E. coli* that possess myosin and actin-like properties (Niemark, 1977; Minkoff and Damadian, 1976; Nakamura *et al.*, 1978; Beck *et al.*, 1978; Casaregola *et al.*, 1991) and tubulin and actin-like proteins have been identified in archaebacteria (Sioud *et al.*, 1987). The activities of DNAses *in vitro* are known to depend on the presence of particular divalent metal cations. The *recBC* nuclease of *E. coli*, which is involved in the general recombination and post-replication DNA repair mechanisms, requires Ca^{2+} concentrations in excess of 1 mM to support its activity *in vitro* (Rosamund *et al.*, 1979).

4.2. Ca^{2+} and Gene Expression

Several gene expression events in prokaryotes have been found to involve Ca^{2+}. The three virulent species of the genus *Yersinia*, including *Y. pestis*, the aetiological agent of bubonic plague, possess a virulence characteristic known as the low Ca^{2+} response. The virulence determinants, a set of diverse proteins called Yops, are encoded on a plasmid of ~70 kbp. Expression of the *yop* genes has been shown to be both temperature and Ca^{2+} dependent. At temperatures above 34°C and in a Ca^{2+}-free medium, maximum expression of the *yop* genes occurs. However, these conditions also caused a cessation of growth and metabolic shutdown within two generations. When the Ca^{2+} concentration of the growth medium exceeded 2.5 mM, the growth restriction was relieved, but *yop* gene expression was repressed. Addition to the growth medium of nucleotides such as ATP, which are not transported into the cell, was able to substitute partially for Ca^{2+} in relieving growth restriction and down-regulating *yop* gene expression. This observation has been used to support the interpretation that Ca^{2+} acts outside the cell membrane as an environmental signal. However, a decrease in the calcium concentration of *Yersinia* cultures was reported to cause major changes in the nucleoid structure (Perry and Brubaker, 1987), which argues that the intracellular Ca^{2+} content may also be involved. Several genes have been identified as participating in the positive induction and negative repression

of *yop* gene expression in response to the temperature and Ca^{2+} signals. However, the mechanism by which Ca^{2+} influences growth, metabolism and *yop* gene expression has not yet been elucidated. It may involve an outer membrane sensor protein such as YopN (Bergman *et al.*, 1991) and/or an inner membrane protein such as the *lcrD* gene product (Plano *et al.*, 1991). Whatever the mechanism, it is clear that, by virtue of the genes on the virulence plasmid, these species of *Yersinia* are capable of sensing the external Ca^{2+} concentration and responding by modifying transcriptional activity.

The haemolysin of the *Actinobacillus pleuropneumoniae* is another virulence factor that requires Ca^{2+} for its expression. Free Ca^{2+} concentrations in the growth medium in excess of 3 mM were found to be required for maximum expression of haemolytic serotype 1 activity. Unlike the haemolysin A of *E. coli*, that of *A. pleuropneumoniae* does not require Ca^{2+} as a co-factor for the lysis of erythrocytes. Experiments with rifampicin suggested that the effect of Ca^{2+} on the expression of the *A. pleuropneumoniae* haemolysin occurs at the level of transcription. Notably the minimum concentration of free Ca^{2+} required for the expression of the haemolysin, 0.7 mM, corresponds to the Ca^{2+} concentration in blood serum (Frey and Nicolet, 1988).

LipA is the name given to an *Arthrobacter photogonimus* cell surface protein which may be a pilin. Expression of the *lipA* gene is induced by exposure of the cells to near UV light (Hoober, 1978) or exposure to visible light in the presence of a photodynamic dye (methylene blue or neutral red) (Hoober and Franzi, 1983). On induction, the rate of synthesis of LipA was found to increase by 50-fold and reach 20% of the total protein synthesis in the cell, although the rate of synthesis of other proteins was not diminished. The expression of *lipA* was repressed by the presence of Ca^{2+} in the growth medium. Unlike the other known bacterial induction or repression mechanisms that are sensitive to millimolar concentrations of Ca^{2+} in the growth medium, *lipA* gene expression was shown to be repressed by a Ca^{2+} concentration of only 1.0 μM. Consequently, a high rate of expression of *lipA* was achieved in the laboratory by the use of Ca^{2+}-chelating agents to control the Ca^{2+} concentration in the culture medium. In contrast to the repressive effect of Ca^{2+} on *lipA* gene expression, Ca^{2+} or other divalent metal cations such as Mn^{2+}, Cu^{2+} or Zn^{2+} were required for the accumulation of the light inducible protein on the cell surface. This requirement can be substituted by the use of protease inhibitors indicating that the divalent cations are required to allow LipA to mature to a protease resistant form.

The sensitivity of *lipA* gene expression to micromolar concentrations of Ca^{2+} suggests that the regulatory mechanism involves a sensor protein(s) that has a very high affinity for Ca^{2+}, with a dissociation constant an order of magnitude less than that of eukaryotic calmodulin. Whether this sensor is

present outside the cell membrane, like those of the two-component or PTS regulatory mechanism, or is present within the cell and monitors free i[Ca^{2+}] is not known (Phinney and Hoober, 1991). Repression by a Ca^{2+} concentration of 1 μM implies that in most natural environments the expression of the *lipA* gene by *A. photogonimos* would be severely restricted.

Two S-layer proteins of *Bacillus brevis*, the outer wall protein and the middle wall protein, are encoded by an operon with three promoters: P1, P2 and P3. Promoter P3 allows initiation of transcription of the operon during exponential growth while promoter P2 serves both exponential and stationary phase expression. Transcription from the P2 promoter was markedly enhanced during the early part of the stationary phase, but this enhancement was inhibited by the addition of either 5 mM Mg^{2+} or Ca^{2+} to the growth medium. The addition of the divalent cations also inhibited the accumulation of the S-layer proteins in the growth medium during the stationary phase. Adachi *et al.* (1991) have proposed a mechanism whereby the action of Ca^{2+} or Mg^{2+} on the P2 promoter function is indirect. They suggest that the divalent cations maintain the integrity of the S-layers and so prevent the loss of S-layer proteins from the surface. Through an unknown mechanism that senses the requirement for S-layer proteins, the transcription from P2 is consequently decreased.

The pectate lyase of the soft rot pathogen *Pseudomonas fluorescens*, which causes the decay of stored fruit and vegetables, is thought to play a role in the invasion of plant tissue. Expression of the pectate lyase has been shown to be regulated by Ca^{2+}. In the presence of glucose, glycerol or polygalacturonates, 1 mM Ca^{2+} in the growth medium was required to obtain constitutive expression (Liao, 1991). In the absence of Ca^{2+} the organism failed to use polygalacturonates as a carbon source and produced very low levels of pectate lyase. Of the little pectate lyase that was made in the absence of Ca^{2+}, the majority was found in the cytoplasm suggesting that Ca^{2+} is also required for the secretion of the enzyme. A recombinant *E. coli* carrying a plasmid clone of the pectate lyase gene, *pel*, expressed pectate lyase without regard to the presence of either the carbon source or Ca^{2+}. This implies that Ca^{2+} and the carbon source induce a negative repression mechanism that is absent in the recombinant *E. coli*. Genes that are homologous to *pel* exist in two other *Pseudomonas* species, *P. viridiflora* and *P. putida*.

Streptococcus pneumoniae is another pathogen for which Ca^{2+} is an obligatory growth supplement, but also exhibits a membrane-associated development, namely the induction of competence, that is regulated by Ca^{2+}. Growth in 1 mM Ca^{2+} was found to promote the export of a protein known as the competence factor and result in the acquisition by the cells of competence for genetic transformation. The acquisition of competence is accompanied by culture lysis in early stationary phase (Trombe *et al.*, 1992). Both the induction of competence and lysis in the presence of 1 mM Ca^{2+} were prevented by addition to the culture of the Ca^{2+} channel inhibitor

2′,4′-dimethylbenzamil (DMB). Kinetic analysis of the effects of the inhibitor suggested that the mode of action involves a Ca^{2+}/Na^{+} antiporter, the activity of which was required for both growth and the induction of competence. Ca^{2+} induces a stress response that is regulated by the competence factor (CF) factor (Trombe *et al.*, 1994). DNA transport across the membranes of competent cells is described as a homeostatic response that prevents autolysis and involves cooperative Ca^{2+} transport.

The role of calcium in the induction of gliding motility in the myxobacteria (Womack *et al.*, 1989) and in the induction and repression of putative Ca^{2+} binding proteins in *E. coli* (Laoudj *et al.*, 1994) have been described above. It is possible that Ca^{2+} may also be involved in the regulation of cytodifferentiation events. Sporulation in several bacterial species is known to require Ca^{2+} (Campbell, 1983) and that in *B. subtilis* appears to involve a calmodulin-like protein and to be inhibited by promethazine and trifluoperazine (Fry *et al.*, 1991). Sporulation is also known to require Ca^{2+} for selective protein degradation during proteolysis (O'Hara and Hageman, 1990). Heterocyst differentiation in the filamentous cyanobacteria is affected by Ca^{2+}. In *Nostoc* PCC 6720 the proportion of heterocysts to vegetative cells in the filament, induced by the removal of a source of combined nitrogen, was increased by treatments that increase the Ca^{2+} content of the cell (Smith *et al.*, 1987a, b; Smith and Wilkins, 1988). However, calmodulin inhibitors appear to have little or no effect on the proportion of heterocysts (Onek and Smith, 1992). In contrast, Zhao *et al.* (1991) reported briefly that the antagonist W7 or Ca^{2+} depletion increased the heterocyst proportions in *Anabaena* PCC 7120 cultures. Whether the effects of Ca^{2+} on bacteria sporulation and cyanobacterial heterocyst differentiation are directly on gene expression is not known. The finding that the control of heterocyst proportion involves a protein, PetA, that shares homology with CheY (Liang *et al.*, 1992) offers one possible mechanism that is worthy of further investigation.

5. CONCLUDING REMARKS

This review began with the propositions that: (1) since calcium was present during the early stages of the evolution of life, all cells might be expected to be able to guard against the toxic effects of Ca^{2+} and; (2) the roles that Ca^{2+} came to play in the early cells may have been conserved during subsequent evolution. The work of Rosen and associates (Gangola and Rosen, 1987) has done much to support these proposals. *E. coli* has been shown to maintain a low free intracellular Ca^{2+} concentration, similar to that found in eukaryotic cells, against large changes in external Ca^{2+} concentrations. The metabolic apparatus that serves this function involves Ca^{2+} channels (Matsushita *et al.*, 1989), Ca^{2+} antiporters (Gangola and Rosen,

1987) and ATP-dependent Ca^{2+} pumps (Gambel *et al.*, 1992), together with intracellular, Ca^{2+}-binding buffers such as the *S. erythraea* protein, calerythrin (Bylsma *et al.*, 1992). Although a detailed comparison at the molecular level is often lacking, there appears on the information available to have been considerable conservation of these functions between prokaryote and eukaryote. However, such conservation is not so obvious in the roles that Ca^{2+} performs in bacteria. Along with other divalent cations, Ca^{2+} clearly plays a structural role in prokaryotic cell walls (Garduno *et al.*, 1992). Less well defined, in eukaryotes as well as prokaryotes, are the interactions between Ca^{2+} and cell membranes. The work of Murthy and associates (Reddy *et al.*, 1992) with mycobacteria indicates that Ca^{2+} can play a role in regulating lipid synthesis and consequently membrane composition. The putative role of Ca^{2+} in regulating the formation and activity of lipid and protein domains (Norris, 1989a) within membranes is more speculative, though not without supporting evidence (Cullis and de Kruijff, 1979; Zachowski, 1987). Particularly relevant are the observed effects of Ca^{2+} on sulphite reduction in *D. gigas* (Chen *et al.*, 1991) and PSII function in *Synechococcus* (Debus, 1992). Our understanding of protein and lipid domains is at an early stage (Shapiro, 1993); however, it is apparent that they could play significant roles in cell structure and function, not least perhaps in cell replication and division (Norris, 1989a). The hypotheses of Norris, Holland and associates (Norris *et al.*, 1991; Norris, 1992; Holland *et al.*, 1990) serve to illustrate the significance of the roles Ca^{2+} might play in domain structure and function. The supplementary evidence that indicates that Ca^{2+} is involved in nucleoid structure and the function of proteins required for DNA replication contributes to the concept (Norris *et al.*, 1991).

A major problem in assessing the role of Ca^{2+} in the prokaryote arises from the difficulty in determining the relevance of disparate data. The phosphoenolpyruvate carboxykinase (PCK) of *E. coli* provides an example (Goldie and Sanwal, 1980). This enzyme catalyses the phosphorylation and decarboxylation of oxaloacetate to form phosphoenolpyruvate, a reaction that is considered a key step in bacterial gluconeogenesis and, in contributing to the phosphoenolpyruvate pool, may possibly influence the PTS regulatory system. The *E. coli* enzyme possesses a Ca^{2+} binding site and is activated by Ca^{2+} through an allosteric mechanism. However, the K_d of the site for Ca^{2+} and the Ca^{2+} concentration required for activation of the enzyme *in vitro* are such that activation *in vivo* appears an unlikely event. Consequently, the relevance of the Ca^{2+} site and its conservation in distinct bacterial species (Delbaere *et al.*, 1991) is an enigma that offers little to the wider questions of the role of Ca^{2+}. Similar problems extend to the observed Ca^{2+}-mediated effects *in vitro* on protein kinase activity (Cozzone, 1993; Stowe *et al.*, 1992) and, while observations of protein kinase C-like activities in bacteria are suggestive (Norris *et al.*, 1991; Sandler *et al.*, 1989), their relevance will remain obscure until further data are available.

In comparison, the work of Tisa and Alder (1992) stands well to the fore. There is good evidence to indicate that the mechanisms of bacterial motility are genetically related and that the ubiquitous involvement of Ca^{2+} reflects this conservation. In demonstrating *in vivo* that the intracellular Ca^{2+} concentration affects key proteins regulating the chemotactic response, Tisa and Alder (1992) have presented prime evidence for Ca^{2+}-mediated regulation in a prokaryote that is free from the complications that afflict the interpretation of *in vitro* studies. Moreover, if one such mechanism exists, others might also.

The investigations of putative calmodulin-like and other Ca^{2+} binding proteins in prokaryotes have demonstrated that proteins are present that are able to activate calmodulin-dependent proteins (Onek and Smith, 1992). Whether they serve this function in bacteria remains to be proven. Excepting the single report of the *Anabaena* sp. adenylate cyclase (Bianchini *et al.*, 1990) and the perhaps special case of the *Bordetella* adenylate cyclase toxins (Gordon *et al.*, 1989) there is no evidence for bacterial enzymes that are calmodulin activated. In contrast there is good evidence that both the EF hand motif and the amphipathic α helical site of calmodulin binding (Moncreif *et al.*, 1990) are part of the genetic repertoire of the prokaryotic kingdom. On the assumption that the presence of these motifs in bacterial proteins serves a function, the existence of prokaryotic calmodulin-like proteins supports the notion of prokaryotic Ca^{2+}-mediated regulation. However, the approach adopted by Holland and associates (Laoudj *et al.*, 1994) for the identification of Ca^{2+} "associated" proteins in *E. coli*, which does not depend on assumed similarities to eukaryotic proteins, may serve to provide a better understanding of the relevance of Ca^{2+} binding proteins in bacteria.

The evidence in favour of Ca^{2+} as a mediator of regulatory phenomena in prokaryotes continues to accumulate. The extent and importance of such regulation is still unclear, but the potential in the forms of protein phosphorylation, nucleoid structure, cell division and membrane domains is large. Equally there is potential for medical (Ratnakar and Murthy, 1992) and commercial applications. However, as recognized by Norris *et al.* (1991), substantial advances will depend on a wider acceptance among biologists of all guises that the involvement of Ca^{2+} in cellular responses is worthy of evaluation.

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Cationic Bactericidal Peptides

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1. INTRODUCTION

Over the past decade many naturally occurring (poly) cationic peptides from a variety of species have been isolated and studied, with respect to their activity, structure and genetic organization. These peptides possess antimicrobial activity against many species including bacteria (Gram-positive and -negative), fungi, and viruses and, in the case of the more potent peptides,

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can have a lytic activity on mammalian cells. The main thrust of research in this area has been to understand the relationship between structure and the biological activity that these peptides exert. These analyses have been substantiated by the design and characterization of synthetic chemical peptides based on studies of these naturally occurring peptides.

Cationic peptides can be classified into several groups on the basis of sequence similarities, secondary and tertiary structure, function and origin. In this review, we are attempting to provide a detailed overview of the known polycationic peptides, with emphasis on those of less than 100 amino acids and with a net charge greater than +2 (Table 1).

2. OCCURRENCE OF CATIONIC PEPTIDES IN NATURE

2.1. Peptides Involved in Mammalian Host Defence Mechanisms

The oxygen-independent microbicidal host defence mechanism of mammals involves several proteinaceous molecules that are cationic in nature. These include lysozyme, bactericidal/permeability increasing factor (BPI), cathepsin G, CAP-37, lactoferrin, defensins and the eosinophil-derived proteins: the major basic protein (MBP) and the eosinophil cationic protein (ECP) (Elsbach and Weiss, 1988). All of these proteins (except lysozyme) are granule-associated and reside in neutrophils, including polymorphonuclear leukocytes and eosinophils. Most of these polypeptides are larger than the polycationic peptides discussed here although a synthetic 24 amino acid cationic domain of CAP-37 has been shown to be the probable bactericidal domain of CAP-37 (Pereira *et al.*, 1993) and a 21 amino acid cationic domain to be the antibacterial domain of CAP-18 (Tossi *et al.*, 1994).

Defensins represent a class of small (29–35 amino acids), arginine- and cysteine-rich peptides which have been isolated from rat, rabbit, guinea pig and human leukocytes (Couto *et al.*, 1992; Eisenhauer and Lehrer, 1992). These molecules, found primarily within the cytoplasmic granules of neutrophils, may constitute between 5 and 15% of the total cellular protein. Two defensins, MCP-1 and MCP-2, are expressed in elicited rabbit alveolar macrophages (Selsted *et al.*, 1983). Cryptdins, the name given to mouse defensins, are found in the Paneth cells of the small intestine (Ouellete *et al.*, 1989), whereas the defensins HNP-5 and HNP-6 were found in the human cell counterpart (Jones and Bevins, 1992). All defensins share amino acid sequence similarities and possess a specific conserved array of six cysteine residues which form three disulphide bridges. Members of the defensin family all possess a secondary structure rich in β-pleated sheet stabilized by these intramolecular disulphide bonds, as discussed in further detail later in this review.

Defensins kill a wide variety of bacteria (being generally more lethal against Gram-positives than Gram-negatives), fungi, spirochaetes and viruses. They exert not only microbicidal activity due to permeabilization of biological membranes but they also possess chemotactic and endocrine regulatory activities (Lehrer *et al.*, 1990).

Synthesis of defensins is under tissue specific, developmental and immune regulation. The cDNA of human defensin clones shows that each defensin is synthesized as a 93–95 amino acid preprodefensin comprising a 19 amino acid signal sequence for targeting to the endoplasmic reticulum and a 40–45 amino acid anionic propiece. It has been proposed that this anionic segment acts to neutralize the cationic charge of the defensin thereby rendering the peptide inactive until cleavage occurs, releasing this segment (Michaelson *et al.*, 1992). This appears to be a general mechanism of synthesis with these biologically active cationic peptides. Piers *et al.* (1993) have supported this finding as they found that cloning a synthetic cationic peptide by fusion to a negatively charged carrier protein or inclusion of the negatively charged pro sequence used in defensin synthesis in eukaryotes, resulted in the stable production in bacteria of an inactive fusion protein, which, on cleavage from the anionic carrier protein, regained activity.

Recently, a new subset of defensins, termed β-defensins, have been characterized from bovine neutrophils (Selsted *et al.*, 1993). This family of 13 structurally homologous peptides, although possessing the six invariantly spaced cysteines forming three disulphide bridges, is distinct from other neutrophil defensins owing to their unique consensus sequences. In addition, the bovine antimicrobial peptide TAP, isolated from the trachea, contains the same triple disulphide motif as β-defensins and is synthesized as a preproprotein (Diamond *et al.*, 1991). Within the large granules of bovine neutrophils are three arginine-rich peptides, the bactenecins (Frank *et al.*, 1990; Romeo *et al.*, 1988) two of which can be subgrouped due to their high proline content. The third is a novel tryptophan-rich, 13-amino acid peptide, indolicin (Selsted *et al.*, 1992). In contrast to indolicin, which is stored in granules in its mature form, bactenecins are present as inactive proforms which are processed into their active form when the granules containing them and protease-containing azurophilic granules interact (Zanetti *et al.*, 1990).

2.2. Insect Defence Peptides

The antibacterial response of insects has been well characterized over the last 20 years. A range of inducible antimicrobial cationic peptides has been isolated, including attacins (Hultmark *et al.*, 1983), cecropins (Steiner *et al.*, 1981), coleoptericin (Bulet *et al.*, 1991), diptericins (Dimarcq *et al.*, 1988), drosocin (Bulet *et al.*, 1993), phormicins (Lambert *et al.*, 1989), sarcotoxins

Table 1 The natural cationic peptides.

Peptide	Origin	Sequence (size)	Accession number	Reference	Activity
Abaecin	Honey bee (*Apis mellifera*)	YVPLPNVPQPGRRPFPTFPGQG PFNPKIKWPQGY	P15450	Casteels *et al.* (1990)	B
Ac-AMP1	Amaranth (*Amaranthus caudatus*)	VGECVRGRCPSGMCCSQFGY	98045[a]	Broekaert *et al.* (1992)	B+ F
Ac-AMP2	,,	VGECVRGRCPSGMCCSQFGYC GKGPKYCGR	98046[a]	Broekaert *et al.* (1992)	B+ F
Adenoregulin	Two-coloured leaf frog (*Phyllomedusa bicolor*)	GLWSKIKEVGKEAAKAAAKAA GKAALGAVSEAV	P31107	Daly *et al.* (1992)	B F
AFP1	Rape (*Brassica napus*)	QKLCERPSGTWSGVCGNNNAC KNQCINLEKARHGSCNYVFPAH K	P30225	Terras *et al.* (1992)	F
AFP2	Turnip (*Brassica rapa*)	QKLCERPSGTXSGVCGNNNAC KNQCIR	P30228	Terras *et al.* (1992)	F
Andropin	Fruit fly (*Drosophila melanogaster*)	VFIDILDKVENAIHNAAQVGIGF AKPFEKLINPK	P21663	Samakovlis *et al.* (1991)	B+
Apidaecin IA	Lymph fluid of honey bee (*Apis mellifera*)	GNNRPVYIPQPRPPHPRI	P11525	Casteels *et al.* (1989)	B−
Apidaecin IB	,,	GNNRPVYIPQPRPPHPRL	P11526	Casteels *et al.* (1989)	B−
Apidaecin II	,,	GNNRPIYIPQPRPPHPRL	P11527	Casteels *et al.* (1989)	B−
AS-48	*Streptococcus faecalis* subsp. *liquefacines* S-48	7.4 kDa		Gálvez *et al.* (1989)	B+
Bactenecin	Cytoplasmic granules of bovine neutrophils	RLCRIVVIRVCR	A33799	Romeo *et al.* (1988)	B
Bac5	Cytoplasmic granules of bovine neutrophils	RFRPPIRRPPIRPPFYPPFRPPI RPPIFPPIRPPFRPPLRFP	B36589	Frank *et al.* (1990)	B−
Bac7	,,	RRIRPRPPRLPRPRPRPLPFP RPGPRPIPRPLPFPRPGPRPIP RPLPFPRPGPRPIPRP	A36589	Frank *et al.* (1990)	B−
Bactericidin B2	Tobacco hornworm larvae hemolymph (*Manduca sexta*)	WNPFKELERAGQRVRDAVISA APAVATVGQAAAIARG*	P14662	Dickinson *et al.* (1988)	B C

Bactericidin B-3	,,	WNPFKELERAGQRVRDAIISA GPAVATVGQAAAIARG*	P14663	Dickinson *et al.* (1988)	B C
Bactericidin B-4	,,	WNPFKELERAGQRVRDAIISA APAVATVGQAAAIARG*	P14664	Dickinson *et al.* (1988)	B C
Bactericidin B-5P	,,	WNPFKELERAGQRVRDAVISA AAVATVGQAAAIARGG*	P14665	Dickinson *et al.* (1988)	B C
Bacteriocin C3603	*Streptococcus mutans*	4.8 kDa		Takada *et al.* (1984)	B+
Bacteriocin IY52	*Staphylococcus aureus*	5 kDa		Nakamura *et al.* (1983)	B+
Bacteriocin plantaricin A	*Lactobacillus plantarum*	AYSLQMGATAIKQVKKLFKKW	P80214	Nissen-Meyer *et al.* (1993)	B
BNBD-1	Bovine neutrophils	DFASCHTNGGICLPNRCPGHMI QIGICFRPRVKCCRSW	127951	Selsted *et al.* (1993)	B
BNBD-2	,,	VRNHVTCRINRGFCVPIRCPGR TRQIGTCFGPRIKCCRSW	127952	Selsted *et al.* (1993)	B
BNBD-3	,,	PEGVRNHVTCRINRGFCVPIRC PGRTRQIGTCFGPRIKCCRSW	127953	Selsted *et al.* (1993)	B
BNBD-4	Bovine neutrophils	PERVRNPQSCRWNMGVCIPFL CRVGMRQIGTCFGPRVPCCRR	127954	Selsted *et al.* (1993)	B
BNBD-5	,,	PEVVRNPQSCRWNMGVCIPIS CPGNMRQIGTCFGPRVPCCR	127955	Selsted *et al.* (1993)	B
BNBD-6	,,	PEGVRNHVTCRIYGGFCVPIRC PGRTRQIGTCFGRPVKCCRRW	127956	Selsted *et al.* (1993)	B
BNBD-7	,,	PEGVRNFVTCRINRGFCVPIRC PGHRRQIGTCLGPRIKCCR	127957	Selsted *et al.* (1993)	B
BNBD-8	,,	VRNFVTCRINRGFCVPIRCPGH RRQIGTCLGPQIKCCR	127958	Selsted *et al.* (1993)	B
BNBD-9	,,	PEGVRNFVTCRINRGFCVPIRC PGHRRQIGTCLGPQIKCCR	127959	Selsted *et al.* (1993)	B
BNBD-10	,,	PEGVRSYLSCWGNRGICLLNR CPGRMRQIGTCLAPRVKCCR	127960	Selsted *et al.* (1993)	B
BNBD-11	,,	GPLSCRRNGGVCIPIRCPGPMR QIGTCFGRPVKCCRSW	127961	Selsted *et al.* (1993)	B
BNBD-12	,,	GPLSCGRNGGVCIPIRCPVPMR QIGTCFGRPVKCCRSW	127962	Selsted *et al.* (1993)	B
BNBD-13	,,	SGISGPLSCGRNGGVCIPIRCP VPMRQIGTCFGRPVKCCRSW	127963	Selsted *et al.* (1993)	B

Table 1—continued

Bombinin	Yellow-bellied toad (*Bombina variegata*)	GIGALSAKGALKGLAKGLA ZHFAN*	P01505	Csordas and Michl (1970)	B
BLP-1	Asian toad (*Bombina orientalis*)	GIGASILSAGKSALKGLAKG LAEHFAN*	M76483	Gibson *et al.* (1991)	B
BLP-2	,,	GIGSAILSAGKSALKGLAKG LAEHFAN*	B41575	Gibson *et al.* (1991)	B
BLP-3	,,	GIGAAILSAGKSALKGLAKG LAEHF*	M76484	Gibson *et al.* (1991)	B
BLP-4	,,	GIGAAILSAGKSIIKGLANGL AEHF*	D41575	Gibson *et al.* (1991)	B
Bombolitin BI	Bumblebee venom (*Megabombus pennsylvanicus*)	IKITTMLAKLGKVLAHV*	P10521	Argiolas and Pisano (1985)	B C
Bombolitin BII	,,	SKITDILAKLGKVLAHV*	P07493	Argiolas and Pisano (1985)	B C
Bombolitin BIII	,,	IKIMDILAKLGKVLAHV*	P07494	Argiolas and Pisano (1985)	B C
Bombolitin BIV	,,	INIKDILAKLVKVLGHV*	P07495	Argiolas and Pisano (1985)	B C
Brevinin-1E	European frog (*Rana esculenta*)	FLPLLAGLAANFLPKIFCK ITRKC	S33729	Simmaco *et al.* (1993)	B C
Brevinin-2E	,,	GIMDTLKNLAKTAGKGALQS LLNKASCKLSGQC	S33730	Simmaco *et al.* (1993)	B
Cecropin	Silk moth (*Bombyx mori*)	RWKIFKKIEKVGQNIRDGIVKA GPAVAVVGQAATI	P14666	Qu *et al.* (1987)	B
Cecropin (lepidopteran A)	Silk moth (*Bombyx mori*)	RWKIFKKIEKMGRNIRDGIVKA GPAIEVIGSAKAI	P04142	Teshima *et al.* (1986)	B
Cecropin A	Silk moth (*Hyalophora cecropia*)	KWKLFKKIEKVGQNIRDGIIKA GPAVAVVGQATQIAK*	M63845	Gudmundsson *et al.* (1991)	B
Cecropin B	Silk moth (*Hyalophora cecropia*)	KWKVFKKIEKMGRNIRNGIV KAGPAIAVLGEAKAL*	X07404	Xanthopoulos *et al.* (1988)	B
Cecropin C	Fruit fly (*Drosophila melanogaster*)	GWLKKLGKRIERIGQHTRDATI QGLGIAQQAANVAATARG*	Z11167	Tryselius *et al.* (1992)	B
Cecropin D	Silk moth pupae (*Hyalophora cecropia*)	WNPFKELEKVGQRVRDAVISA GPAVATVAQATALAK*	P01510	Hultmark *et al.* (1982)	B–
Cecropin P_1	Pig small intestine (*Sus scrofa*)	SWLSKTAKKLENSAKKRISEGI AIAIQGGPR	P14661	Lee *et al.* (1989)	B–

Cecropin 1	Mediterranean fruit fly (*Ceratitis capitata*)	GWLKKIGKKIERVGQHTRDATI AVAQQAANVAATARG	JT0673	Rosetto *et al.* (1993)	
Cecropin 2	,,	GWLKKIGKKIERVGQHTRDAT IQTIGVAQQAANVAATIKG	JT0674	Rosetto *et al.* (1993)	
Charybdotoxin	Scorpion venom (*Leiurus quin-questriatus hebraeus*)	ZFTNVSCTTSKECWSVCQRLH NTSRGKCMNKKCRCYS	P13487	Schweitz *et al.* (1989)	
Coleoptericin	Beetle (*Zophobas atratus*)	8.1 kDa	A41711	Bulet *et al.* (1991)	B
Crabrolin	European hornet venom (*Vespa crabro*)	FLPLILRKIVTAL*	A01781	Argiolas and Pisano (1984)	C
Crambin	Crambe plants (*Crambe abyssinica*)	TTCCPSIVARSNFNVCRIPGTP EAICATYTGCIIIPGATCPGDYAN	P01542	Teeter *et al.* (1981)	
Cryptdin 1 (defensin)	Mouse intestine (*Mus musculus*)	LRDLVCYCRSRGCKGRERM NGTCRKGHLLYTLCCR	A43279	Selsted *et al.* (1992)	
Cryptdin 2 (defensin)	,,	LRDLVCYCRTRGCKRRERM NGTCRKGHLMYTLCCR	B43279	Selsted *et al.* (1992)	
Cryptdin 3 (defensin)	,,	LRDLVCYCRKRGCKRRERM NGTCRKGHLMYTLCCR	C43279	Selsted *et al.* (1992)	
Cryptdin 4 (defensin)	,,	GLLCYCRKGHCKRGERVRGT CGIRFLYCCPR	D43279	Selsted *et al.* (1992)	
Cryptdin 5 (defensin)	,,	LSKKLICYCRIRGCKRRERVF GTCRNLFLTFVFCC	E43279	Selsted *et al.* (1992)	
Defensin 4K	Scorpion (*Leiurus quinquestriatus*)	GFGCPLNQGACHRHCRSIRRR GGYCAGFFKQTCTCYRN	JN0613	Cociancich *et al.* (1993b)	B+
Dermaseptin	South American arboreal frog (*Phyllomedusa sauvagii*)	ALWKTMLKKLGTMALHAGKA ALGAADTISQTQ	P24302	Mor *et al.* (1991)	F
Dermaseptin 1	Sauvage's leaf frog (*Phyllomedusa sauvagei*)	ALWKTMLKKLGTMALHAGKA ALKAAADTISQGTQ	P80277	Mor *et al.* (1991)	B F
Dermaseptin 2	,,	ALWFTMLKKLGTMALHAGKA ALGAAANTISQGTQ	P80278	Mor *et al.* (1991)	F
Dermaseptin 3	,,	ALWKNMLKGIGKLAGKAALG AVKKLVGAES	P80279	Mor *et al.* (1991)	F
Dermaseptin 4	,,	ALWMTLLKKVLKAAAKALNA VLVGANA	P80280	Mor *et al.* (1991)	F

Table 1—continued

Dermaseptin 5	,,	GLWSKIKTAGKSVAKAAAKA AVKAVTNAV	P80281	Mor *et al.* (1991)	F
Dermaseptin B1	,,	AMWKDVLKKIGTVALHAGKA ALGAVADTIS	P80282	Mor and Nicolas (1994)	F
Diptericin	Nestling-suckling blowfly (*Phormia terranovae*)	9 kDa	X15851	Reichhardt *et al.* (1989)	B−
Drosocin	Fruit fly (*Drosophila melanogaster*)	GKPRPYSPRPTSHPRPIRV	S35984	Bulet *et al.* (1993)	B
Endozepine	Domestic pig (*Sus scrofa domestica*)	KQATVGDINTERPDILDKGKAK WDAWNGLKGTSKEDAMKAYI NKVEELKKKYGI	S36839	Agerberth *et al.* (1993)	B
Esculentin	European frog (*Rana esculenta*)	GIFSKLGRKKIKNLLISGLKNV GKEVGMDVVRTGIDIAGCKIK GEC	S33731	Simmaco *et al.* (1993)	B
Gastric inhibitory peptide (GIP)	Domestic pig (*Sus scrofa domestica*)	ISDYSIAMDKIRQQDFVNWLLA QKGKKSDWKHNITQ	S36840	Agerberth *et al.* (1993)	B
GNCP-1 (defensin)	Guinea pig (*Cavia cutteri*)	RRCICTTRTCRFPYRRLGTCIF QNRVYTFCC	S21169	Yamashita and Saito (1989)	B
GNCP-2 (defensin)	,,	RRCICTTRTCRFPYRRLGTCLF QNRVYTFCC	X63676	Yamashita and Saito (1989)	B
Hiastadin 1	Crab eating primate (*Macaca fascicularis*)	DSHEERHHGRHGHHKYGRKFH EKHHSHRGYRSNYLYDN	P34084	Xu *et al.* (1990)	F
Histadin 2	Human (*Homo sapiens*)	MKFFVFALILALMLSMTGADSH AKRHHGYKRKFHEKHHSHRGY RSNYLYDN	292146[a]	Sabatini and Azen (1989)	F
HNP-1 (defensin)	Azurophil granules of human neutrophils	ACYCRIPACIAGERRYGTCIYQ GRLWAFCC	P11479	Lehrer *et al.* (1991)	B F C
HNP-2 (defensin)	,,	CYCRIPACIAGERRYGTCIYQ GRLWAFCC	P11479	Lehrer *et al.* (1991)	B F C
HNP-3 (defensin)	,,	DCYCRIPACIAGERRYGTCIYQ GRLWAFCC	P11479	Lehrer *et al.* (1991)	B F C
HNP-4 (defensin)	,,	VCSCRLVFCRRTELRVGNCLI GGVSFTYCCTRVD	X65977	Wilde *et al.* (1989)	B F C

HNP-5 (defensin)	Human Paneth cells	SQARATCYCRTGRCATRESLS GVCEISGRLYRLCCR	M97925	Jones and Bevins (1992)	B F C
HNP-6 (defensin)	,,	STRAFTCHCRRSCYSTEYSYG TCTVMGINHRFCCL	M98331	Jones and Bevins (1993)	B F C
Indolicidin	Bovine neutrophils	ILPWKWPWWPWRR*	A42387	Selsted *et al.* (1992)	B
Insect defensin	Dragonfly larvae (*Aeschna cyanea*)	GFGCPLDQMQCHRHCQTITGR SGGYCSGPLKLTCTCYR	P80154	Bulet *et al.* (1992)	B+
Lactoferricin B	N-terminal region of bovine lactoferrin	FKCRRWQWRMKKLGAPSITC VRRAF	M63502	Bellamy *et al.* (1992b)	B
Lepidopteran C	Silkworm (*Bombyx mori*)	RWKLFKKIEKVGRNVRDGLIKA GPAIAVIGQAKSL	225797[a]	Teshima *et al.* (1987)	
Leukocin A-Ual 187	*Leuconostoc gelidum* UAL 187 (bacterium)	KYYGNGVHCTKSGCSVNW GEAFSAGVHRLANGGNGFW	S65611	Hastings *et al.* (1991)	B−
Magainin I	Amphibian skin (*Xenopus laevis*)	GIGKFLHSAGKFGKAFVGEIMKS	A29771	Zasloff (1987)	B F E
Magainin II	,,	GIGKFLHSAKKFGKAFVGEIMNS	A29771	Zasloff (1987)	B − Ev
Mastoparan	Wasp venom (*Vespula lewisii*)	INLKALAALAKKIL*	P01514	Bernheimer and Rudy (1986)	B+
MBP-1	Maize (*Zea mays*)	RSGRGECRRQCLRRHEGQPWE TQECMRRCR	P28794	Duvick *et al.* (1992)	F
MCP1 (defensin)	Rabbit alveolar macrophages (*Oryctolagus cuniculus*)	VVCACRRALCLPRERRAGFC RIRGRIHPLCCRR	M28883	Selsted *et al.* (1983)	B F C
MCP2 (defensin)	,,	VVCACRRALCLPLERRAGFC RIRGRIHPLCCRR	M28073	Ganz *et al.* (1989)	B+ F
Melittin	Bee venom (*Apis mellifera*)	GIGAVLKVLTTGLPALISWIK RKRQQ	P01504	Tosteson and Tosteson (1984)	B C F
Mj-Amp1	*Mirabilis jalapa*	QCIGNGGRCNENVGPPYCCSG FCLRQPGQGYGYCKNR	243904[a]	Cammue *et al.* (1992)	B+ F
Mj-AMP2	,,	CIGNGGRCNENVGPPYCCSGFC LRQPNQGYGVCRNR	78217[a]	Cammue *et al.* (1992)	B+ F
Nisin	*Lactococcus lactis* subsp. *lactis* (bacterium)	ITSISLCTPGCKTGALMGCN MKTATCHCSIHVSK	P13068	Hurst (1981)	B+
Nisin Z	,,	ITSISICTPGCKTGALMGCNM KTATCNCSIHVSK	44047[a]	Mulders *et al.* (1991)	B+

Table 1—continued

NP-1 (defensin)	Rabbit neutrophils (*Oryctolagus cuniculus*)	VVCACRRALCLPRERRAGFC RIRGRIHPLCCRR	P01376	Ganz *et al.* (1989)	B F C
NP-2 (defensin)	,,	VVCACRRALCLPLERRAGFCR IRGRIHPLCCRR	P01377	Ganz *et al.* (1989)	B F C
NP-3A (defensin)	,,	GICACRRRFCPNSERFSGYCR VNGARYVRCCSRR	M64599	Michaelson *et al.* (1992)	B F C
NP-3B (defensin)	Rabbit neutrophils (*Oryctolagus cuniculus*)	GRCVCRKQLLCSYRERRIGDC KIRGVRFPFCCPR	M64600	Michaelson *et al.* (1992)	B F C
NP-4 (defensin)	,,	VSCTCRRFSCGFGERASGSCT VNGVRHTLCCRR	M64601	Michaelson *et al.* (1992)	B F C
NP-5 (defensin)	,,	VFCTCRGFLCGSGERASGSCT INGVRHTLCCRR	M64602	Michaelson *et al.* (1992)	B F C
Pep 5	*Staphylococcus epidermidis*	TAGPAIRASVKQCQKTLKATR LFTVSCKGKNGCK	P19578	Kaletta *et al.* (1989)	B+
Peptide 3910	Domestic pig (*Sus scrofa domestica*)	RADTQTYQPYNKDWIKEKIYVL LRRQAQQAGK	S36841	Agerberth *et al.* (1993)	B
PGLa	Amphibian skin (*Xenopus laevis*)	GMASKAGAIAGKIAKVALKAL*	X13388	Kuchler *et al.* (1989)	B
PGQ	Amphibian stomach (*Xenopus laevis*)	GVLSNVIGYLKKLGTGALNAVLKO		Moore *et al.* (1991)	B F
Phormicin A	Nestling-suckling blowfly (*Phormia terranovae*)	ATCDLLSGTGINHSACAAHCL LRGNRGGYCNGKGVCVCRN	P10891	Lambert *et al.* (1989)	B+
Phormicin B	,,	ATCDLLSGTGINHSACAAHCLL RGNRGGYCNRKGVCVRN	P10891	Lambert *et al.* (1989)	B+
Polyphemusin I	Atlantic horseshoe crab (*Limulus polyphemus*)	RRWCFRVCYRGFCYRKCR*	P14215	Miyata *et al.* (1989)	B
Polyphemusin II	,,	RRWCFRVCYKGFCYRKCR*	P14216	Miyata *et al.* (1989)	B
Protegrin I	Porcine leukocytes (*Sus scrofa*)	RGGRLCYCRRRFCVCVGR	S34585	Kokryakov *et al.* (1993)	B F E
Protegrin II	,,	RGGRLCYCRRRFCICV	S34586	Kokryakov *et al.* (1993)	B F E
Protegrin III	,,	RGGGLCYCRRRFCVCVGR	S34587	Kokryakov *et al.* (1993)	B F E
RatNP-1 (defensin)	Rat neutrophils (*Rattus norvegicus*)	VTCYCRRTRCGFRERLSGAC GYRGRIYRLCCR	A60113	Eisenhauer *et al.* (1989)	F B

RatNP-2 (defensin)	,,	VTCYCRSTRCGFRERLSGACG YRGRIYRLCCR		Eisenhauer *et al.* (1989)	F B
RatNP-3 (defensin)	,,	CSCRTSSCRFGERLSGACRLN GRIYRLCC	B60113	Eisenhauer *et al.* (1989)	F B
RatNP-4 (defensin)	,,	ACYCRIGACVSGERLTGACGL NGRIYRLCCR	C60113	Eisenhauer *et al.* (1989)	F B
Royalisin	Royal jelly (*Apis mellifera*)	VTCDLLSFKGQVNDSACAANCL GKAGGHCEKGVCICRKTSFKD LWDKYF	P17722	Fujiwara *et al.* (1990)	B+
Rs-AFP1	Radish (*Raphanus sativus*)	QKLCERPSGTWSGVCGNNNACK NQCINLEKARHGSCNYVFPAHK	109570[a]	Terras *et al.* (1992)	B+ F
Rs-AFP2	,,	QKLCQRPSGTWSGVCGNNNACI NQCIRLEKARHGSC	109572[a]	Terras *et al.* (1992)	B+ F
Sapecin	Flesh fly (*Sacophaga peregrina*)	ATCDLLSGTGINHSACAAHCLL RGNRGGYCNGKAVCVCRN	J04053	Hanzawa *et al.* (1990)	B
Sapecin B	,,	ITCEIDRSLCLLHCRLKGYLRA YCSQQKVCRCVQ	P31529	Yamada and Natori (1993)	B+ F
Sapecin C	,,	ATCDLLSGIGVQHSACALHCVF RGNRGGYCTGKGICVCRN	P31530	Yamada and Natori (1993)	B+ F
Sarcotoxin IA	Flesh fly (*Sacrophaga peregrina*)	GWLKKIGKKIERVGQHTRDAT IQGLGIAQQAANVAATAR*	P08375	Okada and Natori (1985b)	B
Sarcotoxin IB	,,	GWLKKIGKKIERVGQHTRDAT IQVIGVAQQAANVAATAR*	P08376	Okada and Natori (1985b)	B
Sarcotoxin IC	,,	GWLRKIGKKIERVGQHTRDAT IQVLGIAQQAANVAATAR*	P08377	Okada and Natori (1985b)	B
Seminalplasmin	Bovine seminal plasma (*Bos taurus*)	SDEKASPDKHHRFSLSRYAKL ANRLANPKLLETFLSKWIGDRG NRSV	S08184	Reddy and Bhargava (1979)	B F C
Sillucin	*Rhizomucor pusillus* (fungus)	ACLPNSCVSKGCCCGBSGYWC RQCGIKYTC	P02885	Bradley and Somkuti (1979)	B+
Subtilin	*Bacillus subtilis* (bacterium)	MSKFDDFDLDVVKVSKQDSK ITPQWKSESLCTPGCVTGALQ TCFLQTLTCNCKISK	P10946	Banerjee and Hansen (1988)	
Tachyplesin I	Horseshoe crab (*Tachypleus tridentatus*)	KWCFRVCYRGICYRRCR*	P23684	Nakamura *et al.* (1988)	B F

Table 1—continued

Tachyplesin II	,,	**R**WCF**R**VCY**R**GICY**RK**CR*	P14214	Muta *et al.* (1990)	B F
Tachyplesin III	Southeast Asian horseshoe crab (*Tachypleus gigas*)	**K**WCF**R**VCY**R**GICY**RK**CR	P18252	Muta *et al.* (1990)	
TAP	Bovine tracheal mucosa (*Bos taurus*)	NPVSCV**R**N**K**GICVPI**R**CPGSM **K**QIGTCVG**R**AV**K**CC**RK**K	P25068	Diamond *et al.* (1991)	B F
Thionin	Rabbitwood (*Pyrularia pubera*)	**K**SCC**R**NTWA**R**NCYNVC**R**IPGTI S**R**EICA**KK**CDC**K**IISGTTCPSDY P**K**	P07504	Vernon *et al.* (1985)	B F
Thionin BTH6	Barleyleaf (*Hordeum vulgare*)	**K**SCC**K**DTLA**R**NCYNTC**R**FAG GS**R**PVCAGAC**RCK**IISGP**K**CPS DYP**K**	P09618	Bohlmann *et al.* (1988)	
Toxin 1	Waglers pit viper venom (*Trimeresurus wagleri*)	GG**K**PDL**R**PCHPPCHYIP**RPK**P**R**	P24335	Schmidt *et al.* (1992)	
Toxin 2	Sahara scorpion (*Androctonus australis Hector*)	V**K**DGYIVDDVNCTYFCG**R**NAY CNEECT**KLK**GESGYCQWASPY GNACYC**K**LPDHV**RTK**GPG**R**CH	P01484	Bontems *et al.* (1991)	
XPF	Amphibian skin (*Xenopus laevis*)	GWAS**K**IGQTLG**K**IA**K**VGL**K**ELI Q**PK**	P07198	Sures and Crippa (1984)	B

Bold type, positively charged amino acids.
*, amidated C terminus.
[a], NCBI sequence identification number. All other accession numbers refer to the Swiss–Prot database.
B, active on Gram-positive and Gram-negative bacteria.
B+, only active on Gram-positive bacteria.
B−, only active against Gram-negative bacteria.
BNBD, bovine neutrophil β-defensin.
C, cytotoxic to mammalian cells.
Ev, lytic towards enveloped viruses.
F, active against fungi.
HNP, human neutrophil peptide (defensin).
MCP, macrophage cationic peptide (defensin).
TAP, trachael antimicrobial peptide.
XPF, xenopsin precursor fragment.

(Okada and Natori, 1985a), sapecins (Matsuyama and Natori, 1988a) and insect defensins (Lambert *et al.*, 1989). These antibacterial peptides have been isolated and studied from silk moths (*Hyalophora cecropia*), flesh flies (*Sarcophaga peregrina*), blow flies (*Phormia terranovae*), fruit flies (*Drosophila melanogaster*) and beetles (*Zophobus atratus*). The synthesis of molecules involved in the immune response in insects occurs primarily in the fat body.

Two forms of attacins have been isolated from *Hyalophora cecropia*. Both have molecular masses of approximately 20 kDa and have 80% amino acid similarity, but differ in their isoelectric point, one being acidic and the other basic (Hultmark *et al.*, 1983). A protein with homology to the basic attacin, sarcotoxin IIA, has been isolated from *Sarcophaga peregrina* and shown to possess the same antibacterial activity towards Gram-negative bacteria (Ando and Natori, 1988). It has been postulated that attacin works to increase the permeability of the membrane thereby making it more susceptible to the action of lysozyme and cecropins (Engström *et al.*, 1984). *Phormia terranova* possesses antibacterial activity that can be divided into three groupings, diptericins, insect defensins (phormicins) and cecropins, based on the activity and abundance of these molecules. Lysozyme has not been detected in this species and cecropins are not a major component. The diptericins act on Gram-negative bacteria and insect defensins primarily on Gram-positives, with both of these molecules being produced from cells of mesodermal origin (Dimarcq *et al.*, 1990). So far, no homologues to diptericins have been identified in vertebrates, in contrast to the defensins. For production of the mature diptericin peptide, two functions are required: cleavage from a pro molecule and amidation of the C-terminus (Reichhart *et al.*, 1989). Although both of these molecules are present in unchallenged *Diptera*, the level of transcription is greatly induced upon challenge or trauma (Dimarcq *et al.*, 1990).

Recently, a new and novel 19-residue, proline-rich peptide has been isolated and characterized from *Drosophila*. This peptide, known as drosocin, is unique in that it appears to require an O-glycosylation modification before it is active (Bulet *et al.*, 1993).

Defensin-like molecules, also known as sapecin, phormicin and sarcotoxin I, have been isolated from several different insect species (Dimarcq *et al.*, 1990; Lambert *et al.*, 1989; Matsuyama and Natori, 1988a). Although termed defensins and possessing six cysteine residues, these molecules have different tertiary structures when compared with the mammalian defensin family, because the intramolecular disulphide bridges are in a distinct arrangement resulting in a different solution structure (Hoffman and Hetru, 1992). Initially it was thought that the insect and mammalian defensins evolved from a common ancestor, based on sequence similarity seen between a rabbit defensin and the insect defensin molecule isolated from *Phormia* (Lambert *et al.*, 1989). However, on elucidation of the structural data, this does not

seem to be the case. Instead it appears that insect defensins have greater similarity to royalisin from bees, charybdotoxin from scorpion venom and the newly isolated scorpion defensin (Bontems *et al.*, 1991; Cociancich *et al.*, 1993b; Fujiwara *et al.*, 1990). These molecules not only share amino acid similarity with respect to the cysteine array, but also possess a similar tertiary structure (Bontems *et al.*, 1991; Hoffman and Hetru, 1992).

As with the mammalian system, insect defensins are synthesized as preproproteins requiring cleavage to produce the mature peptide (Dimarcq *et al.*, 1990). The genetic structure of the defensin gene in *Drosophila* is known and appears to contain an upstream transcriptional control region which is related to that involved in inducing mammalian interleukin 6 (Isshiki *et al.*, 1991). Recently, a similar system has been identified for cecropin genes. A kappa B-motif has been identified which in mammals binds transcription factor NF-kappa B thereby regulating the immune and acute phase responses (Engström *et al.*, 1993).

One of the main groups of antibacterial components in the insect humoral response is the cecropins. These molecules constitute a class separate from those described above and differ from other bacteriolytic peptides produced by insects by not lysing mammalian cells. Cecropins contain 31–39 residues, do not possess cysteine residues and therefore do not form any disulphide bridges. They can be classified into different groups based on their amino acid sequence, which usually differs by only a few residues (Hultmark *et al.*, 1982). As with other molecules, cecropins are known by a variety of names depending on their origin (Dunn *et al.*, 1985; Okada and Natori, 1985a; Teshima *et al.*, 1986). Similar to diptericin and other antibacterial peptides, the C-terminus contains an amidated residue but non-amidated variants of cecropins have been found in *Hyalophora* (Hultmark *et al.*, 1982), *Sarcophaga* (Matsuyama and Natori, 1988b) and *Drosophila* (Samakovlis *et al.*, 1990). Nevertheless, there are indications that C-terminal amidation may be required for activity against Gram-positive bacteria (Callaway *et al.*, 1993). The cecropin family, members of which have a totally different tertiary structure to the defensin family (see following section), have similarities to another basic amphipathic molecule, melittin, the toxic component of bee stings. Cloning of cDNA corresponding to cecropin genes has revealed the presence of a signal peptide and a pro region which is cleaved off (von Hofsten *et al.*, 1985).

Cecropin-like peptides have also been isolated from pig intestine (Lee *et al.*, 1989). This 31-amino acid peptide differs from insect cecropins by not containing an amidated C-terminus, and by its solution structure (Sipos *et al.*, 1992), discussed in the following section.

2.3. Bee-derived Peptides

The honeybee *Apis mellifera* possesses some novel antimicrobial peptides, namely abaecin (Casteels *et al.*, 1990), apidaecins (Casteels *et al.*, 1989),

hymenoptaecin (Casteels *et al.*, 1993), magainins (Zasloff, 1987), royalisin (Fujiwara *et al.*, 1990) and a bee defensin (Casteels *et al.*, 1993). Apidaecins (IA, IB and II) identified by Casteels and co-workers (1989) from the immune haemolymph of honeybees, are a family of proline-rich peptides which are primarily active against Gram-negative and plant-associated bacteria. They are specifically induced and, owing to their high proline content, are stable at high temperatures and low pH. Similar to other peptides, apidaecins are synthesized as an inactive form with additional residues (8–10) at the N-terminus, requiring the action of a dipeptidyl aminopeptidase for maturation. Recently, the genetic structure of the apidaecin precursor has been characterized in detail (Casteels-Josson *et al.*, 1993). It appears from these studies that the apidaecin precursor contains multiple copies of the mature peptide, with a common prepro structure. Casteels-Josson *et al.* (1993) have postulated that the processing of the preproprotein into its biologically active form is via a mechanism reminiscent of that described for the yeast pheromone system (Singh *et al.*, 1983).

Abaecin, a 34-amino acid peptide containing 30% proline, with sequence similarity to the apidaecins, has been classified into a new group based on its different spectrum of anti-bacterial activity and its delayed activity in contrast with the immediate action of apidaecin (Casteels, 1990). Hymenoptaecin, a recently identified peptide, is composed of 93 amino acids with a glycine content of 19%. It exhibits no amino acid homology to other previously characterized molecules in bees, as it does not possess a high proline content and, unlike defensins, is devoid of cysteines (Casteels *et al.*, 1993).

The representative of the insect defensin family in bees is the 51-residue peptide royalisin isolated from royal jelly (Fujiwara *et al.*, 1990). This peptide appears to contain the characteristic array of cysteine residues forming three disulphide bridges. The N-terminal half of the molecule is hydrophobic, whereas the C-terminal half is hydrophilic. Bees also contain an α-helical peptide, mellittin, where the polarity of the hydrophobic versus the charged domains is reversed compared with cecropins (Suchanek and Kreil, 1977). Melittin is the major component (50%) of bee venom. It is a 26-residue peptide which has a wide spectrum of biological effects, including antibacterial activity (Piers and Hancock, 1994), membrane permeabilization leading to cell lysis, and interference with various enzymatic activities (Habermann, 1972). Melittin has been very well characterized with respect to its tertiary structure and its mode of action (discussed in detail later). Analogues of melittin and hybrid molecules with cecropins have shed some light on the functional residues within these molecules (Sipos *et al.*, 1991, 1992).

Yet another class of antimicrobial peptides has been isolated from the venom of the bumblebee, *Megabombus pennsylvanicus*. These five structurally related peptides called bombolitins possess a high percentage of hydrophobic amino acids (Argiolas and Pisano, 1985). Bombolitins share

functional similarities to other venom peptides including melittin from bees, mastoparan from wasps, and crabrolin from hornets (Argiolas and Pisano, 1985).

2.4. Amphibian Peptides

It has been discovered that the skin of frogs contains a wide array of biologically active peptides in glands located in the skin and also in the gastric mucosa (Erspamer and Melchiorri, 1980; Moore *et al.*, 1991). One peptide was originally identified in the species *Bombina* and was subsequently named bombinin (Csordas and Michl, 1970). Homologues of this 26-amino acid cationic peptide, named bombinin-like peptides (BLPs), have been found in different species of *Bombina*. The significant difference between these peptides and the original bombinin is that BLPs possess no haemolytic activity (Gibson *et al.*, 1991). The genes encoding BLPs have been cloned and analysed, revealing that peptides within this family are expressed as a precursor protein (Gibson *et al.*, 1991; Simmaco *et al.*, 1991). These peptides have a predicted alpha-helical structure reminiscent of the cecropin family.

Magainins, another family of amphibian cationic peptides, produced in the African clawed frog (*Xenopus laevis*), are also amphipathic and alpha-helical in structure (Chen *et al.*, 1988). They have been well characterized structurally, and chemical synthesis of synthetic magainin analogues has helped understand the structures required for the biological activity (Chen *et al.*, 1988; Cuervo *et al.*, 1988). The cDNA for magainin has been cloned, as have those corresponding to PGLa, PGQ and xenopsin, three related amphibian antibiotic peptides (Hoffman *et al.*, 1983; Moore *et al.*, 1991; Sures and Crippa, 1984; Terry *et al.*, 1988; Zasloff, 1987). As with the peptides described above, magainins are also synthesized as precursor molecules containing a signal sequence which shows considerable homology throughout the magainin family. They all possess a common processing motif and are produced with an acidic N-terminal pro region.

In 1991, Mor and colleagues isolated a novel antimicrobial peptide from the skin of the South American arboreal frog (*Phyllomedusa sauvagii*) and named it dermaseptin because of its antiseptic activity. This peptide has no sequence homology to the other amphibian cationic peptides; however, it is thought to permeabilize membranes in a similar fashion, owing to its amphipathic nature (Mor *et al.*, 1991). It is unique in its spectrum of antimicrobial activity as it inhibits the growth of pathogenic moulds (Mor *et al.*, 1991).

Simmaco and co-workers (1993) isolated three antimicrobial peptides from the skin of the European green frog (*Rana esculenta*). Two of these peptides, brevinin 1-E and 2-E, share homology and all three possess a single

C-terminal disulphide bond. These peptides differ in their antibacterial spectrum (Simmaco *et al.*, 1993).

2.5. Plant Peptides

Plants have been shown to combat infections by the production of specific cationic peptides, thionins. For example, barley produces a leaf-specific thionin, BTH6 (Bohlmann *et al.*, 1988). A wide variety of plants produce proteins that belong to the superfamily of highly basic, cysteine-rich peptides, which include thionins and mammalian and insect defensins. These include peptides from the seeds of *Amaranthus caudatus*, Ac-AMP (Broekaert *et al.*, 1992) and *Mirabilis jalapa*, Mj-AMP (Cammue *et al.*, 1992). As with defensins, these plant peptides are toxic to fungi and are more active against Gram-positive bacteria than Gram-negative bacteria.

2.6. Peptides from Other Species

There are various other antimicrobial peptides that do not possess homology to any other family of peptides. Bovine seminalplasmin, a 47-amino acid protein, has been studied to identify the residues responsible for its antibacterial activity. A synthetic peptide corresponding to a 13-amino acid hydrophobic region has been found to possess the same activity as the intact protein (Sitaram and Nagaraj, 1990).

Bacteria also naturally produce antimicrobial peptides. The Gram-positive bacterium *Staphylococcus epidermidis* produces a tricyclic antibiotic pep5, containing the unusual amino acids dehydrobutyrine, lanthionine and 3-methyllanthionine (Kaletta *et al.*, 1989; Weil *et al.*, 1990). This molecule is classified into a group of peptide antibiotics termed lantibiotics that are synthesized using multienzyme complexes rather than ribosomes and mRNA templates. Also included in this group is nisin, a bacteriocin from *Lactococcus lactis* (Hurst, 1981). Antibacterial peptides have also been isolated from fungi. *Rhizomucor pusillus*, a thermophilic fungus, produces a defensin-like peptide, sillucin, that is active against Gram-positive bacteria (Bradley and Somkuti, 1979).

Horseshoe crabs (*Limulus polyphemus*, *Carcinoscorpius rotundicauda* and *Tachypleus gigas*), in response to bacterial infection, produce two classes of antimicrobial peptides, tachyplesins and polyphemusins, which are contained in cytoplasmic granules (Miyata *et al.*, 1989; Muta *et al.*, 1990; Ohta *et al.*, 1992). Tachyplesins contain 17 amino acids with a C-terminal arginine amide; polyphemusins consist of 18 residues. Both groups contain cysteines that participate in two disulphide linkages. These peptides are abundant within the haemolymph and act on Gram-negative and Gram-positive bacteria and fungi (Nakamura *et al.*, 1988).

Recently, a class of peptides produced in porcine leukocytes has been identified and shown to possess not only the characteristics of tachyplesin but also of the mammalian defensins (Kokryakov *et al.*, 1993). Protegrins, as they are called, consist of 16–18 residues and contain two intramolecular disulphide linkages.

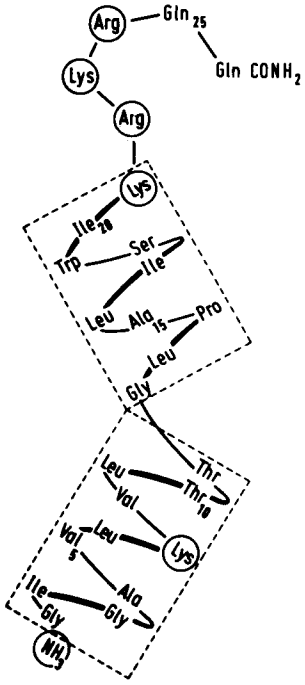
3. STRUCTURE–FUNCTION RELATIONSHIPS

3.1. Structure of Polycationic Peptides

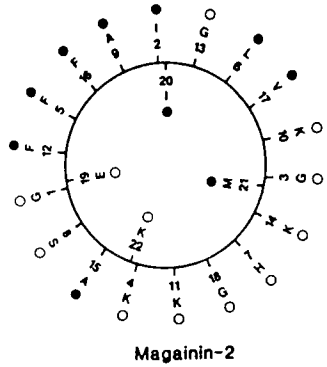
There are basically two major structural classes of natural polycationic peptides: those that form an α-helical structure in membranes, but are often disordered in aqueous solution, and those that form an antiparallel β-sheet containing β-hairpin turns (Figs 1, 2). The former helical structure often comprises a helix–turn–helix arrangement with a 9–16 amino acid amphiphilic α-helix near the N-terminus, a 2–4 residue turn and an 11–14 amino acid hydrophobic helix near the C-terminus (Fig. 1A). Examples of peptides that have been shown by two-dimensional nuclear magnetic resonance (NMR) to possess such a configuration include cecropins A and B (Holak *et al.*, 1988), melittin (Bazzo *et al.*, 1988), the magainins (Marion *et al.*, 1988) and a synthetic cecropin–melittin hybrid (Sipos *et al.*, 1991). Another variation on the theme is provided by cecropin P1 which comes from the pig intestine. This seems to have an uninterrupted amphiphilic helix of 24 residues bounded

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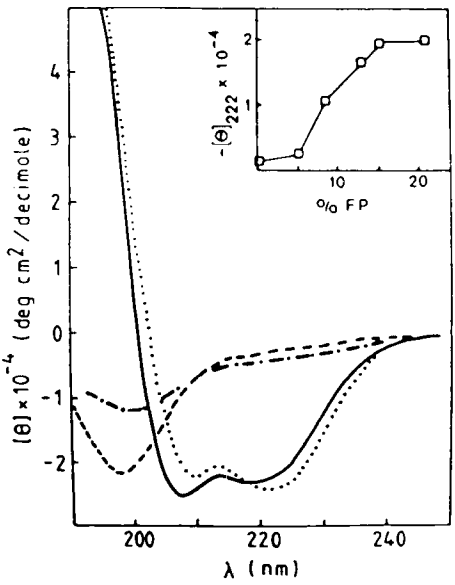
Figure 1 Structure of the α helical peptides. (A) Monomeric membrane-associated melittin showing the helix–hinge–helix structure. Charged residues are circled. (Reproduced by copyright permission from Vogel and Jähnig (1986). © Biophysical Society.) (B) Helical wheel diagram (axial projection) for magainin-2 demonstrating the amphipathic nature of the α helix. Solid circles represent hydrophobic residues and open circles represent hydrophilic residues including the five clustered lysine (K) residues. Single letter code is used for amino acids. (Reproduced by copyright permission from Kini and Evans (1989) © *International Journal of Peptide and Protein Research.*) (C) Transition in cecropin structure dependent on the suspension medium. Circular dichroism spectra are shown for cecropin A in buffer (- - -; showing largely random coil structure); cecropin A in 20% 1,1,1,3,3,3-hexafluoro-2-propanol (FP) (–; showing approximately 81% α-helix); cecropin B in a liposone containing solution (–·–·; showing approximately 20% helix). A simulated spectrum of cecropin A containing 81% α-helix, 7% β-sheet and 12% random coil is shown (. . . . .). The inset shows the increase in α-helix content (ellipticity θ at 222 nm) as a function of the % FP (reflecting the hydrophobicity of the environment). (Reproduced by permission of Steiner (1982) © Federation of European Biomedical Societies.)


Arg
Gln 25
Gln CONH2
Lys
Arg
Lys
Ile 20
Trp
Ser
Ile
Leu
Ala 15
Pro
Leu
Gly
Leu
Thr
Val
Thr 10
Val 5
Leu
Lys
Ile
Gly
Ala
Gly
NH2

(A)


Magainin-2

(B)


[θ] x 10^-4 (deg cm^2 / decimole)
λ (nm)
200
220
240
4
3
2
1
0
-1
-2
-(θ)222 x 10^-4
2
1
10
20
% FP

(C)

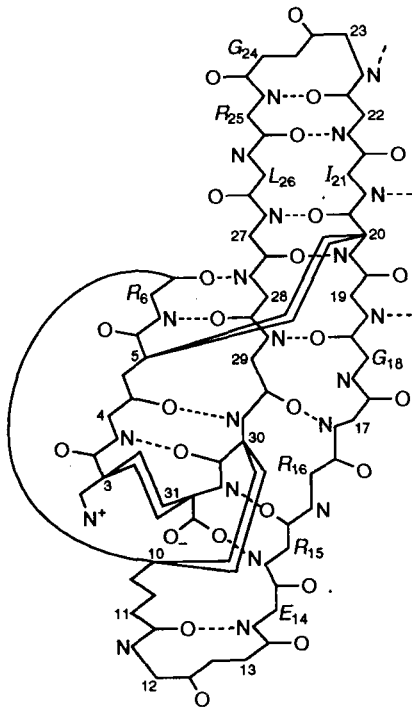


Figure 2 Model of the mammalian defensin structure. The triple stranded anti-parallel β-sheet structure of an HNP-3 monomer. The disulphide bonds are represented as "lightning bolts". Charged residues are indicated as R = arginine and E = glutamate. (Reproduced by copyright permission of Hill *et al.* (1991) © American Association for the Advancement of Science.)

by 2–4 residues at the N- and C-termini (Sipos *et al.*, 1992). Several other peptides can be fitted in part to a helical wheel diagram (Kini and Evans, 1989) which shows a tendency to form an α-helix with one hydrophobic face and one positively charged face (Fig. 1B). In the case of cecropins A and B and magainins, it has been determined that the peptides are random in aqueous solution and 80% α-helical in organic solvents (Fig. 1C) (Steiner, 1982; Marion *et al.*, 1988; Bechinger *et al.*, 1992).

The second class of structures, typified by the defensins, shows a disulphide-linked β-sheet structure (Zhang *et al.*, 1992). This structure (Fig. 2) contains an antiparallel β-sheet in addition to a short region of triple-stranded β-sheet, with the β-strands interconnected by β-turn regions. Defensins have been crystallized (HNP3) and studied by two-dimensional NMR techniques (HNP1, NP2, NP5) with quite similar results (Pardi *et al.*, 1988; Hill *et al.*, 1991; Zhang *et al.*, 1992), despite differences in amino acid composition including numbers of basic amino acids. From a three-

Table 2 Influence of selected amino acid changes on the minimal inhibitory concentrations (MIC) of synthetic cecropin A analogues. Data selected from Andreu *et al.* (1985).

	MIC (μM)			
Cecropin A analogue	*E. coli*	*P. aeruginosa*	*B. megaterium*	*M. luteus*
Native	0.4	2.6	0.6	1.4
Lys^1 Trp^2 deletion	2.6	90	13	>110
$Trp^2 \rightarrow Phe^2$	0.3	3.5	0.8	7.4
$Trp^2 \rightarrow Glu^2$	3.2	170	39	>170
$Leu^4 \rightarrow Pro^4$	0.4	8.1	11	87
$Lys^6 \rightarrow Leu^6$	0.6	120	0.8	7.3
$Lys^6 \rightarrow Glu^6$	0.6	34	2.2	4.7
$Ile^8 \rightarrow Pro^8$	0.5	15	31	80

dimensional perspective, defensins have a predominant hydrophobic face with the charged residues spread out along one face of the structure (Zhang *et al.*, 1992). NMR evidence suggests that they dimerize. The insect defensin, sapecin, has, like animal defensins, three disulphide bonds, but differs substantially in sequence and by the presence of lysines and histidines as positively charged amino acids in the former (Hanzawa *et al.*, 1990). Moreover, the solution structure of sapecin is quite different, containing a flexible loop, an 8-amino acid residue α-helix and two extended regions (Hanzawa *et al.*, 1990). This difference in structure has been attributed to the different arrangement of cysteine disulphides in the animal and insect defensins. However, scorpion toxins with an arrangement of disulphides similar to the animal defensins have a hybrid structure containing both a short region of triple-stranded β-sheet as well as a 9-amino acid α-helix (Bontems *et al.*, 1991). It is important to note that sapecin has, like the animal defensins, a hydrophobic surface and a region rich in basic residues.

3.2. Structure–Function Studies

Several studies have examined the influence of deletion or modification of specific amino acids on the activity of specific families of peptides. One example is the cecropins (Table 2). This and other studies have revealed the following general principles. (1) There is considerable specificity in the way that changes in amino acid sequence influence activity (Andreu *et al.*, 1985; Blondelle and Houghten, 1991). For example, introduction of a turn-promoting proline at positions 4 or 8 in the first α-helical segment of cecropins has a substantial effect on activity against *M. luteus*, lesser effects on *B. megaterium* and *P. aeruginosa*, and no effect against *E. coli* (Table 2).

(2) For the α-helical peptides, changes that increase the tendency to form an α-helix in aqueous solution tend to increase activity (Andreu *et al.*, 1985; Steiner *et al.*, 1988; Frohlich and Wells, 1991; Blondelle and Houghten, 1991). (3) There is no absolute relationship between the numbers of positive charges and activity, although the position of specific positive charges is important (Blondelle and Houghten, 1991). (4) Enantiomers (i.e. all D-amino acids *vs.* all L-amino acids) have equal activity (Bessalle *et al.*, 1990; Wade *et al.*, 1990) showing that chirality is not important. (5) There is no absolute relationship between ability to lyse liposomes or ability to bind to bacterial cells and minimal inhibitory concentration (MIC) against bacteria, although trends are observable (i.e. decreased lysis or binding tends to correlate with decreased MIC) (Steiner *et al.*, 1988). (6) Finally, for the disulphide-bonded peptides, reduction of the cysteine disulphides destroys activity (Kagan *et al.*, 1990).

Three studies have indicated general methods of enhancing activity. The reduction in size of cecropin–melittin hybrids from 26 amino acids to 14 amino acids did not influence activity so long as these compounds maintained an α-helical structure (Andreu *et al.*, 1992). In a study of magainins, it was demonstrated that the addition of 10 or more basic amino acids to the N- or C-termini, but not 4 basic or 10 non-polar amino acids, resulted in a 10-fold enhancement of antibacterial activity (Bessalle *et al.*, 1992). In contrast, addition of two positive charges to the carboxy terminus (hydrophobic domain) of a cecropin–melittin hybrid protein actually decreased the MIC for some bacteria while enhancing the interactions with endotoxin and with bacterial outer membranes (Piers *et al.*, 1994). As above, treatments that enhanced α-helicity also increased activity. In a third study, it was demonstrated that human defensin HNP4 had increased hydrophobicity compared with other human defensins and a 100-fold greater potency against *E. coli* (Wilde *et al.*, 1989).

4. INTERACTIONS WITH LIPIDS AND MEMBRANES

4.1. Lipid Interactions

Where studied, antibacterial peptides undergo a conformational change on interaction with liposomes and/or apolar solvents (Knoppel *et al.*, 1979; Andreu *et al.*, 1985; Lee *et al.*, 1986; Marion *et al.*, 1988; Williams *et al.*, 1990; Agawa *et al.*, 1991; Bechinger *et al.*, 1992; Jackson *et al.*, 1992). The process is initiated by binding of the positively charged peptides to lipids (Batenburg *et al.*, 1987; Matsuzaki *et al.*, 1991; Sekharam *et al.*, 1991). Binding to negatively charged lipids is extremely rapid (Sekharam *et al.* 1991). The extent of binding corresponds to the zeta potential of the lipids

involved and is inhibited by salt, leading one to conclude that it is electrostatic in nature (Matsuzaki *et al.*, 1991; Sekharam *et al.*, 1991). In contrast, binding to zwitterionic lipids is slower and, in the case of melittin, demonstrates negative cooperativity (presumably because the interaction of the cationic peptides with the surface of such lipids increases the surface positive charge, causing charge repulsion of other peptide molecules; Sekharam *et al.*, 1991).

Subsequently, the cationic peptides insert into the lipid bilayer (in many cases under the influence of an appropriate membrane potential (Cruciani *et al.*, 1991; de Kroon *et al.*, 1991)) and undergo a conformational change. In the case of the "α-helical" peptides such as melittin (Vogel and Jähnig, 1986), magainins (Bechinger *et al.*, 1992; Williams *et al.*, 1990) and cecropins (Andreu *et al.*, 1985), the transition is from unstructured or β-sheet conformation to α-helix. At the same time, the lipids themselves undergo changes in phase and/or motion (Smith *et al.*, 1992). In some cases these peptides are thought to end up spanning the bilayer (Steiner, 1982; Vogel and Jähnig, 1986; Sipos *et al.*, 1992; Andreu *et al.*, 1992) although, as discussed below, they appear to form channels owing to assembly into multimeric complexes. Other peptides are considered to be too short to span the bilayer and, in these cases, aggregation or multimerization may be critical to permit the spanning of membranes and channel formation (Williams *et al.*, 1990; Agawa *et al.*, 1991; Andreu *et al.*, 1992).

In many cases (Lee *et al.*, 1986; Steiner *et al.*, 1988; Katsu *et al.*, 1990; Frohlich and Wells, 1991; Matsuzaki *et al.*, 1991; Grant *et al.*, 1992) it has been demonstrated that interaction with biomembranes leads to leakiness (i.e. permeabilization) of these membranes and, with more extreme treatments, lysis. A common assay for measuring liposome leakiness is leakage of carboxy fluorescein. There is a general correlation between ability to disrupt model liposomes and activity against the most sensitive target bacteria (Steiner *et al.*, 1988; Agawa *et al.*, 1991). In more complex eukaryotic cell membranes, Bashford *et al.* (1986) argued for a common mechanism of membrane damage by cationic proteins and peptides, as well as complement, viruses, toxins and detergents. They found the following common features of these agents when applied to Lettre cells: sensitivity to changes in ionic strength and divalent cations, positive cooperativity, synergy between diverse agents and a nearly identical sequence of permeability changes. These authors concluded that the action of these agents (including melittin and polylysine) was a detergent-like disruption of permeability, although this presumably reflects the lytic action of these agents as distinct from the formation of defined channels, as discussed in the next section. Kini and Evans (1989) also suggested that peptides that function as cytolysins have common features. Another factor that has been suggested to enhance lytic capability is the spontaneous aggregation of magainin 2 and a melittin analogue into large oligomers (Urrutia *et al.*, 1989; John and Jähnig, 1992).

4.2. Planar Lipid Bilayer Studies

Several cationic peptide channels have been examined in planar lipid bilayers. In this system a lipid bilayer is constituted across a hole in a teflon divider separating two aqueous compartments, each of which contains an electrode. Addition of specific cationic peptides to one of the aqueous compartments (e.g. the cis side), and application of a negative voltage to the trans side (such that the positive ions would tend to move from the cis to the trans compartments), leads to an observed increase in conductance as the cationic peptides enter the membrane and form channels (Hanke *et al.*, 1983; Christensen *et al.*, 1988; Kordel *et al.*, 1988; Kagan *et al.*, 1990; Cociancich *et al.*, 1993a). In several cases studied, the reversal of the sign of the voltage not only prevents or substantially decreases the rate of formation of channels, but actually results in an exponential decrease in the conductance of membranes into which peptides had already been inserted, with a half-time of around 30 s (Christensen *et al.*, 1988). Thus, channel formation may actually be "driven" by electrophoresis of the cationic peptide towards the membrane and reversed by electrophoresis towards an aqueous compartment. This is consistent with the situation in bacterial cytoplasmic membranes, in which the $\Delta\psi$ (electrical potential gradient) is oriented interior-negative (see below). Alternatively, Kordel *et al.* (1988), on the basis of chemical modification experiments, suggested that the requirement for a trans-negative voltage for Pep5 channel formation reflected the orienting action of the transmembrane voltage.

Formation of channels is generally voltage-dependent, as observed with both the β-structured defensins (Kagan *et al.*, 1990) and the helix–turn–helix structured melittin (Tosteson *et al.*, 1987), cecropin (Christensen *et al.*, 1988) and magainin (Duclohier *et al.*, 1989) peptides. In one case, that of Pep5, there is actually a threshold (turn-on) potential of approximately -100 mV that must be applied before channel formation is observed (Kordel *et al.*, 1988). However, this is unusual. Far more usual is the observation that, as the voltage increases, there is an exponential, rather than linear, increase in current. This could be due to voltage-induced gating (i.e. opening of channels), or the effects of voltage on the rate of channel formation or the rate of aggregation of channels in the membrane. The voltage dependence of the formation of α-helical peptide channels may be related to the existence of a flexible (turn) segment between the N-terminal amphipathic and the C-terminal hydrophobic regions, because synthetic variants of the cecropins, which lacked the turn segment, were not voltage-dependent (Christensen *et al.*, 1988).

The interaction of the peptides with membranes is related to the charge and folding of the cationic peptides. Thus, succinylated Pep5 forms channels with a greater voltage dependence (Kordel *et al.*, 1988), whereas reduced and carboxymethylated defensins do not form channels (Kagan *et al.*, 1990).

In addition, positively charged phospholipids and cholesterol decrease cecropin channel formation by 5- and 60-fold, respectively (Christensen *et al.*, 1988). Since eukaryotic membranes are rich in cholesterol (which affects both the fluidity and dipole potential of bilayers), this may explain in part the selectivity of several cationic peptides for bacterial cells, which lack cholesterol and have very low levels of positively charged phospholipids.

Examination of the influence of peptide concentration on the conductance of membranes reconstituted with defensins or melittin reveals a linear relationship when conductance induced by the peptide is plotted as a function of peptide concentration, with a slope of 1.5 to 4 pS/mg ml^{-1} (Tosteson *et al.*, 1987; Kagan *et al.*, 1990). This suggests that the functional units are oligomers rather than monomers (in this latter case, a slope of 1 would be predicted). Where examined, these channels have a weak preference for chloride over sodium (i.e. 2:1) (e.g. Kagan *et al.*, 1990; Kordel *et al.*, 1988). This means that the positive charges of the individual subunits of the oligomeric channel must be distant from one another, because closely spaced charges would tend to make the channel anion-specific. This could be caused by the formation of large channels, as suggested by certain authors (Kordel *et al.*, 1988; Christensen *et al.*, 1988), with the charges spaced at distances of up to 1 nm (i.e. similar to the spacings observed for the weakly selective bacterial porins; Cowan *et al.*, 1992).

When the increases in current are examined more closely, they can be resolved into smaller increments, i.e. single channels. Single channel conductances for a given cationic peptide generally vary between 10 and 2000 pS (Hanke *et al.*, 1983; Kordel *et al.*, 1988; Duclohier *et al.*, 1989; Kagan *et al.*, 1990), the latter being similar to the value for porins (Benz *et al.*, 1985) (cf. the cecropins, Christensen *et al.*, 1988; Wade *et al.*, 1990). This multistate channel behaviour has been observed for the channel-forming peptide alamethicin (Boheim, 1974), for which it has been proposed that after monomers of alamethicin are induced by voltage to span the membrane, the monomers then associate or dissociate with various rate constants, resulting in aggregates of different sizes or lifetimes. These aggregates are then proposed to align like the staves of a barrel, with a central channel that represents the conducting pore. The size of this pore and the resultant conductance would depend on the number of monomers (staves) making up the conducting unit. The lifetime of individual channels is in the order of several milliseconds to seconds (e.g. Kordel *et al.*, 1988).

A credible model to explain the above events for cecropins is presented in Fig. 3. This suggests alignment of the positive charges of cecropins with the negatively charged lipid head groups, followed by insertion of the hydrophobic segment into the membrane, and a major (voltage-induced) conformational rearrangement which results in channel formation. This is rather analogous to the proposal for alamethicin (Boheim, 1974).

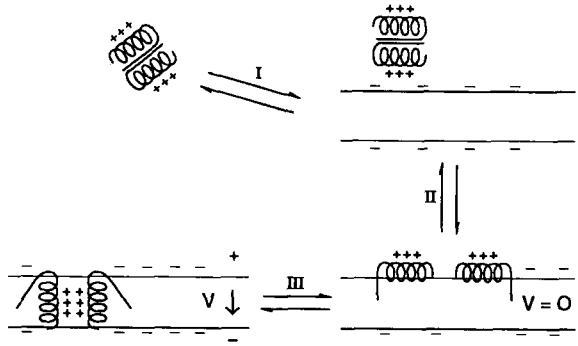


Figure 3 Tentative model for the interaction of cecropins with a lipid bilayer membrane. Aggregates adsorb to the bilayer–water interface by electrostatic forces (I). Only a dimer is sketched for the sake of simplicity, but larger aggregates are likely to occur. The next step (II) would be insertion of the hydrophobic segment into the membrane core. Upon application of voltage (positive on the side of the peptide addition), a major conformational rearrangement takes place (III), which results in channel formation. This rearrangement could be insertion of the positively charged amphipathic helix into the membrane or opening of preformed, closed channels. (Reproduced by copyright permission from Christensen *et al.* (1988) © The National Academy of Sciences of the United States of America.)

4.3. Channel Structure in Lipids

As discussed above, several of the cationic peptides undergo a conformational change to an α-helical configuration when placed in solvents with reduced water activity (i.e. ones that create a hydrophobic membrane-like environment), such as hexafluoropropanol. An ability to form discrete conductance units (channels) as opposed to an erratic increase in membrane conductance was found only for those α-helical model peptides that were long enough to span the membrane (i.e. >20 residues, Agawa *et al.*, 1991). In the case of melittin, there is a glycine at residue 12 that causes a kink in the α-helix, giving the resultant channel the appearance of a tetramer of bent α-helices with charged and hydrophilic residues pointing into the channel of the tetramer, and hydrophobic residues facing the non-polar membrane core (Fig. 4), as modelled from Raman spectroscopy and fluorescence transfer data (Vogel and Jähnig, 1986). The cecropins, which are thematically similar to melittin, have been modelled by Durell *et al.* (1992) at an atomic scale. They propose two types of channels comprising a star-shaped arrangement of six dimers with a 0.56 nm internal channel, and a circular arrangement of six dimers with a 1.1–1.5 nm channel, a model consistent with the two discrete conductance increments (0.4 and 1.9 nS) reported by Christensen *et al.* (1988).

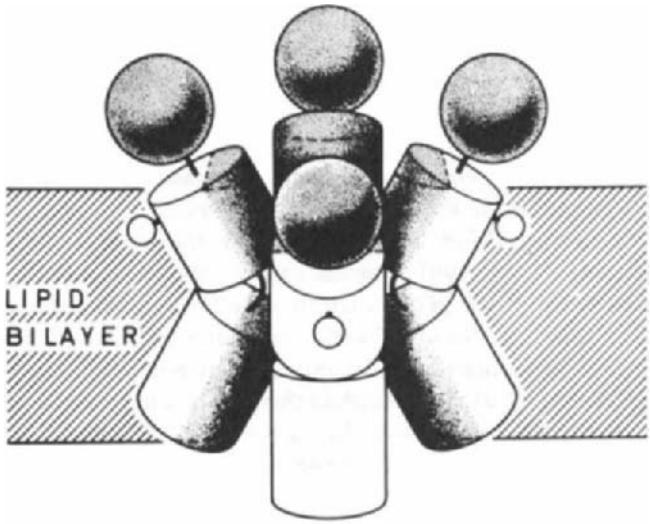


Figure 4 Model of a membrane-associated tetramer of melittin containing a central channel. The orientation of each melittin monomer is identical to that depicted in Fig. 1C. The shaded areas represent hydrophilic amino acid residues and the unshaded areas hydrophobic residues. (Reproduced by copyright permission from Vogel and Jähnig (1986) © Biophysical Society.)

Williams *et al.* (1990) have used Raman spectroscopy to follow the interaction of magainin 2a and PGLa with negatively charged liposomes. The basic interaction scheme proposed was quite similar to that based on planar bilayer studies.

4.4. Interactions with Bacterial Outer Membranes

The outer membranes of Gram-negative bacteria constitute a semi-permeable barrier to penetration of substances from the external medium (Nikaido and Vaara, 1985; Hancock, 1987, 1991; Benz, 1988; Vaara, 1992). The existence of channel-forming proteins, called porins, gives outer membranes their characteristic size-dependent exclusion limit. Thus, with one prominent exception, hydrophilic substances below a certain size can permeate the weakly ion-selective, chemically non-selective water-filled channels of these porins, whereas substances exceeding this size will not. For *E. coli*, for example, it has been suggested that substances equal to, or larger than, tetrasaccharides (e.g. stachyose) or pentapeptides (e.g. pentalysine) will diffuse very slowly or not at all across the outer membrane (Payne and Gilvarg, 1968; Nikaido and Vaara, 1985). In addition, many Gram-negative

bacteria limit the rate of uptake of hydrophobic substances by virtue of the tight packing and divalent cation stabilization of their surface glycolipid, lipopolysaccharide (LPS) molecules (Hancock, 1984; Nikaido and Vaara, 1985). An exception to these generalizations is provided by the cationic peptides described here, which are too large and bulky to pass through the porins of, for example, *E. coli*, whose crystal structure is now known (Cowan *et al.*, 1992). Instead, these molecules have been proposed to cross the outer membrane via a non-porin pathway termed the self-promoted uptake pathway (Hancock, 1984, 1991; Sawyer *et al.*, 1988).

Early studies on polymyxin B, a cationic cyclic peptide with a fatty acyl tail, suggested that it interacted with Gram-negative outer membranes, causing structural perturbations and increased outer membrane permeability (Schindler and Teuber, 1975). To explain these data and results with a mutant of *P. aeruginosa* that was cross-resistant to EDTA, aminoglycosides and polymyxins, it was proposed that these compounds promoted their own uptake across the outer membrane (Nicas and Hancock, 1980; Hancock *et al.*, 1981; Young *et al.*, 1992). This hypothesis was later extended to embrace the cationic peptides (Hancock, 1984; Sawyer *et al.*, 1988), melittin and two other α-helical peptides (Piers and Hancock, 1994; Piers *et al.*, 1994).

Self-promoted uptake is initiated by the interaction of the cationic antibiotic with anionic, divalent cation-binding sites on LPS. Direct interaction of defensins (Sawyer *et al.*, 1988), magainins (Rana *et al.*, 1991) and melittin (David *et al.*, 1992) with LPS has been demonstrated. Using dansyl polymyxin as a probe, it was shown that the interaction of defensins (Sawyer *et al.*, 1988), melittin and two cecropin–melittin-derived hybrids (Piers and Hancock, 1994; Piers *et al.*, 1994) occurs at divalent cation-binding sites on LPS. However, the above peptides, for example, have binding affinities that are three orders of magnitude higher than the normally resident divalent cations, making binding to this LPS site an efficient process. Interestingly, the kinetics of probe displacement by defensins from purified LPS exactly mirrored the kinetics of displacement from intact cells, suggesting that LPS binding could explain binding to Gram-negative bacteria. It was demonstrated that addition in CEMA of two positive charges to the carboxyl terminus of a cecropin melittin hybrid CEME, enhanced the interaction of CEMA with LPS and with the outer membrane (Piers *et al.*, 1994). By Fourier transform-infrared spectroscopy studies of *S. typhimurium* mutant LPS molecules of different chain lengths, it was concluded that magainin interaction with LPS (and with the cells from which these LPS molecules are derived) depends on the magnitude of LPS charge rather than chain length *per se* (Rana *et al.*, 1991). ^{31}P-NMR studies were consistent with others that suggest that the LPS binding sites comprise negatively charged phosphate residues (Rana *et al.*, 1991; Peterson *et al.*, 1985, 1987; Schindler and Osborn, 1979), of which there are 5–15 per molecule of LPS.

Interaction of cationic substances, including magainins, with purified LPS

causes the LPS to undergo a conformational change including an alteration in the mobility of both the hydrophilic portion and the fatty acyl chains (Peterson *et al.*, 1985, 1987). This disorganization can have one of two observable consequences. In electron micrographs of cells treated with sarcotoxin (Okada and Natori, 1984) or defensins (Sawyer *et al.*, 1988; Lehrer *et al.*, 1989), it was manifested as structural perturbations that have been visualized in one case as blebbing of the outer membrane (Sawyer *et al.*, 1988). Similar blebbing has been observed in cells treated with the cationic antibiotic polymyxin and some, but not all, other polycations (Gilleland and Murray, 1976; Vaara and Vaara, 1983; Vaara, 1992). In some cases, LPS is actually released from cells by treatment with polycations (Vaara and Vaara, 1983), although we consider this to be a more extreme manifestation of the blebbing phenomena and one that generally occurs at higher polycation concentrations. It has been proposed that these structural perturbations reflect the formation of transient cracks (Martin and Beveridge, 1986). A more easily assayed phenomenon is breakdown of the outer membrane permeability barrier, which has been assessed as increased permeation of hydrophobic fluorescent probes such as 1-N-phenylnapthylamine, which are normally excluded (Hancock and Wong, 1984; Sawyer *et al.*, 1988; Piers *et al.*, 1994), lysozyme (Piers and Hancock, 1994) and chromogenic β-lactams such as pyridium-2-azo-p-dimethylanaline cephalothin (PADAC) and nitrocefin, which normally have limited access to periplasmic β-lactamase (Sawyer *et al.*, 1988; Lehrer *et al.*, 1989; Skerlavaj *et al.*, 1990). The ability of cationic peptides to promote their own uptake has not as yet been formally demonstrated, although this seems to be a plausible hypothesis.

It must be stated that ability to permeabilize the outer membrane to probe molecules is not equivalent to self-promotion of uptake. As discussed in detail by Vaara (1992), molecules such as the deacylated derivative of polymyxin B, polymyxin B nonapeptide (PMBN), are effective at permeabilizing outer membranes at concentrations orders of magnitude below the MIC. This probably reflects the inability of PMBN to transfer to and/or from channels in the cytoplasmic membrane, since its parent compound polymyxin B has a similar outer membrane permeabilizing concentration (0.3–1 μg/ml) but a far lower MIC (1 *vs.* $\geqslant$300 μg/ml). Consistent with this proposal, polymyxin B but not PMBN causes voltage-dependent channels in asymmetric planar bilayers (Schroeder *et al.*, 1992). Thus, it is not surprising that human defensins (which carry three to four net positive charges) permeabilize the outer membrane of *E. coli* at concentrations close to the minimal growth inhibitory concentration (Sawyer *et al.*, 1988; Vaara *et al.*, 1988). Similar results were observed for a cecropin–melittin hybrid peptide CEME (Piers and Hancock, 1994). Such results are consistent with the concept that self-promoted passage across the outer membrane is the rate-limiting step for many of these peptides. In the case of rabbit defensins, at low pH values which inhibited the killing of cells (Lehrer *et al.*, 1983), permeabilization

actually increased (Sawyer *et al.*, 1988). These data suggest a potential relevance *in vivo* for the phenomenon of permeabilization, because the release of defensins into phagocytic vacuoles containing bacteria, i.e. phagosomes, is accompanied by rapid vacuole acidification (Cech and Lehrer, 1984). Thus, permeabilization at low pH may be required to permit penetration of other potentially bactericidal substances that would otherwise be excluded by the outer membrane. Permeabilization may also have clinical relevance. Darveau *et al.* (1991) demonstrated that magainins were therapeutically ineffective against systemic *E. coli* infections, but worked synergistically with sub-inhibitory doses of the β-lactam antibiotic, cefepime.

Some cationic peptides are relatively ineffective against Gram-negative bacteria. Three of these, nisin, mastoparan and melittin, have been shown to have enhanced efficacy in wild-type cells after treatment of outer membranes with EDTA or, in two cases against mutant cells, with truncated lipopolysaccharides (Katsu *et al.*, 1985; Rana *et al.*, 1991; Stevens *et al.*, 1992). These treatments would be expected to enhance the accessibility of LPS-binding sites; indeed, melittin has been shown to bind with reasonably high affinity to lipid A ($K_d = 2.5 \times 10^{-6}$ M; David *et al.*, 1992) and LPS (Piers *et al.*, 1994). Thus, the reason for the limited or poor activity of these and similar compounds against Gram-negative bacteria is that they have limited ability to access divalent cation-binding sites on cell surface LPS and thus cannot initiate cooperative binding/permeabilization. A similar explanation was used to explain the generalized polycation resistance of *P. cepacia* (Moore and Hancock, 1986).

The intrinsic resistance of some strains of *S. typhimurium* to the peptides melittin and protamine has been found to be determined by an ATP-binding cassette (ABC) transporter SapABCDF encoded by the *sapABCDF* gene (Parra-Lopez *et al.*, 1993). The proteins SapBCDF are thought to be associated with the inner membrane while SapA is believed to be a periplasmic binding protein. Resistance requires the presence of all five subunits and is believed to be involved in transport of the toxic peptide to the cytoplasm.

4.5. Bacterial Cytoplasmic Membranes

The cytoplasmic membranes of Gram-negative bacteria have a major role in maintaining cytoplasmic integrity, vectorial transport of substrates into and out of the cytoplasm, exclusion of many non-substrate molecules, synthesis and export of molecules found external to the cytoplasmic membrane, generation and maintenance of cellular energization including synthesis of ATP and macromolecules, maintenance of a transmembrane proton gradient, and energization of transport (Cronan *et al.*, 1987). As described above,

cationic peptides form weakly anion-selective channels in planar lipid bilayers. Thus, it is no surprise that these peptides have a dramatic effect on bacterial cytoplasmic membrane integrity and that their antibacterial action probably, in part, reflects this, despite the rather complex phenotypic changes that arise from altered cytoplasmic membrane integrity.

Bacteria maintain across their cytoplasmic membranes a protonmotive force of approximately −170 mV (Bakker and Mangerich, 1981). According to Mitchell's chemiosmotic hypothesis, this protonmotive force comprises two individual forces that reflect the properties of protons, namely the electrical potential gradient, $\Delta\psi$, and ΔpH. These gradients are oriented so that the cytoplasm is negatively charged and alkaline (pH 7.8) relative to the external face of the cytoplasmic membrane. Treatment of cells with cations such as magainins (Juretic *et al.*, 1989; Westerhoff *et al.*, 1989), sarcotoxin (Okada and Natori, 1985b), insect defensin (Cociancich *et al.*, 1993a), nisin or pep5 (Kordel and Sahl, 1986) leads to dissolution of the $\Delta\psi$ as revealed by increased uptake of the lipid soluble cation triphenyl phosphonium. This apparently occurs at concentrations approaching the minimal effective concentration. The decrease in the protonmotive force is also manifest as an increased respiration rate as the cells attempt to compensate with an increase in respiration-driven proton pumping (Juretic *et al.*, 1989). This increased respiration rate occurs as a sigmoidal function of peptide concentration suggesting that magainins act in a cooperative fashion on cytoplasmic membranes. Another manifestation of this breakdown in cell integrity is K^+ leakage that has been shown to occur on treatment of Gram-positive bacteria with mastoparan or melittin (Katsu *et al.*, 1990). Mastoparan is relatively ineffective against Gram-negative bacteria. However, destruction of outer membrane integrity with EDTA causes a similar effect on susceptibility to killing and K^+ release in Gram-positive and Gram-negative bacteria (Katsu *et al.*, 1990). These data strongly support the hypothesis that loss of cytoplasmic membrane integrity is responsible for cell death, and that the resistance of Gram-negative bacteria to mastoparan is mediated by the outer membrane.

One phenomenon associated with cationic peptides is their generally weaker antibacterial activity at low pH (5.5) compared with that at mid-range pH values (7.5) (Lehrer *et al.*, 1983; Kordel *et al.*, 1988). This is probably not due to effects on the outer membrane, because Sawyer *et al.* (1988) demonstrated that the ability of rabbit defensins to permeabilize the outer membrane actually increases at low pH. One plausible hypothesis might relate to the relatively lower $\Delta\psi$ that exists at low pH, possibly due to a pH-sensitive K^+ pump which maintains the overall magnitude of the protonmotive force by causing a compensatory decrease in $\Delta\psi$ in response to an increase in ΔpH (Yamasaki *et al.*, 1980; Bakker and Mangerich, 1981). The $\Delta\psi$ in this case would be required for uptake of cationic peptides, in

keeping with the data discussed above. Similarly, the role of anaerobiosis and/or energy inhibitors in protecting bacterial cells (Walton and Gladstone, 1976) might reflect decreases in $\Delta\psi$. In the case of the model compound 48/80, it has been shown that membrane permeability changes occur only above the phase transition for *E. coli* lipids, a result consistent with reduced uptake of this polycation into gel-phase lipid bilayers (Katsu *et al.*, 1985).

Loss of cytoplasmic membrane integrity has also been followed by examining uptake of the normally excluded substrate ortho-nitrophenylgalactoside permitting cleavage by the cytoplasmic enzyme β-galactosidase in *E. coli*. However, this phenomenon, which occurs in cells treated with defensins (Lehrer *et al.*, 1989) and seminalplasmin (Sitaram *et al.*, 1992), has been demonstrated only at high concentrations of peptide and, in the case of defensins, after a considerable lag time. Thus it may not reflect a primary action of these cationic peptides. Similarly, the influence of cationic peptides on DNA, RNA and protein synthesis (Lehrer *et al.*, 1989) may reflect a decrease in cellular ATP levels or other secondary manifestations of ion leakage.

Seminalplasmin may lead to cellular lysis through activation of intrinsic autolysin activity (Chitnis *et al.*, 1990). Similar activation of autolytic activity has been observed after treatment with the polycationic antibiotics polymyxin B and the aminoglycosides (Nicas and Hancock, 1980) and with other cationic peptides (Piers *et al.*, 1994).

4.6. Effects on Mammalian Cells

Some of the antibacterial compounds have very weak activity against mammalian cells. Others are quite toxic. For example, indolicidin and, to a lesser extent, bactenecin are strongly toxic to rat and human T lymphocytes (Schluesener *et al.*, 1993). The determinative factor appears to be the ability to bind to (Steiner *et al.*, 1988) and/or enter into (Christensen *et al.*, 1988) the membranes of mammalian cells. In the latter case, the partitioning of peptides into membranes has been shown to depend on lipid charge, lipid composition (Christensen *et al.*, 1988; Sekharam *et al.*, 1991) and/or transmembrane potential (de Kroon *et al.*, 1991). Nevertheless, it is possible to design synthetic compounds with excellent antibacterial activity and weak haemolytic activity (Steiner *et al.*, 1988; Boman *et al.*, 1989). Of assistance may be a detailed comparison of the structural features of peptides that favour cytolytic activity (Kini and Evans, 1989). Another significant therapeutic consideration is the demonstrated ability of polycations to enhance phagocytosis of bacteria (Peterson *et al.*, 1984; Sawyer *et al.*, 1988), whereas the ability of those polycations to bind to lipid A/LPS makes them candidates as anti-endotoxins (since endotoxin = lipid A).

5. OUTLOOK

Cationic peptides represent the first novel antibiotic structures in 20 years. They have several features that confer some advantages over existing antibiotics. They are broad in their antimicrobial spectrum, including action against known antibiotic-resistant clinical isolates, do not induce resistant mutants at measurable frequencies, have bonus activities that include permeabilizer, anti-endotoxin and antifungal activities and can be manufactured recombinantly. We believe that the next 5–10 years will see the first marketing of these antibiotics, and that studies of structure–activity relationships will result in steady improvements of activity. These studies will expand beyond the β-structured and α-helical classes to other classes (loops, tryptophan or proline rich, etc.). Furthermore, intensive future studies should help to define what we believe will prove to be an important and currently undervaluated role in human non-specific defences against infection.

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Methylglyoxal and Regulation of its Metabolism in Microorganisms

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1. INTRODUCTION

Several environmental stresses are known to trigger intracellular alterations in organisms, such as synthesis of stress-inducible proteins or other cellular responses. Organisms of all types show the synthesis of stress-inducible

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proteins, and the most advanced understanding of such proteins has been obtained from the study of heat shock proteins (hsp). A sudden increase in temperature of the environment in which cells are growing induces increased synthesis of a set of heat shock mRNAs and proteins in the cells. When *Escherichia coli* cells are shifted from 30°C to 42°C, the intracellular concentration of σ^{32} increases 15- to 20-fold. The σ factor is one of the components of RNA polymerase of *E. coli* and substitution of a vegetative σ factor (σ^{70}) to σ^{32} changes the RNA polymerase to recognize specifically the hsp-encoding genes. In eukaryotes, a heat shock transcription factor, which is synthesized constitutively, is trimerized, modified and translocated by heat shock and binds to a heat shock element in the promoter region of heat shock genes to activate transcription (for reviews, see Lindquist, 1986; Lindquist and Craig, 1988; Morimoto *et al.*, 1990).

Intracellular stresses also affect the expression of hsp genes. For example, active oxygen species, which are generated during respiration in aerobic organisms, are known to induce the synthesis of some hsps. All aerobic organisms use molecular oxygen for respiration or the oxidation of nutrients to acquire the energy to live. Molecular oxygen is reduced to water through the acceptance of four electrons. During the reduction of molecular oxygen, several active oxygen species are formed, i.e. acceptance of one, two and three electrons to form, respectively, the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO·). Such reactive oxygens have been reported to be causative agents in several degenerative diseases (Ames, 1983; Ames *et al.*, 1993; Cerutti, 1985). Such species attack almost all cell components, DNA, protein and lipid membrane, and sometimes cause lethal damage to the cells. Both prokaryotic and eukaryotic cells have defensive mechanisms against such oxidative damage. *E. coli* and *Salmonella typhimurium* have the *oxyR*-controlled regulon of hydrogen peroxide-inducible genes (Christman *et al.*, 1989; Storz *et al.*, 1990). *E. coli* cells also have a *soxRS*-regulon which is induced by the superoxide radical (Greenberg *et al.*, 1990; Tsaneva and Weiss, 1990; Nunoshiba *et al.*, 1992; Wu and Weiss, 1991). The yeast *Saccharomyces cerevisiae* has a cytochrome *c* peroxidase (Yonetani, 1970) as well as superoxide dismutase-catalase systems. Occurrence of lipid hydroperoxides in membranes may be one of the major cases of oxidative damage to cells. *S. cerevisiae* has an oxidative stress-resistant gene as the mechanism for adaptation to oxidative stress caused by lipid hydroperoxides (Inoue *et al.*, 1993b, c). The yeast *Hansenua mrakii* has a membrane-bound glutathione peroxidase that is induced by lipid hydroperoxide, as well as active oxygen species such as superoxide radical and hydroxy radical, protecting the membrane phospholipids from peroxidation (Inoue *et al.*, 1990c, 1993c; Tran *et al.*, 1993a, b).

On the other hand, some normal metabolites possessing cytotoxicity affect the growth or proliferation of cells. Methylglyoxal is a typical cellular 2-oxoaldehyde. Formation of this compound occurs both enzymati-

cally and non-enzymatically, and the major precursors of methylglyoxal are dihydroxyacetone phosphate or glyceraldehyde-3-phosphate which are derived from glycolysis, a ubiquitous energy-generating system (Cooper and Anderson, 1970). Though methylglyoxal is a normal metabolite, it arrests the growth of various organisms at millimolar concentrations. Because the compound is toxic, its synthesis and degradative pathway must be strictly controlled in cells to avoid the overaccumulation of methylglyoxal. The study of metabolism of methylglyoxal was started in 1913.

Neuberg (1913) and Dakin and Dudley (1913a) independently found that methylglyoxal is metabolized to lactic acid (Fig. 1). Dakin and Dudley (1913b) named the enzyme catalyzing this reaction glyoxalase. In the 1920s, the enzyme was extensively studied; methylglyoxal and D-lactic acid were thought to be an intermediate of glycolysis. In 1932, Lohmann proved that methylglyoxal was not an intermediate of glycolysis. Because the glyoxalase required glutathione as a co-factor, however, glutathione did not affect the activity of enzymes involved in glycolysis (Lohmann, 1932).

Hopkins and Morgan (1945) suggested that glyoxalase would have some biological functions because this enzyme activity was distributed in various kinds of organisms. In the 1950s, Racker found that methylglyoxal is metabolized to D-lactic acid via *S*-D-lactoylglutathione (Racker, 1951, 1954) and that "glyoxalase" consisted of two enzymes, i.e. glyoxalase I (*S*-D-lactoylglutathione methylglyoxal-lyase; EC 4.4.1.5) and glyoxalase II (*S*-2-hydroxyacylglutathione hydrolase; EC 3.1.2.6).

In the mid-1960s, Szent-Gyorgyi and his co-workers proposed the hypothesis that the compounds involved in the glyoxalase system controlled the growth of cells (Egyud and Szent-Gyorgyi, 1966b; Szent-Gyorgyi *et al.*, 1967; Fodor *et al.*, 1967). Szent-Gyorgyi *et al.* thought that the glyoxalase enzymes might be growth-promoting. They found that six carbon-ketoaldehydes such as 2-keto-3-deoxyglucose are growth inhibiting, and thought that enzymes that release the cells from inhibition caused by ketoaldehydes would have growth-promoting activity. They studied the action of methylglyoxal and some of its derivatives on the division of bacteria, on cells in tissue culture and on fertilized sea urchin eggs, and found that in all cases cell division was inhibited by low concentrations of these chemicals. If 2-oxoaldehydes do act in the regulation of cell division, the glyoxalase system might do so. However, Otsuka and Egyud (1968) and Jellum (1968b) reported that 2-keto-3-deoxyglucose was not the natural substance in the cells and glyoxalase enzymes were not active toward this compound. Despite these negative data, it was believed that methylglyoxal would be concerned with the regulation of cell growth. In fact, Jerzykowski *et al.* reported that no glyoxalase II activity was detected in approximately two-thirds of the tumors studied, while in the remaining one-third it was lower compared with the activity in normal tissues (Jerzykowski *et al.*, 1975; Winter *et al.*, 1978).

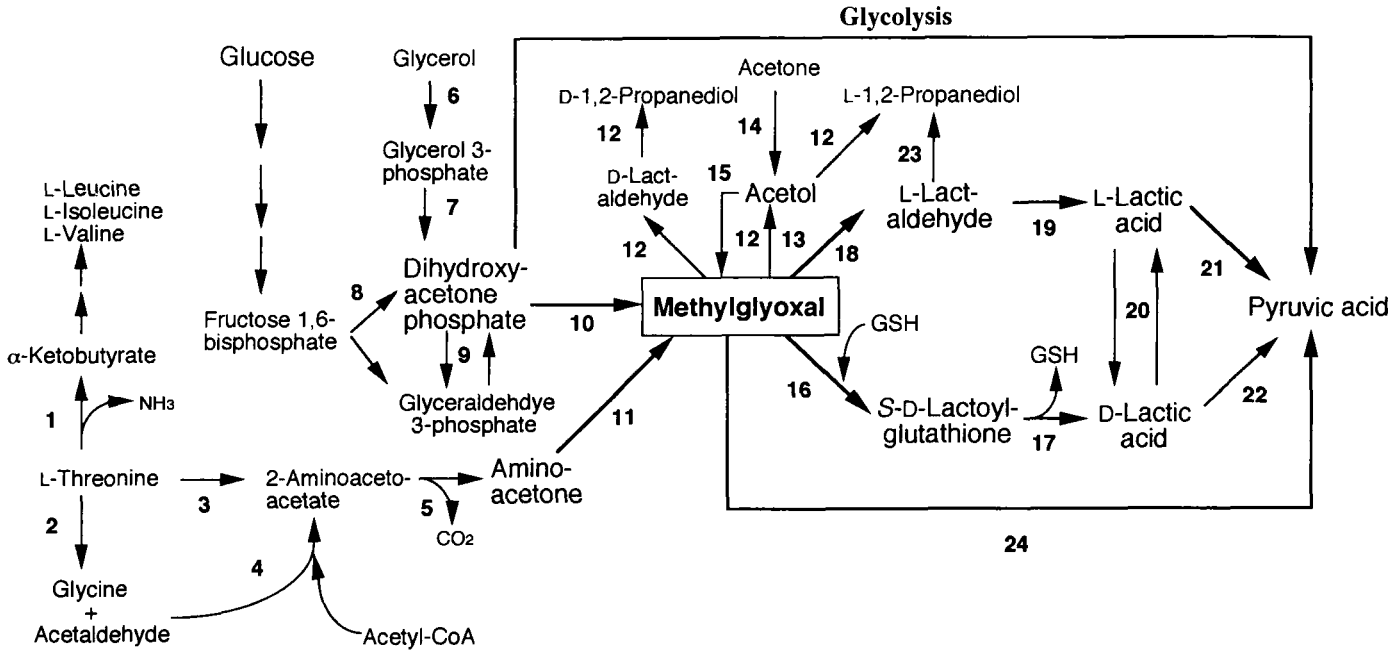


Figure 1 Metabolism of methylglyoxal. The enzymes and steps indicated by the numbers are: (1) threonine deaminase; (2) threonine aldolase; (3) threonine dehydrogenase; (4) aminoacetone synthase; (5) spontaneous decarboxylation; (6) glycerol kinase; (7) glycerol 3-phosphate dehydrogenase; (8) fructose 1,6-bisphosphate aldolase; (9) triosephosphate isomerase; (10) methylglyoxal synthase; (11) monoamine oxidase; (12) aldose reductase; (13) aldehyde reductase; (14) acetone monooxygenase; (15) acetol dehydrogenase; (16) glyoxalase I; (17) glyoxalase II; (18) methylglyoxal reductase; (19) lactaldehyde dehydrogenase; (20) lactate racemase; (21) L-lactate dehydrogenase; (22) D-lactate dehydrogenase; (23) propanediol oxidoreductase; (24) 2-oxoaldehyde dehydrogenase.

On the other hand, Gillespie hypothesized that the function of the glyoxalase system is to regulate the level of *S*-D-lactoylglutathione, a product of the glyoxalase I reaction, and provided evidence that an increased level of *S*-D-lactoylglutathione is related to the tumor-promoting properties of the compound (Gillespie, 1978, 1979, 1981). The growth-inhibiting effect of 2-oxoaldehydes and the growth-promoting effect of *S*-D-lactoylglutathione explain why inhibitors of glyoxalase I have been thought to be promising anti-cancer chemicals (Vince and Daluge, 1971; Vince *et al.*, 1971; Hall *et al.*, 1977). The carcinostatic activity of various glyoxal derivatives toward certain tumors and leukemia supports this possibility. Thus, regulation of the synthesis and degradation of methylglyoxal in mammalian, plant and microbial cells is of special interest, in particular in terms of the regulation of cell division. Recently, Thornalley *et al.* have studied the relationship between the metabolism of methylglyoxal and diabetes (Thornalley, 1990, 1993).

In this review, we will verify the multilateral effects of methylglyoxal, and discuss the biological significance and function of methylglyoxal with respect to metabolic pathways, regulation of enzyme activity and expression of the gene in some microorganisms. We will also describe the production of *S*-D-lactoylglutathione, an intermediate of the glyoxalase system, and its physiological activity.

2. PROPERTIES OF METHYLGLYOXAL

2.1. Formation

2.1.1. *Enzymatic Formation*

(a) *Formation by methylglyoxal synthase.* The major route for formation of methylglyoxal in microorganism cells is catalyzed by methylglyoxal synthase (EC 4.2.99.11). The process by which methylglyoxal is formed *in vivo* has been clarified by isolation of methylglyoxal synthase from *E. coli* (Hopper and Cooper, 1972), *Pseudomonas saccharophilia* (Cooper, 1974), *Proteus vulgaris* (Tsai and Gracy, 1976) and *S. cerevisiae* (Murata *et al.*, 1985c). The same enzyme activity was reported in goat liver (Ray and Ray, 1981). The properties of these enzymes have been well documented and are similar in terms of substrate specificity and susceptibility to inorganic phosphate (Pi). The synthase is specific for dihydroxyacetone phosphate and converts it to methylglyoxal without formation of the Schiff base (Hopper and Cooper, 1972). The relative molecular weight (M_r) of the enzyme varies depending on its source. The M_r of the methylglyoxal synthases from *E. coli* and *P. saccharophilia* is 67 000. The enzyme from *P. vulgaris* has an M_r of

135 000 and is composed of two identical subunits. The M_r of the *S. cerevisiae* enzyme is 26 000, significantly lower than the bacterial enzymes. The M_r of goat liver enzyme has not been reported.

Hopper and Cooper (1971, 1972) and Cooper (1984) discussed the function of methylglyoxal synthase in triosephosphate catabolism. They indicated that Pi regulates the diversion of triosephosphate to 1,3-diphosphoglycerate or methylglyoxal because methylglyoxal synthase is inhibited by a Pi concentration that is close to the K_m for the Pi used as the substrate for 3-phosphoglycerate dehydrogenase. Regulation by Pi is not likely in the case of the yeast enzyme because the methylglyoxal synthase from yeast cells is relatively insensitive to Pi, about 5 mM Pi being required to produce 50% inhibition of the enzyme *in vitro* (Murata *et al.*, 1985c).

(b) *Formation in threonine catabolism.* L-Threonine is oxidized to 2-aminoacetoacetate by NAD-dependent threonine dehydrogenase (EC 1.1.1.103), and the compound is spontaneously decarboxylated to give aminoacetone. L-Threonine is also degraded to glycine and acetaldehyde by the action of threonine aldolase (EC 2.1.2.1). Glycine formed is condensed with acetyl-coenzyme A to form 2-aminoacetoacetate by aminoacetone synthase, and the 2-aminoacetoacetone thus formed is decarboxylated to aminoacetone (Fig. 1).

Formation of methylglyoxal from aminoacetone in *Staphylococcus aureus* was suggested as part of a cycle for oxidation of L-threonine and glycine (Elliott, 1959). Similar enzymatic synthesis of methylglyoxal from aminoacetone was reported in pseudomonads (Higgins and Turner, 1969). *S. cerevisiae* cells degrade L-threonine added to the medium as a sole source of nitrogen (Murata *et al.*, 1986b). Concomitant with the disappearance of L-threonine from the medium, large amounts of aminoacetone are accumulated. Aminoacetone is converted to methylglyoxal by monoamine oxidase (EC 1.4.3.4). We found that aminoacetone accumulated in the medium during the catabolism of L-threonine by *S. cerevisiae* decreased rapidly; in parallel with this decrease of aminoacetone in the medium, glyoxalase I activity in yeast cells was found to increase approximately two-fold. One possible explanation of this phenomenon is that glyoxalase I was induced to obviate the cytotoxicity of methylglyoxal formed during the catabolism of L-threonine.

Oxidation of aminoacetone to methylglyoxal in mammals was reported by Elliot (1960) using ox plasma and by Ray and Ray (1983) using goat plasma; the reaction is catalyzed by plasma amine oxidase. Ray and Ray (1987) have also reported that methylglyoxal is synthesized from aminoacetone by aminoacetone oxidase in goat liver. The enzyme catalyzing this reaction is different from plasma amine oxidase, mitochondrial monoamine oxidases (Guha and Krishana, 1965; Nara *et al.*, 1966; Huang and Faulkner, 1981; Katz *et al.*, 1984) and diamine oxidases (Holtta *et al.*, 1973; Pegg and McGill, 1978) judging from the substrate specificity or behaviour to inhibitors.

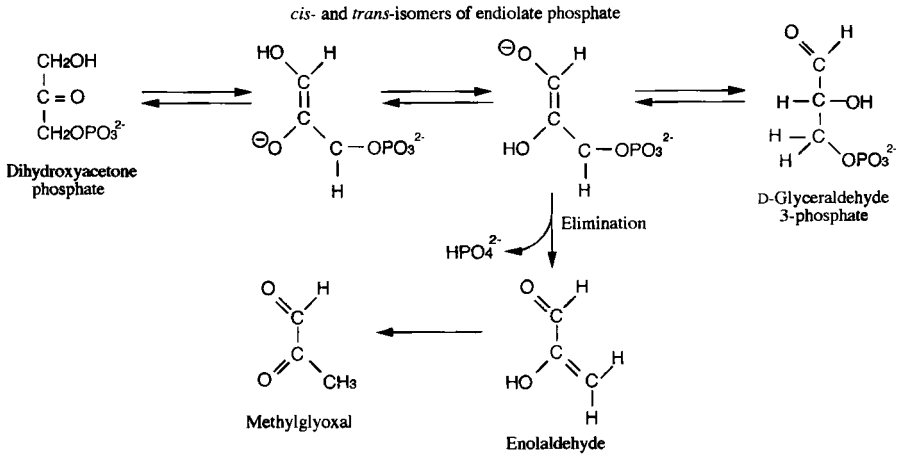


Figure 2 Synthesis of methylglyoxal in an elimination reaction by triosephosphate isomerase.

(c) *Formation by triosephosphate isomerase.* Triosephosphate isomerase (EC 5.3.1.1) catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. This enzyme is one of the components of glycolysis and is present in the cell at extremely high concentrations to maintain the large glycolytic flux to generate energy (ATP) (Shonk and Boxer, 1964). Endiol(ate) phosphate is synthesized as an intermediate by triosephosphate isomerase. This compound is highly labile and Pi is eliminated to give enolaldehyde that is then tautomerized to methylglyoxal (Fig. 2) (Iyengar and Rose, 1981; Richard, 1991). Although the elimination reaction is slow relative to the isomerization reaction, triosephosphate isomerase is an extremely effective catalyst so that the triosephosphate isomerase catalyzed-formation of methylglyoxal in the cells might have a physiological significance. Richard (1991, 1993) calculated the cellular formation of methylglyoxal in rat tissue to be 0.4 mM/day. Sato *et al.* (1980) have also reported that methylglyoxal is enzymatically synthesized from dihydroxyacetone phosphate or D-glyceraldehyde 3-phosphate using dialyzed rat liver homogenates. The major route of formation of methylglyoxal in microorganisms is catalyzed by methylglyoxal synthase, whereas methylglyoxal synthase activity was not reported in mammalian systems, except for one report of goat liver (Ray and Ray, 1981). Therefore, the elimination reaction by triosephosphate isomerase is considered to be the primary source of methylglyoxal in mammalian cells.

(d) *Formation of fructose 1,6-bisphosphate aldolase.* Grazi and Trombetta (1978) found that methylglyoxal and Pi were formed slowly from dihydroxyacetone phosphate when the compound was incubated with rabbit muscle fructose 1,6-bisphosphate aldolase (EC 4.1.2.13). Iyengar and

Rose (1981) also confirmed the formation of methylglyoxal and Pi from dihydroxyacetone phosphate by rabbit muscle fructose 1,6-bisphosphate aldolase, whereas yeast aldolase did not catalyze this reaction.

(e) *Formation in acetone metabolism.* It is well known that acetone is used by living systems. Sakami proposed two different pathways: one route involves degradation of acetone to formate and acetate (Sakami, 1950), and the other route is conversion of acetone to a 3-carbon gluconeogenic precursor (Sakami and Lafaye, 1951). Casazza *et al.* (1984) proposed that acetone can be oxidized to methylglyoxal via acetol (1-hydroxyacetone) by acetone-inducible monooxygenase in rat liver microsomes. Both reactions required oxygen and NADPH. Reichard *et al.* (1986) have also suggested oxidation of acetone to methylglyoxal by acetone-inducible cytochrome P-450 monooxygenase in the hepatic pathway of mammals, including man (Fig. 1).

Taylor *et al.* (1980) reported that acetol was oxidized to methylglyoxal by acetol dehydrogenase in the presence of NAD using cell extracts of Gram-positive bacteria isolated from soil. The enzyme was induced approximately 20-fold when the cells were cultured in acetone-containing medium. Since the enzyme was labile, the enzyme was not purified. On the other hand, we found that methylglyoxal could be converted to acetol by NADH-dependent aldehyde reductase of the yeast *H. mrakii* and the enzyme was partially purified. However, the reaction catalyzed by the enzyme was irreversible and formation of methylglyoxal from acetol by the enzyme was not observed (Inoue *et al.*, 1992a). This enzyme activity has not been detected so far in the yeast *S. cerevisiae*.

2.1.2. *Non-enzymatic Formation*

(a) *Formation from D,L-glyceraldehyde and dihydroxyacetone.* Dische and Robbins (1934) failed to obtain appreciable conversion of glyceraldehyde or dihydroxyacetone to methylglyoxal in the presence of 0.067 M neutral phosphate. On the other hand, Needham and Lehmann (1937) reported that methylglyoxal is formed non-enzymatically from D,L-glyceraldehyde or dihydroxyacetone in the presence of Ringer's bicarbonate buffer. Meyer (1953) demonstrated that non-enzymatic formation of methylglyoxal from glyceraldehyde was not accelerated by the addition of muscle extracts. Riddle and Lorenz (1968) reported that methylglyoxal is formed from dihydroxyacetone and from D,L-glyceraldehyde at physiological pH and temperature, catalyzed by Tris and by certain polyvalent anions such as phosphate and tetraborate. Biological substances such as glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-bisphosphate also catalyzed the formation of methylglyoxal from these trioses.

(b) *Formation from L-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate.* L-Glyceraldehyde 3-phosphate spontaneously isomerizes to

dihydroxyacetone phosphate through an enediolate intermediate, which gives enolaldehyde, and then methylglyoxal and Pi (Richard, 1984). Richard (1991, 1993) estimated that the rate of the spontaneous elimination reaction of L-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate to form methylglyoxal was $v_N = 0.1$ mM/day in rat tissue. As described in the section on triosephosphate isomerase, he estimated the rate of enzymatic formation of methylglyoxal (v_E) from these triosephosphates to be 0.4 mM/day. The calculated value of $v_E/v_N = 4.0$ shows the similarity in the rates of enzymatic formation and spontaneous formation of methylglyoxal from D-glyceraldehyde 3-phosphate ($v_E/v_N = 2.8$) and from dihydroxyacetone phosphate ($v_E/v_N = 8.8$) in dialyzed whole-cell homogenates of rat liver (Sato *et al.*, 1980).

(c) *Formation in glycerol metabolism.* In metabolism, glycerol is phosphorylated to glycerophosphate by glycerol kinase, and the compound is converted to dihydroxyacetone phosphate (Fig. 1). This triosephosphate is dephosphorylated by phosphatase to give dihydroxyacetone. From these, dihydroxyacetone phosphate and dihydroxyacetone, methylglyoxal may be formed by non-enzymatic reactions. Therefore, if cells are exposed to glycerol-rich conditions, the flux to methylglyoxal inevitably increases and several physiological effects are observed. For example, respiration of avian spermatoza is inhibited by incubation with glycerol or dihydroxyacetone, and methylglyoxal is accumulated (Riddle and Lorenz, 1973). Growth arrest of a glyoxalase I-deficient mutant of *S. cerevisiae* in glycerol medium was supposedly due to the accumulation of toxic levels of methylglyoxal in cells (Penninckx *et al.*, 1983). On the other hand, glyoxalase I activity in *S. cerevisiae* cells increased several-fold when the cells were cultured in glycerol medium (Kosugi *et al.*, 1988). It is interesting that methylglyoxal fed in the medium arrests the growth of yeast but that methylglyoxal formed *in vivo* induces the glyoxalase I activity.

In *E. coli*, glycerol kinase activity is negatively regulated by fructose 1,6-bisphosphate (Zwaig and Lin, 1966). A mutant which is insensitive to this feedback regulation shows a lethal phenotype when cultured in glycerol medium (Zwaig *et al.*, 1970). In such a mutant, the rate of glycerol metabolism is much faster than in wild-type cells and over-accumulation of dihydroxyacetone phosphate in the cells is expected. Hopper and Cooper (1971) reported that methylglyoxal synthase was inhibited by 1 mM Pi but the inhibition could be overcome by excess dihydroxyacetone phosphate. Therefore, growth inhibition by glycerol of the desensitized mutant of glycerol kinase was due to the large flux of glycerol to methylglyoxal by both enzymatic and non-enzymatic pathways. Krymkiewicz *et al.* (1971) identified a lethal product in a glycerol kinase mutant of *E. coli* as methylglyoxal.

(d) *Formation from lipid hydroperoxides.* Intracellular lipid hydroperoxides are synthesized non-enzymatically by several reactive oxygen species such as hydroxy radical (HO·) or hydroperoxy radical (HOO·). Lipid

hydroperoxides are also synthesized enzymatically by the action of lipoxygenase. Lipid hydroperoxides are degraded in the presence of transition metal ions to yield radicals such as alkoxy and peroxy radicals, and also aldehydes and ketones. We screened several yeast strains that showed resistance to lipid hydroperoxides and found that *H. mrakii* IFO 0895 was highly resistant to linoleic acid hydroperoxide (Inoue *et al.*, 1990c). The yeast also showed resistance to methylglyoxal (Inoue *et al.*, 1991a); it could grow in medium containing methylglyoxal up to 25 mM, whereas the growth of *S. cerevisiae* was inhibited at 2 mM (Murata *et al.*, 1986b). Malondialdehyde is one of the well-known degradation products of lipid hydroperoxide, although direct evidence for the formation of methylglyoxal from lipid hydroperoxide has not been suggested.

2.2. Toxicity, Mutagenicity and Carcinogenicity of Methylglyoxal

2.2.1. *Toxicity*

(a) *Methylglyoxal and nucleic acids.* Staehelin (1959) first reported that 2-oxoaldehydes such as glyoxal and kethoxal (β-ethoxy-α-ketobutyraldehyde) react with RNA of tobacco mosaic virus and inactivate it. Tiffany *et al.* (1957) noted that these chemicals, as well as methylglyoxal, had anti-viral activity. Interaction between glyoxal and DNA was reported by several investigators (Brooks and Klamerth, 1968; Nakaya *et al.*, 1968), and interaction between glyoxal and guanine residues in RNA was also reported by Kochetkov *et al.* (1967). Interaction of kethoxal and yeast tRNA was also extensively studied and specific introduction of α-ethoxypropionyl groups onto the guanine amino groups of RNA was demonstrated (Litt and Hancock, 1967; Litt, 1969, 1971; Litt and Greenspan, 1972; Shapiro *et al.*, 1970). Methylglyoxal reacts with guanine residues in both DNA and RNA to form an adduct, N^2-alkylguanine (3,5,6,7)-tetrahydro-6,7-dihydroxy-6-methylimidazo[1,2,-α]purine-9(8H)one (Krymkiewicz, 1973; Shapiro *et al.*, 1969).

(b) *Inhibition of protein synthesis by methylglyoxal.* Egyud and Szent-Gyorgyi (1966a, b) reported that methylglyoxal inhibits protein synthesis. Zwaig and Dieguez (1970) found that bactericidal products of a mutant of *E. coli*, which was insensitive to feedback inhibition of glycerol kinase by fructose 1,6-bisphosphate, inhibited protein synthesis *in vivo*. The bactericidal product was later identified as methylglyoxal. Krymkiewicz *et al.* (1971) reported that methylglyoxal strongly inhibited synthesis of DNA, RNA and protein *in vivo*. They also demonstrated inhibition of protein synthesis *in vitro* by methylglyoxal. They observed that methylglyoxal reacts with guanine residues of nucleic acids and with GTP, but not with ATP, to inhibit synthesis of macromolecules.

(c) *Modification of proteins.* Interactions of 2-oxoaldehydes, such as glyoxal and methylglyoxal, with proteins are also one of the causes of cytotoxicity of these compounds (Abdulnur, 1976). Pethig (1978) reported a complex between methylglyoxal and casein; incorporation of methylglyoxal into protein produced a charge-transfer interaction and resulted in the creation of mobile "electron holes". Gascoyne (1980) also reported the formation of a complex between methylglyoxal and bovine serum albumin. Riordan *et al.* (1977) reported that all glycolytic enzymes, except for triosephosphate isomerase, have arginine residues in their catalytic sites and that those enzymes were rapidly inactivated by methylglyoxal or 2,3-butanedione. The inactivation was presumably due to interaction between the guanidino group of arginyl residues and methylglyoxal. Indeed, methylglyoxal has been shown to react with arginine and arginyl residue in proteins (Takahashi, 1977a, b; Cheung and Fonda, 1979). Selwood and Thornalley (1993) showed that incubation of [^{14}C]methylglyoxal and blood plasma for 3 weeks led to the labeling of plasma proteins; about 50% of the methylglyoxal existed in a protein-bound form.

(d) *Glycation of protein.* Non-enzymatic modification of proteins with sugar (glycation) is distinguished from enzyme-catalyzed protein glycosylation. The Maillard reaction *in vivo* is hypothesized to contribute to the glycation or cross-linking of several proteins; long-lived proteins such as collagen and lens crystallin in particular are highly glycated in diabetes and aging (Monnier and Cerami, 1981; Kohn and Monnier, 1987; Vlassara *et al.*, 1987). Glycation of proteins causes a reduction or loss of biological activity. For example, the activity of superoxide dismutase, myosin-ATPase, RNase, *N*-acetylglucosaminidase and catepsine B is reduced by glycation. On the contrary, the activity of some proteins such as aldose reductase, alcohol dehydrogenase and NADPH-dependent 2-oxoaldehyde reductase is elevated by glycation, supposedly due to an increase of affinity of substrate and enzyme (Hayase, 1993).

Lysine residues of proteins are modified by 5-hydroxymethyl-1-alkylpyrrole-2-carboaldehyde (pyrraline), which is formed from 3-deoxyglucosone, a product of the Maillard reaction, to give an adduct of protein and the pyrraline (Hayase *et al.*, 1989). Miyata and Monnier (1992) demonstrated that a rat treated with streptozotocin to induce diabetes showed a threefold higher level of pyrraline, suggesting that, in diabetes, the advanced stage of the Maillard reaction progressed much faster. Vander Jagt *et al.* (1992) compared the abilities of glucose, methylglyoxal and acetol to produce covalent modification of albumin, and found that methylglyoxal and acetol were much more reactive than glucose. Thornalley (1988) reported that incubation of red blood cells with glucose *in vitro* led to an increase in flux to D-lactic acid via methylglyoxal and *S*-D-lactoylglutathione. Therefore, in diabetes a flux to 3-deoxyglucosone and its derivative (pyrraline) or methylglyoxal and its reductant (acetol) from glucose may increase, and cellular proteins are

subsequently glycated by these carbonyl compounds to reduce or lose biological activity.

(e) *Radical formation from methylglyoxal.* Gascoyne *et al.* (1982) reported free radical-producing reactions with cysteine and dicarbonyl compounds such as glyoxal and methylglyoxal, and identified the radical as complex substituted pyrazine cation radicals by electron spin resonance.

(f) *Carcinostatic activity of methylglyoxal.* Methylglyoxal is a growth-inhibitory substance; Szent-Gyorgyi and his co-workers were among the first to consider its biological effects (Egyud and Szent-Gyorgyi, 1966a). Growth of *E. coli* was inhibited by 0.2–1.0 mM methylglyoxal and its effect was supposed to be due to inhibition of protein synthesis, as described above. Effects of methylglyoxal on tumor growth were extensively studied by Apple and Greenberg (1967). Mice were inoculated with tumor cells and were then given an intraperitoneal injection of methylglyoxal. Methylglyoxal inhibited growth of tumor cells by 90–99%. Egyud and Szent-Gyorgyi (1968) also reported that Swiss albino mice inoculated intraperitoneally with sarcoma 180 can be cured by intraperitoneal injection of methylglyoxal. However, Jerzykowski *et al.* (1970) reported that methylglyoxal was relatively ineffective against L-1210 leukemia.

Egyud and Szent-Gyorgyi (1966a, 1968) found that cancer cells in tissue culture were more sensitive to methylglyoxal than were normal cells, raising the possibility of using methylglyoxal for cancer chemotherapy. Detoxification of methylglyoxal to D-lactic acid via *S*-D-lactoylglutathione is catalyzed by glyoxalase I and glyoxalase II. Some investigators, however, reported that the activity of glyoxalase I in tumor tissues is higher, lower or about the same level as in corresponding normal tissues. Glyoxalase II activity is markedly lower in tumors than normal tissues (Jerzykowski *et al.*, 1975, 1978b; Hopper *et al.*, 1987, 1988a, b; Thornalley and Bellavite, 1987).

2.2.2. *Mutagenicity and Carcinogenicity*

(a) *Mutagenicity of methylglyoxal.* Mutagenicity of several dicarbonyl compounds was studied by Nagao *et al.* (1986). Among the dicarbonyls tested, 1 mg of methylglyoxal without S9 mix (a supernatant fluid obtained by centrifuging liver homogenate at 9000 *g* for 10 min) induced 100 000 revertants of *S. typhimurium* TA100 in an Ames test (Ames *et al.*, 1975). Mutagenicity of other dicarbonyl compounds was as follows: glyoxal, 20 000 revertants/mg; ethylglyoxal, 25 000; propylglyoxal, 35 000; diacetyl, 400; and acetol, 0, respectively. Bieldanes and Chew (1979) reported that glyoxal and diacetyl showed mutagenicity, whereas Kasai *et al.* (1982) suggested that mutagenicity of commercially available acetol was due to contamination with small amounts of methylglyoxal.

Mutagenicity of methylglyoxal was abolished by the addition of S9 mix. This was presumably due to action by enzymes of the glyoxalase system in S9 mix.

(b) *Methylglyoxal and other carbonyl compounds in food.* Methylglyoxal has been detected in various foods and beverages such as bread (Wiseblatt and Kohn, 1960), roast turkey (Hrdlicka and Kuca, 1965), boiled potato (Kajita and Senda, 1972), whiskey (Sugimura and Sato, 1983), tea (Nagao *et al.*, 1979) and coffee (Kasai *et al.*, 1982). Occurrence of methylglyoxal in instant coffee increased when it was roasted under aerobic conditions (Y. Suwa, PhD thesis, Shizuoka Prefectural University, 1993). Besides methylglyoxal, instant coffee contains several dicarbonic compounds such as 2,3-pentanedione, 1-phenyl-1,2-propanedione, 3,4-hexanedione, 2,3-hexanedione and 1,2-cyclohexanedione. These compounds showed mutagenicity to *S. typhimurium* TA100 without S9 mix. Non-mutagenic dicarbonyl compounds were also detected in instant coffee, such as 3-methyl-1,2-cyclopentanedione, 2,5-hexanedione and 2,4-pentanedione.

Mutagenicity of methylglyoxal in coffee has been well documented by Sugimura and his co-workers (Nagao *et al.*, 1986). Both instant and freshly brewed coffee induce revertants of *S. typhimurium* TA100, TA102, TA104 and *E. coli* WP2 *uvrA*/pKM101 without metabolic activation by S9 mix (Nagao *et al.*, 1984). Methylglyoxal also induces λ prophage lysogenized in *E. coli* (Kosugi *et al.*, 1983). These genotoxic activities were provoked by extracts of roasted coffee beans, whereas the extracts of green coffee beans show no genotoxicity. Because the mutagenicity of the extracts of roasted coffee beans was suppressed by sulfite and bisulfite (Suwa *et al.*, 1982), the mutagen was supposed to be a carbonyl compound such as an aldehyde or methylethyl ketone. One gram of the powder of instant coffee and roasted coffee beans contains 210 μg and 167 μg of methylglyoxal, respectively (Sugimura and Sato, 1983). Mutagenicity of the extracts of coffee was also suppressed by a cytosol fraction of rat liver (Nagao *et al.*, 1984). Mutagenicity-inactivating factor was partially purified from the rat liver and shown to possess catalase activity (Fujita *et al.*, 1985a). Commercial catalase (bovine liver) and peroxidase (horseradish) also suppressed the mutagenicity of the extracts of coffee; however, methylglyoxal itself was not degraded by either catalase or peroxidase. Therefore, instant coffee and roasted coffee beans may contain hydrogen peroxide, or hydrogen peroxide-generating systems, which enhance the mutagenicity of methylglyoxal. In fact, hydrogen peroxide enhanced the mutagenicity of methylglyoxal approximately 30-fold (Fujita *et al.*, 1985b). Methylglyoxal is known to react with guanine residues of DNA and RNA (see section 2.2.1). Nukaya *et al.* (1990) reported that incubation of 2′-deoxyguanosine with methylglyoxal and hydrogen peroxide produced N^2-acetyl-2′-deoxyguanosine *in vitro*. No appreciable modification of other deoxynucleosides was detected by incubation with

methylglyoxal and hydrogen peroxide. Instant coffee also produced N^2-acetyl-2′-deoxyguanosine by incubation with 2′-deoxyguanosine. In summary, mutagenicity of methylglyoxal in coffee is enhanced by hydrogen peroxide, generated by an unknown system in coffee, and its effect is expressed through the formation of N^2-acetyl-2′-deoxyguanosine from 2′-deoxyguanosine.

(c) *Carcinogenicity of methylglyoxal.* Subcutaneous injection of methylglyoxal (0.2 ml of 10 mg/ml in saline solution, twice a week for 10 weeks) to Fischer 344 rats induced tumors at the injection site after 20 months. The tumors were histologically identified as fibrosarcomas (Nagao *et al.*, 1986).

3. METABOLISM OF METHYLGLYOXAL

3.1. Glyoxalase I

Conversion of methylglyoxal to D-lactic acid via *S*-D-lactoylglutathione is catalyzed by a system comprising glyoxalase I and glyoxalase II in the presence of glutathione. Methylglyoxal is non-enzymatically condensed with glutathione to give an adduct, hemimercaptal (hemithioacetal), which is the intrinsic substrate for glyoxalase I (Fig. 3). The dissociation constant (K_d) for hemimercaptal is 3×10^{-3} M (Vander Jagt, 1993). Concentrations of methylglyoxal in cells are estimated to be in the range 0.1–2 μM (Ohmori *et al.*, 1987a; Thornalley and Tisdale, 1988), whereas intracellular concentrations of glutathione are in the millimolar range; thus the fraction of methylglyoxal that exists as hemimercaptal is dependent on the glutathione concentration.

In our laboratory, systematic studies of methylglyoxal-metabolizing enzymes in microorganisms have been conducted. Because methylglyoxal is cytotoxic, bacteria such as *E. coli* or *Pseudomonas putida* cannot grow in medium containing 1.0–1.2 mM methylglyoxal (Egyud and Szent-Gyorgyi, 1966a; Rhee *et al.*, 1987a). We found that the yeast *H. mrakii* IFO 0895 was highly resistant to methylglyoxal and was able to grow in a medium containing up to 25 mM methylglyoxal (Inoue *et al.*, 1991a). Growth of *S. cerevisiae* is completely arrested in 2.0 mM methylglyoxal-containing medium and cells finally die (Murata *et al.*, 1986b). Specific activity of glyoxalase I in cell extracts of *H. mrakii* (1.5–2.1 units/mg protein) (Inoue *et al.*, 1991a) was higher than in *S. cerevisiae* (0.24 units/mg protein) (Kosugi *et al.*, 1988), *Aspergillus niger* (0.026 units/mg protein) (Inoue *et al.*, 1987), *P. putida* (0.14 units/mg protein) (Rhee *et al.*, 1986) and *E. coli* (0.012 units/mg protein) (Rhee *et al.*, 1987a).

Glyoxalase I is widely distributed in organisms (Hopkins and Morgan, 1945; Jerzykowski *et al.*, 1978b) and has been purified from various sources,

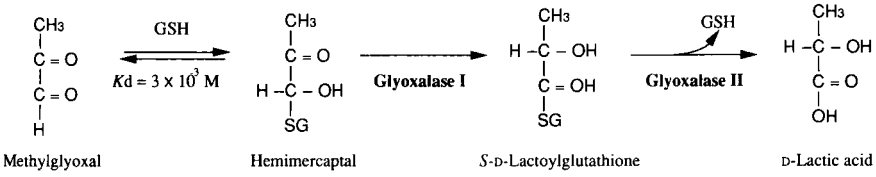


Figure 3 Reaction of glyoxalase system. Methylglyoxal non-enzymatically reacts with glutathione (GSH) to form an adduct hemimercaptal. The compound is an intrinsic substrate for glyoxalase I, and *S*-D-lactoylglutathione formed is hydrolyzed to glutathione and D-lactic acid.

from mammals (Aronsson and Mannervik, 1977; Mannervik *et al.*, 1972; Marmstal and Mannervik, 1979; Uotila and Koivusalo, 1975) to microorganisms (Inoue *et al.*, 1987, 1991a; Marmstal *et al.*, 1979; Rhee *et al.*, 1986). Mammalian glyoxalase I enzymes are dimeric with molecular masses varying from 43 to 48 kDa. On the other hand, microbial glyoxalase I enzymes are monomeric and molecular masses vary from 19 to 38 kDa. Douglas *et al.* (1986) suggested that yeast glyoxalase I contained approximately 0.75% (w/v) carbohydrate.

Each subunit of mammalian glyoxalase I contains one atom of zinc (Aronsson *et al.*, 1978; Marmstal *et al.*, 1979). The zinc ion is located in the catalytic site of the enzyme and can be replaced by other divalent metal ions, such as Mg^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} (Uotila and Koivusalo, 1975; Han *et al.*, 1977; Sellin and Mannervik, 1984; Sellin *et al.*, 1983; Aronsson *et al.*, 1981). The metal ion in the active site is suggested to be coordinated to two nitrogen atoms and to four oxygen atoms (Sellin *et al.*, 1987), although the amino acid residues involved in the coordination were not identified (Mannervik and Ridderstrom, 1993).

The activity of glyoxalase I purified from *S. cerevisiae* was inhibited by EDTA, and the activity of EDTA-inhibited enzyme was restored partially by Mg^{2+} or Ca^{2+}, and slightly by Mn^{2+} (Murata *et al.*, 1986c). Fe^{2+}, Ni^{2+} and Co^{2+} were all without effects on reactivation. Glyoxalase I purified from *P. putida* (Rhee *et al.*, 1986) was not inhibited by EDTA. The enzyme from the yeast *H. mrakii* was also insensitive to divalent metal ion chelators such as EDTA, 1,2-cyclohexane-diaminetetraacetic acid and 8-hydroxyquinoline (Inoue *et al.*, 1991a). As with other microbial glyoxalase I enzymes, the activity of the *A. niger* enzyme was inhibited by Zn^{2+} (Inoue *et al.*, 1987), but protected by the addition of an equimolar amount of EDTA. The reaction of glyoxalase I from this fungus was inhibited by EDTA, and the inhibition was found to be competitive against hemimercaptal (K_i = 1.3 mM), perhaps due to the structural similarity between hemimercaptal and EDTA. Douglas and Shinkai (1985) suggested a model for the reaction of glyoxalase I, in which two oxygen atoms of hemimercaptal, at the positions C-1 and C-2,

chelate Zn^{2+} in the enzyme with the subsequent isomerization of hemimercaptal. EDTA has a similar structure with oxygen atoms able to chelate divalent metal ions. Therefore, EDTA may compete with hemimercaptal at the active site of the mold glyoxalase I. Mannervik *et al.* (1972) suggested that glyoxalase I from mammals contained Zn^{2+} and that the inhibitory effect of EDTA was due to removal of Zn^{2+}; the EDTA-inhibited enzyme could be reactivated by Zn^{2+} and/or other divalent metal ions. Glyoxalase I from *A. niger*, however, was not found to be inactivated either by preincubation with EDTA or by dialysis against buffer containing EDTA. The *A. niger* enzyme, as well as the bacterial (*P. putida*) and yeast (*H. mrakii*) enzymes, may not contain Zn^{2+} in their active sites, or Zn^{2+} might be tightly associated with the enzyme.

Inhibitors of glyoxalase I have been sought as anticancer agents (Kermack and Matheson, 1957; Merster *et al.*, 1974; Kurasawa *et al.*, 1975; Vince and Daluge, 1971; Vince *et al.*, 1973; Phillips and Norton, 1975). Activators of glyoxalase I have not been described. The identification of such activators would give much information about the function of the enzyme. Interestingly, the glyoxalase I from *S. cerevisiae* was activated by ferrous ions and polyamines (Murata *et al.*, 1986c). An approximately six-fold increase in activity was observed in the presence of 4 mM Fe^{2+} or 40 mM spermine. Glyoxalase I from *A. niger* was also activated by Fe^{2+} (Inoue *et al.*, 1987), although the mechanism for activation by these chemicals is not clear. The effect of polyamines on glyoxalase I activity is of physiological interest, because both glyoxalase I and polyamines have been supposed to function in the regulation of cell division.

3.2. Glyoxalase II

Glyoxalase II is an alternative enzyme in the glyoxalase system. It catalyzes the hydrolysis of *S*-D-lactoylglutathione, formed by the reaction of glyoxalase I, to glutathione and D-lactic acid (Fig. 3). Glyoxalase II enzymes have been purified from various sources, such as mammals, birds, fish, snakes, frogs, plants and yeasts (Uotila, 1973a, b, 1979; Ball and Vander Jagt, 1979; Oray and Norton, 1980; Principato *et al.*, 1984, 1985, 1987a, b; Murata *et al.*, 1986a; Talesa *et al.*, 1988, 1989; Inoue and Kimura, 1992b). Mammalian enzymes are distributed in various organs, such as brain and liver and in erythrocytes. The enzymes are found in mitochondria as well as the cytosol, although other organelles are unlikely to contain the enzyme (Jerzykowski *et al.*, 1978a; Uotila, 1989; Talesa *et al.*, 1988). The molecular masses of glyoxalase II are in the range 18–30 kDa. The specific activity of the enzyme is approximately 600–900 μmol/min/mg protein, except for the yeast (*S. cerevisiae*, 1.34 μmol/min/mg protein; *H. mrakii*, 31.9 μmol/min/mg protein) enzymes (Murata *et al.*, 1986a; Inoue and Kimura, 1992b). The K_m value for

S-D-lactoylglutathione is in the range 86–450 μM, except for an extraordinarily low K_m value of the enzyme from *S. cerevisiae* (Murata *et al.*, 1986a). The values of k_{cat} of calf brain and rat erythrocytes were estimated to be $16 \times 10^3\,min^{-1}$ and $17 \times 10^3\,min^{-1}$, respectively, and $k_{cat.}/K_m$ values were about $10^8\,M^{-1}\,mm^{-1}$, suggesting that the hydrolysis of *S*-D-lactoylglutathione by glyoxalase II is diffusion-limited (Guha *et al.*, 1988).

Glyoxalase II can hydrolyse a wide variety of *S*-hydroxyacylglutathione derivatives such as *S*-acetyl, *S*-lactoly, *S*-glycol, *S*-mandelyl, *S*-glycerol and *S*-acetoacetylglutathiones. Glyoxalase II from *S. cerevisiae* was, however, specific to *S*-D-lactoylglutathione. *S. cerevisiae* has other enzymes that are active on *S*-D-lactoylglutathione as well as *S*-acetylglutathione, and one of these enzymes was purified (Murata *et al.*, 1987). The enzyme (P3) showed group-transfer activity and catalyzed a reaction forming acetyl-coenzyme A from *S*-acetyglutathione and coenzyme A. The *S*-D-lactoylglutathione-hydrolyzing activity was not resolved into more than one peak during the purification from the yeast, *H. mrakii* (Inoue and Kimura, 1992b; Inoue *et al.*, 1993a).

Glyoxalase II is inhibited by many derivatives of glutathione (Ball and Vander Jagt, 1979, 1981; Seddon *et al.*, 1980; Hsu and Norton, 1983; Bush and Norton, 1985). Among them *S*-(substituted-carbobenzoxy)glutathione derivatives are the strongest competitive inhibitors, suggesting the occurrence of hydrophobic substrate-binding sites in glyoxalase II (Al-Timari and Douglas, 1986; Principato *et al.*, 1989). Hemimercaptal, a non-enzymatic condensation product between methylglyoxal and glutathione, inhibited glyoxalase II (Uotila, 1973b; Oray and Norton, 1980; Rae *et al.*, 1990, 1991; Murata *et al.*, 1986a; Inoue and Kimura, 1992b). Yeast glyoxalase II enzymes were also inhibited by hemimercaptal, although the activity of P3 enzyme, which hydrolyzed *S*-D-lactoylglutathione, was not affected by hemimercaptal (Murata *et al.*, 1987).

Chemical modifications of mammalian glyoxalase II have revealed that histidine, arginine and lysine residues are concerned with activity (Principato *et al.*, 1985; Ball and Vander Jagt, 1981; Uotila, 1973b). A possible mechanism of action of glyoxalase II was proposed (Thornalley, 1990; Vander Jagt, 1993). A histidine residue at the active site attacks *S*-D-lactoylglutathione and a covalent enzyme (E)-substrate (S) intermediate is formed. The positive charge of the guanidino group of an arginine residue and the amino group of a lysine residue supports the binding of substrate and the enzyme. Glutathione is liberated, and lactoyl-histidine is then hydrolyzed to D-lactic acid.

3.3. Methylglyoxal Reductase

Methylglyoxal-reducing activity in the presence of NADH was first reported by Ting *et al.* (1965) in rat liver homogenates and was designated

D-lactaldehyde dehydrogenase (D-lactaldehyde:NAD^+ oxidoreductase, EC 1.1.1.78). Later, Ray and Ray (1984) reported the enzyme catalyzing the reduction of methylglyoxal to lactaldehyde from goat liver, and these enzymes were distinguished from NADPH-linked aldehyde reductase (Bosron and Prairie, 1972; Davidson and Flyn, 1979; Tulsiani and Touster, 1977). The enzyme activity found in rat liver homogenates preferentially catalyzes the reduction of 2-oxoaldehydes to yield the corresponding 2-hydroxyaldehydes, whereas NADPH-linked aldehyde reductases reduce aldehydes to give alcohols (Fig. 1).

Methylglyoxal reductases purified so far have been mainly obtained from microbial sources, such as *E. coli* (Saikusa *et al.*, 1987), the yeasts *S. cerevisiae* (Murata *et al.*, 1985b) and *H. mrakii* (Inoue *et al.*, 1991b), and the fungus *A. niger* (Inoue *et al.*, 1988). Purification from mammalian sources such as goat liver (Ray and Ray, 1984) and porcine liver (Kato *et al.*, 1988) was also reported. The molecular structures of methylglyoxal reductases from microbial cells are similar to each other. They are monomers with molecular masses varying from 36 to 43 kDa, while the goat and porcine liver enzymes consist of two identical subunits with molecular masses of 89 kDa and 69 kDa, respectively. Methylglyoxal reductase of *S. cerevisiae* contains 5% (w/w) carbohydrate (Murata *et al.*, 1985b). *A. niger* has two kinds of methylglyoxal reductases, MGR-I and MGR-II, which can be separated by hydrophobic column chromatography (Inoue *et al.*, 1988). The molecular masses of MGR-I and MGR-II were estimated by SDS-PAGE to be 36 kDa and 38 kDa, respectively.

The enzymes from rat and goat liver showed broad substrate specificity, and were active toward several aldehydes as well as 2-oxoaldehydes (Ting *et al.*, 1965; Ray and Ray, 1984). The enzyme purified from *E. coli* was also active on 2-oxoaldehydes (glyoxal, methylglyoxal, phenylglyoxal and 4,5-dioxovalerate) and some aldehydes such as glycoaldehyde, D,L-glyceraldehyde, propionaldehyde and acetaldehyde (Saikusa *et al.*, 1987). Methylglyoxal reductases purified from eukaryotic microorganisms such as *S. cerevisiae*, *H. mrakii* and *A. niger* (MGR-I) were almost specific to 2-oxoaldehyde, although MGR-II of *A. niger* was also active on propionaldehyde and acetaldehyde (Inoue *et al.*, 1988). Although the *E. coli* and goat liver enzymes could use NADH as well as NADPH for the reduction of methylglyoxal to lactaldehyde, the enzymes from yeast (*S. cerevisiae* and *H. mrakii*) and *A. niger* (MGR-I and MGR-II) were specific to NADPH.

NADP, one of the reaction products of methylglyoxal reductase, inhibited the activities of methylglyoxal reductases from eukaryotic microorganisms. The inhibition of MGR-I by NADP was competitive and the K_i value for NADP was estimated to be 490 μM. On the other hand, the inhibition of MGR-II and the enzyme from *H. mrakii* were of mixed type and the K_i values for NADP were 45 μM and 250 μM, respectively (Inoue *et al.*, 1988, 1991b).

The K_i value for NADP of the enzyme from *S. cerevisiae* was 70 μM (Murata *et al.*, 1985b).

Methylglyoxal reductases purified from yeasts (*S. cerevisiae* and *H. mrakii*) and *A. niger* (MGR-I and MGR-II) were inactivated by brief incubation with substrates such as glyoxal, methylglyoxal and phenylglyoxal in the absence of NADPH (Murata *et al.*, 1985b; Inoue *et al.*, 1988, 1991b). Inactivation was not observed when the enzyme was preincubated with NADPH prior to exposure to 2-oxoaldehydes. Because methylglyoxal and phenylglyoxal are known to modify arginine residues in proteins (Takahashi, 1968), methylglyoxal reductases from these sources may contain an arginine residue at the NADPH-binding site.

Although *A. niger* has two methylglyoxal reductases (MGR-I and MGR-II), the physiological significance of these two enzymes remains to be discovered. The properties of these two enzymes are similar to each other, as described above. However, MGR-I was most active at pH 9.0, whereas the optimum pH for MGR-II was 6.5. The amino acid composition of MGR-I was slightly different from that of MGR-II (Inoue *et al.*, 1988). Methylglyoxal reductase in *E. coli* cells was suggested to exist in two forms, i.e. an "associated" form and a "dissociated" form, depending on the pH in the cell (Saikusa *et al.*, 1987). However, this has not been demonstrated *in vitro* using the purified enzyme.

3.4. Lactaldehyde Dehydrogenase

In several microbial cells, including bacteria, actinomycetes, yeasts and other fungi (Saikusa *et al.*, 1987), and also in mammalian tissues (Ting *et al.*, 1965; Ray and Ray, 1984), methylglyoxal can be reduced to lactaldehyde by the action of NAD(P)H-dependent methylglyoxal reductase. Further enzymatic conversion of lactaldehyde is then considered to result in either of two products, by reduction to 1,2-propanediol or oxidation to lactic acid (Fig. 1).

The metabolism of lactaldehyde in yeast cells was studied by Neuberg and Vercellone (1935) and Sandman and Miller (1958). Their investigations were designed to study the metabolic relationship between lactaldehyde, acetol (1-hydroxyacetone), 1,2-propanediol and lactic acid. The data obtained indicated that lactaldehyde was converted to 1,2-propanediol, lactic acid and acetol, the acetol probably being derived from 1,2-propanediol. In *E. coli*, the metabolism of lactaldehyde was studied with respect to the dissimilation of L-fucose and L-rhamnose. These methyl pentoses are converted to dihydroxyacetone phosphate and L-lactaldehyde by the sequential actions of corresponding isomerases (Green and Cohen, 1956; Takagi and Sawada, 1964a), kinases (Heath and Ghalambor, 1962; Takagi and Sawada, 1964b) and aldolases (Chiu and Feingold, 1969; Ghalambor and Heath, 1962).

Under aerobic conditions, L-lactaldehyde is oxidized to L-lactic acid by lactaldehyde dehydrogenase, while under anaerobic conditions, L-lactaldehyde is reduced to L-1,2-propanediol by propanediol oxidoreductase (Baldoma and Aguilar, 1988). L-Lactic acid is further oxidized to pyruvic acid by a flavoprotein dehydrogenase, whereas L-1,2-propandiol is excreted as a fermentation product (Cocks *et al.*, 1974).

Sridhara and Wu (1969) purified lactaldehyde dehydrogenase from *E. coli*. The enzyme catalyzes the oxidation of L-lactaldehyde to L-lactic acid in the presence of NAD, and is almost specific to L-lactaldehyde, but other aldehydes such as D-lactaldehyde (relative activity, 5%), pyruvaldehyde (relative activity, 3%), D,L-glyceroaldehyde (relative activity, 3%) and propionaldehyde (relative activity, 12%) are also slightly oxidized by the enzyme. Inoue *et al.* (1985) purified lactaldehyde dehydrogenase from *S. cerevisiae* which was almost completely specific to L-lactaldehyde, i.e. D-lactaldehyde was used only 0.2% by the enzyme compared with that of L-lactaldehyde. The enzyme was specific to NAD, and NADP could not substitute for NAD. The *E. coli* enzyme was also specific to NAD. The optimum pH for the reaction of the *E. coli* enzyme was 10.5–11.0, while the enzyme from *S. cerevisiae* was most active at pH 6.5. The molecular mass of the *E. coli* enzyme was estimated to be 100 kDa (Sridhara and Wu, 1969), whereas Baldoma and Aguilar (1987) reported that the molecular mass of the enzyme was 55 kDa on SDS-PAGE. On the other hand, the enzyme from *S. cerevisiae* was a monomer with a molecular mass of 40 kDa.

Several aldehyde dehydrogenases active on lactaldehyde have been purified from other sources such as *Pseudomonas fluorescens* (Jakoby, 1958), bovine liver (Racker, 1949) and baker's yeast (Black, 1951; Seegmiller, 1953). These aldehyde dehydrogenases showed a broad substrate specificity. Baker's yeast has two aldehyde dehydrogenases: one is a K^+-activated aldehyde dehydrogenase (Black, 1951) and the other is an NADP-dependent aldehyde dehydrogenase (Seegmiller, 1953). The former used both NAD and NADP as co-factor and the latter used NADP; optimum pH values for the reaction were 8.7 and 7.5–8.0, respectively. These aldehyde dehydrogenases from baker's yeast were, therefore, considered to be different from lactaldehyde dehydrogenase purified from *S. cerevisiae*.

3.5. 2-Oxoaldehyde Dehydrogenase

Methylglyoxal is converted to D-lactic acid via *S*-D-lactoylglutathione by the sequential actions of glyoxalase I and glyoxalase II. However, methylglyoxal is metabolized to L-lactic acid via L-lactaldehyde by NAD(P)H-dependent methylglyoxal reductase and NAD-dependent lactaldehyde dehydrogenase. Several microorganisms such as *Clostridium acetobutylicum* (Katagiri *et al.*, 1961), *Clostridium butylicum* (Dennis, 1962; Pepple and Dennis, 1976;

Cantwell and Dennis, 1974), *Staphylococcus ureae* (Katagiri *et al.*, 1961), *Lactobacillus sake* (Hiyama *et al.*, 1968) and *Lactobacillus curvatus* (Setter and Kandler, 1973) have lactate racemase (EC 5.1.2.1) which catalyzes the reversible isomerization between D-lactate and L-lactate. However, D-lactate is poorly utilized by mammals and its isomerization in tissues has not been demonstrated (Monder, 1967). Therefore, D-lactic acid is probably oxidized to pyruvic acid by D-lactate dehydrogenase (EC 1.1.1.28), although the D-lactate dehydrogenase has been found in prokaryotes and lower eukaryotes (Tarmy and Kaplan, 1968a, b; Long and Kaplan, 1968) (Fig. 1).

Direct oxidation of methylglyoxal to pyruvic acid was studied by Monder (1967) and the enzyme was partially purified from sheep liver. He proposed the name of this enzyme as α-ketoaldehyde dehydrogenase. The enzyme activity was later also found in rat and mouse liver, and from malignant tumors (Jellum, 1968a; Jellum and Elgjo, 1970). A partially purified enzyme from sheep liver catalyzed the oxidative conversion of methylglyoxal to pyruvic acid with either NAD or NADP as co-factor. Because the ratio of NAD-linked activity to NADP-linked activity changed at different steps of purification, the occurrence of two forms of the enzyme having different specificity for co-factors was suggested (Monder, 1967). Later, Dunkerton and James (1975) purified the enzyme from sheep liver to a homogeneous state on PAGE; the purified enzyme could use both NAD and NADP for the oxidation of methylglyoxal. The enzyme activity was completely dependent on Tris(hydroxymethyl)aminomethane or amines with similar structure such as L-2-aminopropan-1-ol (Dunkerton and James, 1975). Besides methylglyoxal, sheep liver enzyme was active on other 2-oxoaldehydes such as glyoxal, phenylglyoxal and hydroxypyruvaldehyde (Monder, 1967).

Vander Jagt and Davison (1977) partially purified 2-oxoaldehyde dehydrogenase from rat liver. The enzyme was highly specific for NADP and methylglyoxal. The rat liver enzyme also required certain amines for its enzymatic activity. In the case of sheep liver enzyme, Tris and L-2-aminopropan-1-ol were effective for the activity, although these amines gave little effect on rat liver enzyme, whereas a D,L mixture of 2-aminopropan-1-ol, L-serine methyl ester and D,L-serine methyl ester were effective for enzyme activity.

Ray and Ray (1982) purified two 2-oxoaldehyde dehydrogenases, an NAD-linked enzyme and an NADP-linked enzyme, from goat liver. These two enzymes could be separated by thiol-Sepharose affinity column chromatography. Both enzymes were monomeric with molecular masses of 42 kDa. The NAD-linked enzyme was inhibited by *p*-chloromercuribenzoate, 5,5′-dithiobis(2-nitrobenzoate) and *N*-ethylmaleimide, while the NADP-linked enzyme was not, suggesting the presence of SH group(s) in the active site of NAD-linked enzyme.

2-Oxoaldehyde dehydrogenase was also reported in some microbial cells (Higgins and Turner, 1969; Willetts and Turner, 1970; Taylor *et al.*, 1980;

Rhee *et al.*, 1987b). Rhee *et al.* (1987b) screened several microorganisms (*E. coli*, *Bacillus*, *Pseudomonas*, *Streptomyces*, *Mucor*, *Aspergillus* and *Saccharomyces*) but only *P. putida* IFO 3738 was found to contain the enzyme activity. However, Higgins and Turner (1969) and Willetts and Turner (1970) reported that a pseudomonad and *Bacillus subtilis* possessed an enzyme catalyzing the oxidation of methylglyoxal to pyruvic acid. The enzyme was partially purified from *B. subtilis* (Willetts and Turner, 1970) and purified from *P. putida* (Rhee *et al.*, 1987b). The *Bacillus* enzyme was specific for NAD ($K_m = 0.5$ mM) and the pH optimum was 9.8. The *Pseudomonas* enzyme also required NAD for oxidation of methylglyoxal to pyruvic acid ($K_m = 0.1$ mM), while NADP inhibited the enzyme activity. *The Pseudomonas* enzyme was not specific for 2-oxoaldehyde and glycolaldehyde was also used as substrate with NAD. The molecular mass of the *Pseudomonas* enzyme was 42 kDa and comprised a single subunit. The molecular mass of the *Bacillus* enzyme was estimated to be 500 kDa by Bio-Gel P300 exclusion column chromatography, and the enzyme was suggested to be made up of smaller (inactive) subunits (Higgins and Turner, 1969).

3.6. Aldehyde Reductase (Aldose Reductase)

Reduction of methylglyoxal is expected to form two products, either lactaldehyde or acetol (1-hydroxyacetone). The enzyme reducing methylglyoxal to lactaldehyde is methylglyoxal reductase and has been purified from mammals (Ray and Ray, 1984; Kato *et al.*, 1988) and several microorganisms (Saikusa *et al.*, 1987; Murata *et al.*, 1985b; Inoue *et al.*, 1988, 1991b). The enzyme catalyzing the reduction of methylglyoxal to acetol has been reported in microorganisms (Inoue *et al.*, 1992a), plants (Liang *et al.*, 1990) and mammals (Liang *et al.*, 1991), including man (Vander Jagt *et al.*, 1990a, b, 1992).

A methylglyoxal-reducing enzyme was partially purified from the yeast *H. mrakii* (Inoue *et al.*, 1992a). The enzyme specifically required NADH as co-factor for the reduction of methylglyoxal to acetol. NADPH was also used by the enzyme when acetaldehyde and propionaldehyde were used as substrate, although the relative activities were only 1.2% and 5.9%, respectively, of that with NADH (Inoue *et al.*, 1992a). The molecular mass of the enzyme was estimated to be 90 kDa by gel filtration using HPLC, although the subunit structure was not identified. This enzyme activity has not been detected in *S. cerevisiae* by us.

Kato and his co-workers have been extensively studying the effects of the Maillard reaction and its derivatives on aging of proteins by cross-linking and glycation of proteins *in vitro* and *in vivo*. They proved that 3-deoxyglucosone, a dicarbonyl compound, was a major intermediate of the Maillard reaction and could be reduced to 3-deoxyfructose in the rat when the compound was

administered orally and intravenously (Kato *et al.*, 1990). The enzyme responsible for the reduction of 3-deoxyglucosone to 3-deoxyfructose was purified from porcine liver (Liang *et al.*, 1991). The molecular mass of the purified enzyme was 38 kDa after both gel filtration and SDS-PAGE. The enzyme required NADPH as a co-factor for the reduction of 3-deoxyglucosone; the relative activity was only 4% with NADH. The enzyme also reduced methylglyoxal ($K_m = 3.3$ mM) to acetol as well as 3-deoxyglucosone ($K_m = 2.1$ mM) to 3-deoxyfructose. Other dicarbonyl compounds such as phenylglyoxal, glyoxal and diacetyl were also used by the enzyme; thus, they named the enzyme NADPH-dependent 2-oxoaldehyde reductase. The enzyme inhibited the polymerization of lysozyme and formation of fluorescent compounds by 3-deoxyglucosone *in vitro*. They also purified the NADPH-dependent 2-oxoaldehyde reductase from parsley leaves (Liang *et al.*, 1990). The plant enzyme was a dimer composed of identical subunits with a molecular mass of 35 kDa.

NADPH-dependent oxidoreductases in mammals are thought to comprise a multigene family (Wirth and Wermuth, 1985; Wermuth, 1985; Grimshaw and Muthur, 1989; Carper *et al.*, 1987). The enzymes involved in this family can be tentatively classified into three groups: ALR1, aldehyde reductase (alcohol:$NADP^+$ oxidoreductase, EC 1.1.1.2); ALR2, aldose reductase (alditol:$NADP^+$ oxidoreductase, EC 1.1.1.21); and ALR3 (Vander Jagt *et al.*, 1990a). These enzymes can reduce a wide variety of aldehydes and ketones, some of which are xenobiotics. Thus, one of the physiological functions of this enzyme family may be to detoxify such reactive carbonyl compounds. Recently, ALR2 has been studied with respect to the reduction of glucose to sorbitol during diabetic hyperglycemia and complications (Kador and Kinoshita, 1985; Vander Jagt *et al.*, 1992). Glucose can be reduced to sorbitol by aldose reductase, and this reaction increases during hyperglycemia, especially in the tissues with insulin-independent uptake of glucose (Kador, 1988). Aldose reductase was purified from human skeletal muscle and the apparent molecular mass of the enzyme was 36.7 kDa on SDS-PAGE (Vander Jagt *et al.*, 1990b). Methylglyoxal was, however, the best substrate for human aldose reductase ($k_{cat.} = 142\ min^{-1}$, $k_{cat.}/K_m = 1.8 \times 10^7\ M^{-1}\ min^{-1}$). Reduction of methylglyoxal by human aldose reductase yielded 95% acetol and 5% D-lactaldehyde (Fig. 1) (Vander Jagt *et al.*, 1992). Acetol and D-lactaldehyde are further reduced to L-1,2-propanediol and D-1,2-propanediol by aldose reductase, respectively. Both acetol and L-1,2-propanediol can be accumulated in diabetes. Methylglyoxal and acetol can modify human serum albumin to a much higher extent than glucose *in vitro* (Vander Jagt *et al.*, 1992).

Designation of the enzyme catalyzing the reduction of methylglyoxal to acetol seems to be confusing. 2-Oxoaldehyde reductase purified from porcine liver by Liang *et al.* (1991) and human aldose reductase (Vander Jagt, 1990b) showed similar properties in terms of co-factor requirement (NADPH) and

molecular mass (porcine enzyme, 38 kDa; human enzyme, 36.7 kDa). Yeast aldehyde reductase, however, is different from these enzymes in co-factor requirement and molecular mass (Inoue *et al.*, 1992a). Oxidation of acetol to methylglyoxal with NAD by acetol dehydrogenase was observed in some Gram-positive bacteria isolated from soil (Taylor *et al.*, 1980), although methylglyoxal is further metabolized to pyruvic acid by these bacteria. On the other hand, oxidation of acetone to methylglyoxal via acetol is catalyzed by acetone-inducible cytochrome P450-monooxygenase in mammals (Casazza *et al.*, 1984; Koop and Casazza, 1985; Reichard *et al.*, 1986). This reaction consumes NADPH. Molecular cloning and nucleotide sequence analysis will clarify the confusion of the designation of these enzymes.

4. GENES FOR METABOLIC ENZYMES OF METHYLGLYOXAL IN MICROORGANISMS

4.1. Genes Corresponding to Glyoxalase I in Microorganisms

4.1.1. *Glyoxalase I Gene from* S. cerevisiae *and Regulation of its Expression*

(a) *Molecular cloning of glyoxalase I activation conferring (GAC) gene from* S. cerevisiae. Growth of the yeast *S. cerevisiae* was completely arrested in the presence of 2 mM methylglyoxal (Murata *et al.*, 1986b). The addition of an equimolar amount of glutathione or L-cysteine to a culture containing methylglyoxal eliminated the toxic effect of methylglyoxal, indicating that the cytotoxic effect of methylglyoxal is chemically neutralized by sulfhydryl compounds. However, in the presence of a large amount of methylglyoxal (20 mM) and glutathione (20 mM), the growth of the yeast cells was inhibited. Since a non-enzymatic adduct (hemimercaptal) of methylglyoxal and glutathione is an intrinsic substrate for glyoxalase I, a yeast genomic DNA library was then screened for the glyoxalase I gene by selecting transformants showing rapid growth on minimal medium containing 20 mM methylglyoxal and 20 mM glutathione. Three candidate clones were obtained; the glyoxalase I activity in these clones was appreciably higher (five-fold) than that of a control strain (Murata *et al.*, 1988). Since the phenotypic characters of these clones were identical, the nucleotide sequence of the inserted DNA in the recombinant plasmid from one of the clones was determined. An open reading-frame of 318 bp was identified. The M_r of the gene product deduced from the DNA sequence was calculated to be 14 700 (106 amino acids). However, yeast glyoxalase I is a monomer with an M_r of 32 000. Thus, the open reading-frame found was thought not to correspond to yeast glyoxalase I. We named the gene *GAC* (glyoxalase I activation

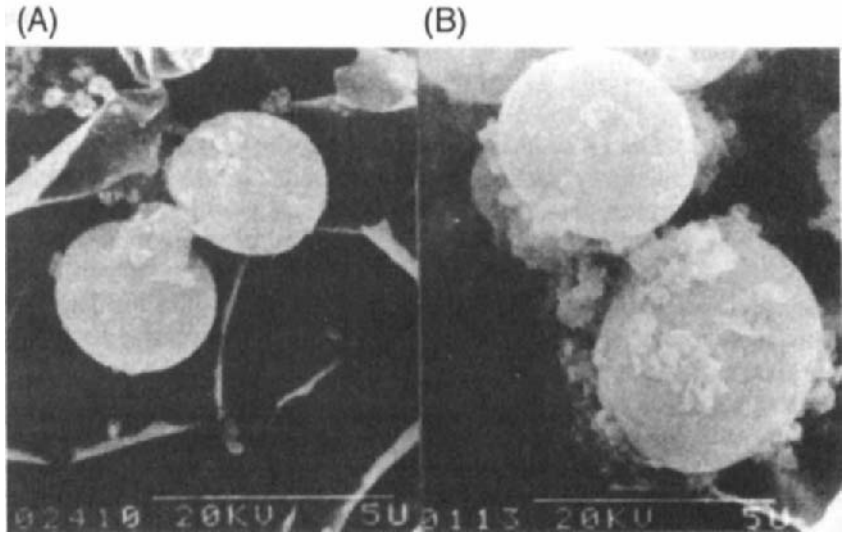


Figure 4 Effect of the *GAC* gene on the cell size of *S. cerevisiae*. (A) *S. cerevisiae* with vector, (B) *S. cerevisiae* dosed with *GAC* gene.

conferring) (Inoue *et al.*, 1990a). The *GAC* gene gave two transcripts of different molecular sizes in *S. cerevisiae* cells.

(b) *Expression of the* GAC *gene in* E. coli. To confirm that the *GAC* gene is not the structural gene for yeast glyoxalase I, the gene was expressed in *E. coli*. The gene was ligated downstream of the *tac* promoter in an *E. coli* expression vector and the resultant plasmid was introduced into *E. coli*. Expression was induced by IPTG (isopropyl-β-D-thiogalactopyranoside). GAC protein was produced to approximately 7% of total cellular protein, although glyoxalase I activity did not increase (Y. Inoue, H. Ginya and A. Kimura, unpublished results). Therefore, the *GAC* gene does not code the structural gene for yeast glyoxalase I.

(c) *Effects of the* GAC *gene on yeast cell growth and cell size.* Cells containing the *GAC* gene on a multicopy plasmid could grow in a medium containing methylglyoxal at a concentration giving complete growth arrest of the control strain, suggesting that the *GAC* gene product was in fact functioning in the detoxification of methylglyoxal; an increase in glyoxalase I activity was observed, which seemed to allow the cells to grow rapidly (Murata *et al.*, 1988). Penninckx *et al.* (1983) reported that the growth rate of a glyoxalase I-deficient yeast mutant was slightly lower than that of the parental cells. Increase in turbidity of the culture of cells expressing the *GAC* gene was not, however, proportional to cell number, reflecting a change of yeast cell size during growth (Fig. 4). In fact, the yeast cells carrying the *GAC*

gene on a multicopy plasmid showed increased cell size (Fig. 4B). Thus, glyoxalase I may have an additional role in the regulation of growth rate or cell size, other than the detoxification of methylglyoxal and other 2-oxoaldehydes.

(d) *Molecular cloning of the structural gene for glyoxalase I from* S. cerevisiae. The structural gene for yeast glyoxalase I (*GLO1*) was cloned from a genomic DNA library constructed with λgt11 using anti-glyoxalase I IgG as a probe. The amino acid sequence deduced from the nucleotide sequence of a positive clone was that of a tryptic fragment of yeast glyoxalase I. Glyoxalase I protein produced as a fusion protein with β-galactosidase of *E. coli* was immunologically identical with that purified from yeast (Inoue *et al.*, 1991c). The amino acid sequence of yeast glyoxalase I showed homology with that of human glyoxalase I, which was recently reported independently by several groups (Ranganathan *et al.*, 1993; Kim *et al.*, 1993; Mannervik and Riddestrom, 1993).

(e) *Effect of the* GAC *gene product on the expression of the* GLO1 *gene in* S. cerevisiae. Although the expression of the *GAC* gene in *E. coli* did not increase the glyoxalase I activity, yeast with the cloned gene showed an increased activity of glyoxalase I and resistance against methylglyoxal. Therefore, the biological function of the *GAC* gene in *S. cerevisiae* cells should be a subject of considerable interest. The mRNA level from the *GLO1* gene in these cells was increased compared with control cells (Fig. 5) (Inoue *et al.*, 1991c). Thus, the *GAC* gene product affected the activity of yeast glyoxalase I at the *GLO1* mRNA level, i.e. by transcriptional activation or enhancing the stability of *GLO1* mRNA.

It is known that the 5′-flanking regions of several yeast genes contain a specific DNA sequence for the binding of a *trans*-activator of transcription, designated UAS (upstream activating sequence) (Guarente, 1984, 1988; Struhl, 1987). Several proteins that bind to the UAS and activate the transcription of yeast genes have been identified (Verdier, 1990). DNA binding proteins often contain a Zn^{2+} ion by tetrahedral coordination by cysteine residues, the domain being termed a zinc finger (Johnson and McKnight, 1989; Johnston, 1987a). The DNA binding protein binds to the specific site of the 5′-flanking region of the gene and accelerates the transcription of DNA through interaction with RNA polymerase (Allison and Ingles, 1989). The Zn^{2+} is essential for the protein to bind DNA (Johnston, 1987b). According to the amino acid sequence deduced from the *GAC* sequence, the gene product is predicted to contain five cysteine residues near the carboxyl terminus, but the spacing of the cysteine residues is not similar to that of the C6 zinc fingers of GAL4 and other yeast activator proteins (Inoue *et al.*, 1990a). However, it bears a similarity to the C5 zinc finger which is characteristic of the hormone receptor superfamily. Recent findings suggest that GAC protein produced in *E. coli* has the capability for DNA binding; gel retardation assays indicate that the GAC protein produced in *E. coli* binds

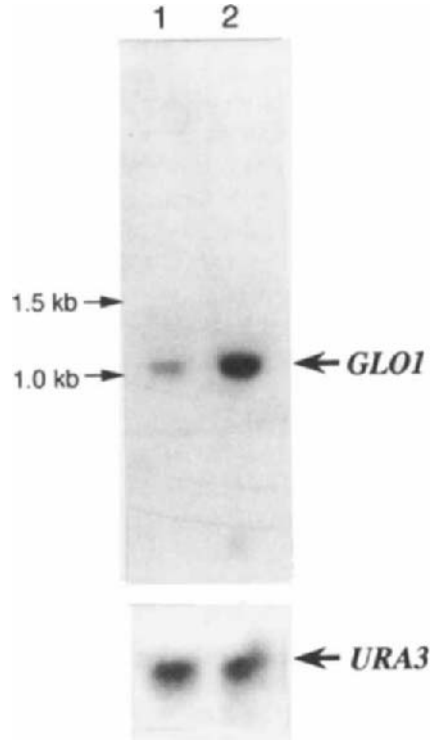


Figure 5 Effect of the *GAC* gene product on *GLO1* mRNA levels in *S. cerevisiae*. Total RNA prepared from *S. cerevisiae* cells transformed with YEp13 (lane 1) and YEp13 + *GAC* (lane 2) were separated in agarose gel containing formaldehyde and transferred onto nylon membrane. mRNA of the *GLO1* gene was detected by the ^{32} P-labeled *Eco*RI-*Eco*RI fragment of λGI10 (Inoue *et al.*, 1991c) as a probe. The amount of RNA loaded in both lanes was confirmed by reprobing the membrane with *URA3* gene as an internal control as indicated at the bottom.

to chromosomal DNA of *S. cerevisiae* (H. Ginya, MSc thesis, Kyoto University, 1991).

4.1.2. *Glyoxalase I Gene from* Pseudomonas putida

The structural gene for *P. putida* glyoxalase I was cloned in *E. coli* (Rhee *et al.*, 1987a) and its nucleotide sequence was determined (Rhee *et al.*, 1988). The M_r of the enzyme deduced from the nucleotide sequence was 18 422; the value was similar to that estimated from the purified enzyme (M_r = 20 000) (Rhee *et al.*, 1986). The *N*-terminal amino acid sequence of the *P. putida* enzyme expressed in *E. coli* was Ser-Leu-Asp-Asn, whereas

the nucleotide sequence of the gene indicated an amino acid sequence of Met-Ser-Leu-Asp-Asn, suggesting post-translational processing of the *N*-terminal methionine of the enzyme in *E. coli*. A cDNA coding for human colon glyoxalase I was recently cloned and the nucleotide sequence was determined (Ranganathan *et al.*, 1993; Kim *et al.*, 1993). The human enzyme showed 51% identity at the nucleotide level and 42% identity at the amino acid level with *P. putida* glyoxalase I. The M_r of human enzyme deduced from the nucleotide sequence was calculated to be 20 774 (183 amino acids). Mammalian glyoxalase I is a dimer (M_r = 43 000 to 48 000) composed of identical subunits and each subunit contains one Zn^{2+} at its catalytic site. Binding frequency of the zinc atom to the proteins was thought to be as follows: His >> Glu > Asp = Cys, and appropriate spacing of these amino acids is considered (Vallee and Auld, 1990). Ranganathan *et al.* (1993) predicted that the amino acids concerned with Zn^{2+} binding are Glu-100 and His-103, with one of Asp-121, His-127, Cys-139 or Glu-143. They also suggested the Zn^{2+} binding motif of bacterial glyoxalase I to be Glu-91 and His-94, with one of Asp-112, His-118, Cys-130 or Glu-132. However, Rhee *et al.* (1986) reported that the *P. putida* glyoxalase I was not inhibited by excess (10 mM) EDTA and suggested no contribution of metal ions to the catalytic function of the bacterial enzyme.

Introduction of the *P. putida* glyoxalase I gene into *E. coli* cells resulted in a 150-fold increase in glyoxalase I activity as well as an increase in resistance to methylglyoxal. When *E. coli* cells harboring only the plasmid vector were incubated in medium containing 1.0 mM methylglyoxal, cell division was inhibited but cells were not killed, and the cells showed an abnormally elongated cell shape (Fig. 6B). However, *E. coli* cells harboring the *P. putida* glyoxalase I gene cloned into the same vector showed normal cell shape (Fig. 6A), presumably due to detoxification of methylglyoxal (Rhee *et al.*, 1987a).

4.2. Methylglyoxal Reductase Gene from *S. cerevisiae*

Methylglyoxal reductase is one of the enzymes that directly act on methylglyoxal in *S. cerevisiae*. A DNA fragment responsible for methylglyoxal reductase was cloned from a genomic DNA library of *S. cerevisiae* (Murata *et al.*, 1985a). The DNA fragment (8.8 kb) in the vector YEp13 was cloned by screening transformants for resistance against 5 mM methylglyoxal. Methylglyoxal reducing activity increased in the transformant carrying the hybrid plasmid (pYMG14) and the enzyme was further induced by the addition of methylglyoxal (5.5 mM) to the medium.

Transformants carrying pYMG14 showed the multidrug-resistant phenotype. Besides methylglyoxal, the transformant carrying pYMG14 was resistant to phenylglyoxal, tetramethylthiuram disulfide, iodoacetamide and

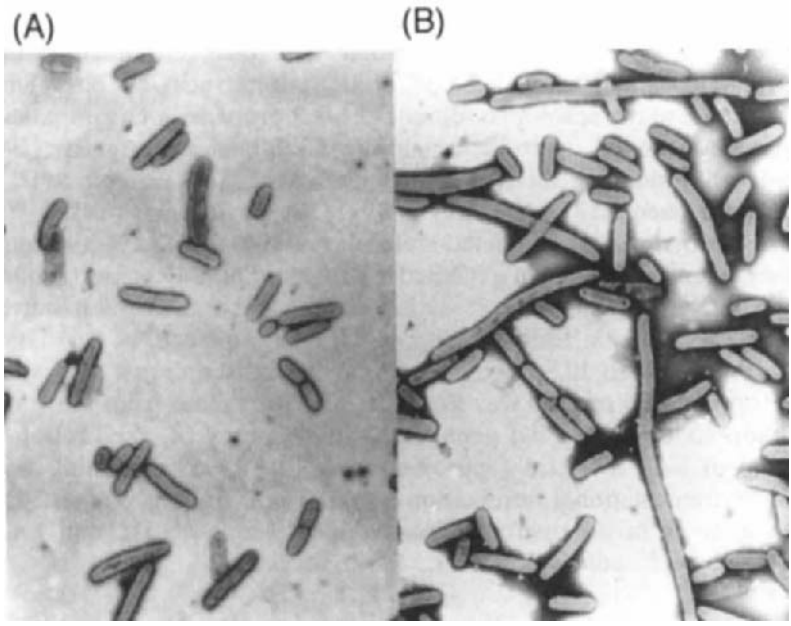


Figure 6 Inhibition of cell division of *E. coli* by methylglyoxal. *E. coli* cells carrying pGI423 (pBR322 + *P. putida glo-I* gene) (A) and pBR322 (B) were incubated in a medium containing 1.0 mM methylglyoxal. Cell division of *E. coli* carrying vector alone was inhibited by methylglyoxal and the cells showed an extremely elongated cell shape.

metal ions such as Zn^{2+}, Cd^{2+} and Co^{2+} (Murata *et al.*, 1985a). One possible reason is that the transformant with pYMG14 had a two-fold higher intracellular glutathione content and also secreted glutathione into the medium. Cytotoxic effects of methylglyoxal and of other chemicals could be neutralized through non-enzymatic interaction with SH group of glutathione. Despite an increase in glutathione concentration in the transformant carrying pYMG14, the enzyme activities responsible for glutathione biosynthesis, γ-glutamylcysteine synthetase and glutathione synthetase (Kimura, 1986a, b, 1989, 1993) did not increase. Although the nucleotide sequence of this DNA fragment has not been described, the restriction map of the fragment was not similar to that of the γ-glutamylcysteine synthetase gene of *S. cerevisiae* (Ohtake and Yabuuchi, 1991). The possibility that several relevant genes are cloned in this 8.8 kb fragment cannot be excluded.

4.3. Lactaldehyde Dehydrogenase Gene from *E. coli*

Lactaldehyde synthesized from methylglyoxal by the action of methylglyoxal reductase can be oxidized to L-lactic acid by NAD-dependent lactaldehyde

dehydrogenase (Sridhara and Wu, 1969; Inoue *et al.*, 1985). In *E. coli*, lactaldehyde is also synthesized through the dissimilation of L-fucose and L-rhamnose, and is oxidized to either L-lactic acid by lactaldehyde dehydrogenase under aerobic conditions or L-1,2-propanediol by propanediol oxidoreductase under anaerobic conditions (Baldoma and Aguilar, 1988). The *ald* gene encoding lactaldehyde dehydrogenase of *E. coli* K-12 was cloned and sequenced (Hidalgo *et al.*, 1991). A single open reading-frame encoding a protein with a predicted molecular mass of 52 278 kDa (479 amino acids) was identified. The *ald* gene had a potential Shine-Dalgarno sequence at a position −11 bp from the initiation codon, and transcription started a further 32 bp upstream from the Shine-Dalgarno sequence. A translational stop codon is located 10 bp upstream from the transcriptional start site. A set of 9 bp inverted repeats was found 10 bp downstream from the translational stop codon of the *ald* gene whose free energy of stabilization was calculated to be −25.8 kcal (approximately −107.9 kJ), suggesting a *rho*-dependent transcriptional termination signal. These findings suggested that the *ald* gene is in an operon. The *ald* locus maps at 31.2 min on the chromosome of *E. coli* (Chen *et al.*, 1987).

5. REGULATION OF GLYOXALASE I ACTIVITY IN YEAST

5.1. Regulation of the Glyoxalase System by Mating Factor in *S. cerevisiae*

5.1.1. *Alteration of Enzyme Activity in the Glyoxalase System by Mating Factor*

The activities of the enzymes involved in methylglyoxal metabolism have some relationships to cell growth or proliferation. For example, when cells of *S. cerevisiae* mutant strain *cdc7* were cultured at 38°C (a non-permissive temperature for this strain) the activity of glyoxalase I decreased, whereas when the cells were transferred to 23°C (a permissive temperature) the activity of glyoxalase I increased (Murata and Kimura, 1987). The gene product of *CDC7* is a protein kinase which is essential for yeast cell growth, and the protein is thought to be involved in signal transduction in yeast (Patterson *et al.*, 1986; Uno, 1989). The signal transduction system in *S. cerevisiae* has been extensively studied through the analysis of the mating system.

S. cerevisiae has two different genes whose gene products are homologous to G protein, *GPA1* and *GPA2* (Nakafuku *et al.*, 1987, 1988). The product of *GPA1* is involved in mating factor-mediated signal transduction (Dietzel and Kurjan, 1987; Miyashita *et al.*, 1987) and that of *GPA2* is involved in

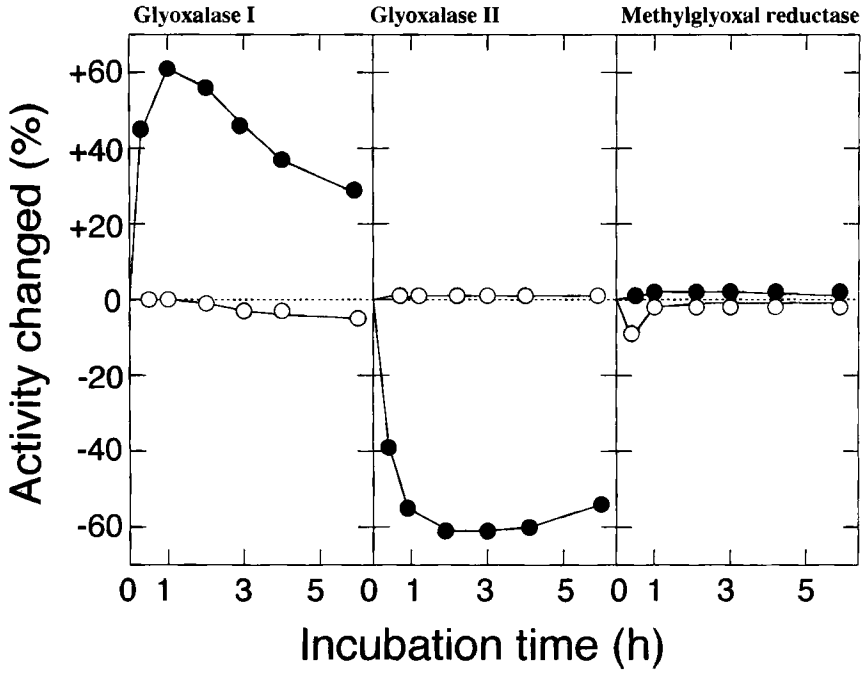


Figure 7 Effect of mating factor on the activities of the enzymes involved in methylglyoxal metabolism. Cells of *S. cerevisiae* S288C (*MATα SUC2 mal mel gal2 CUP1*) were exposed to the culture supernatant of the opposite mating type (*S. cerevisiae* DKD-5D-H (*MAT***a** *leu2-3, 112 his3 trp1*) (•) and the same mating type (*S. cerevisiae* LB1-16A (*MATα SUC2 mal mnn2 gal2 CUP1*) (○), and the enzyme activity was measured periodically.

the regulation of cAMP level (Nakafuku *et al.*, 1988). Both gene products are GTP(GDP)-binding proteins, while the β- and γ-subunits of G protein in *S. cerevisiae* are thought to be encoded by *STE*4 and *STE*18, respectively (Jahng *et al.*, 1988). Haploid cells of budding yeast are of two different sexes, **a** and α, and cells of each type secrete a mating factor, either **a**-factor or α-factor, respectively. There are receptors for the mating factors of opposite type on the surface of haploid cells (Jenness *et al.*, 1983; Haugen *et al.*, 1986) and transmembrane signalling also occurs in yeast cells, as in mammalian cells (Matsumoto *et al.*, 1989).

When α-type cells were exposed to the culture medium in which cells of the opposite mating type were grown, glyoxalase I activity increased and glyoxalase II activity decreased (Fig. 7). The factor that activated glyoxalase I in α-type cells was secreted from **a**-type cells, although the factor could not accelerate glyoxalase I activity in α-type cells which are deficient in **a**-factor receptors (Table 1), indicating that the glyoxalase I activating factor being

Table 1 Effect of the culture supernatant of **a**-type cells on the alteration of methylglyoxal-metabolizing enzyme activity.

Strain	Treatment by supernatant	Specific activity (μmol/min/mg protein)		
		Glyoxalase I	Glyoxalase II	Methylglyoxal reductase
S288C	−	0.390(100)[a]	0.014(100)	0.034(100)
	+	0.634(160)	0.008(57)	0.036(105)
VQ3	−	0.170(100)	0.043(100)	0.019(100)
	+	0.194(114)	0.046(107)	0.022(116)

Cells of *Saccharomyces cerevisiae* S288C (*MATα SUC2 mal mel gal2 CUP1*) and *S. cerevisiae* VQ3 (*MATα ste3-1*) were treated with the culture supernatant of the same (*S.cerevisiae* LB1-16A (*MATα SUC2 mal mnn 2 gal2 CUP1*)) or the opposite (*S. cerevisiae* DKD-5D-H (*MATa leu2-3, 112 his3 trp1*)) type cells. *S. cerevisiae* S288C, VQ3 and LB1-16A were obtained from the Yeast Genetic Stock Center, University of California at Berkeley, USA. VQ3 is a mutant deficient in the receptor for **a**-factor.

[a]Relative activity.

secreted from **a**-type cells may be **a**-factor (Inoue *et al.*, 1989; Inoue and Kimura, 1992c).

Exposure of haploid cells of one sex to the mating factor of the opposite type of cells arrests the cell division cycle at G_1 phase and the cells undergo morphological change (Sakai and Yanagishima, 1972; Fehrenbacher *et al.*, 1978; Konopka *et al.*, 1988; Walton and Yarranton, 1989). In contrast, incubation of human promyelocytic leukemia 60 cells with exogeneous *S*-D-lactoylglutathione induces growth arrest and the cells accumulate in G_0–G_1 phase (Thornalley and Tisdale, 1988). Alteration of enzyme activity in the glyoxalase system by mating factor, i.e. increase of glyoxalase I activity and decrease of glyoxalase II activity, apparently results in an increase in the intracellular concentration of *S*-D-lactoylglutathione, an intermediate of the glyoxalase system. Intracellular levels of *S*-D-lactoylglutathione, therefore, may affect the cell division cycle or differentiation.

5.1.2. *Phosphorylation of Glyoxalase I*

It has been reported that when **a**-type *S. cerevisiae* cells were treated with α-factor, extracellular Ca^{2+} was taken up by the cells and the amount of some calmodulin-binding proteins increased (Tachikawa *et al.*, 1987; Liu *et al.*, 1990). In the case of the heterobasidomycetous yeast, *Rhodosporidium toruloides*, extracellular Ca^{2+} is also taken up by the cells after treatment with rhodotorucine A, the mating factor of *R. toruloides* (Miyakawa *et al.*,

1985, 1986). Calcium ions taken up by the cells bind to calmodulin and then Ca^{2+}-calmodulin-dependent protein kinase is activated through autophosphorylation (Miyakawa *et al.*, 1989). However, the target proteins for this protein kinase have not been identified.

When phosphatase-treated yeast glyoxalase I (M_r = 32 000) was incubated with [γ-^{32}P]ATP and the cell extracts prepared from α-type cells that had been exposed to the culture supernatant of a-type cells, a protein with an M_r of 32 000 was labeled. Several proteins were also phosphorylated in the same mixture; however, the phosphorylation of a protein of this size was not observed when cell extracts prepared from α-type cells that had been treated with the culture supernatant of the same mating type cells were used (Fig. 8). This phosphorylated protein was identified as yeast glyoxalase I using anti-glyoxalase I IgG (Fig. 8) (Inoue *et al.*, 1990b). Calcium ions did not affect the phosphorylation of yeast glyoxalase I *in vitro* (Inoue *et al.*, 1990b).

Glyoxalase I was proved to be able to act as a substrate for protein kinase(s) which are activated during the sexual response. Recently, cDNA encoding human glyoxalase I was cloned and sequenced. The amino acid sequence deduced from the cDNA sequence indicates that four potential phosphorylation sites are present in human glyoxalase I (Ranganathan *et al.*, 1993). Thus, the activation and/or inactivation of glyoxalase I during the sexual response was thought to be controlled through phosphorylation/dephosphorylation of the enzyme. It has been reported that the products of the *STE7* (Teague *et al.*, 1986) and *STE11* (Hunter, 1987) genes show homology to protein kinases. These genes are components of the mating factor response (Walton and Yarranton, 1989) and are, therefore, candidate genes for protein kinases that may act on glyoxalase I. Recently, we observed that the mRNA level of the *GAC* gene (glyoxalase I activation conferring gene) increased in *S. cerevisiae* cells treated with mating factor (Inoue and Kimura, 1990; Kimura and Inoue, 1993). Purification of protein kinase(s) whose substrate is glyoxalase I and whose activity is regulated during the process of the sexual response, and analysis of 5′-flanking region of *GAC* gene would provide more information on the mechanism for signal transduction in budding yeast.

5.2. Effects of Cell–Cell Interaction on the Glyoxalase I Activity in *S. cerevisiae*

Regulation of yeast glyoxalase I activity seems to be concerned with the cell–cell recognition system or with the environment to which the cells are exposed. We found that glyoxalase I activity in yeast could be changed by exposing the cells to high cell-density environments. In fact, when cells of *S. cerevisiae* were exposed to high cell-densities, glyoxalase I activity increased in accord with culture density up to a maximum level when the cells were concentrated four-fold. At this point, the glyoxalase I activity

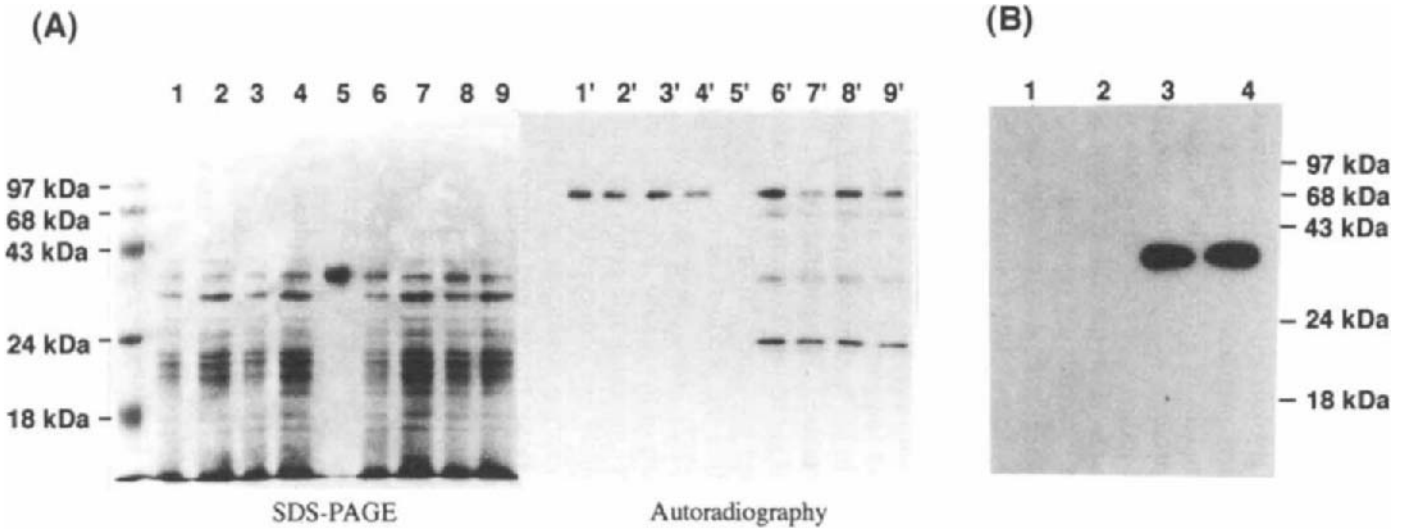


Figure 8 Phosphorylation of yeast glyoxalase I. (A) *In vitro* phosphorylation. Phosphorylation experiments are described in our previous paper (Inoue *et al.*, 1990b). Lanes 1–4, the cell extracts used were prepared from α-type cells that had been exposed to the culture supernatant of α-type cells. Lanes 6–9, the cell extracts used were prepared from α-type cells that had been exposed to the culture supernatant of a-type cells. The concentrations of Ca^{2+} and cell extracts in the phosphorylation mixtures were as follows: Lanes 1 and 6: Ca^{2+}, 0 mM; cell extracts, 100 μg protein. Lanes 2 and 7: Ca^{2+}, 0 mM; cell extracts, 50 μg protein. Lanes 3 and 8: Ca^{2+}, 1 mM; cell extracts, 100 μg protein. Lanes 4 and 9: Ca^{2+}, 1 mM; cell extracts, 50 μg protein. Lane 5, purified glyoxalase I. Lanes 1′–9′ correspond to lanes 1–9, respectively. (B) Identification of phosphorylated protein. For lanes 1 and 2, the cell extracts (100 μg protein) used were prepared from cells of α-type that had been treated with the culture supernatant of α-type cells. For lanes 3 and 4, the cell extracts (100 μg protein) used were prepared from α-type cells that had been exposed to the culture supernatant of a-type cells. For lanes 2 and 4, 1 mM Ca^{2+} was added to the phosphorylation mixture. After SDS-PAGE, autoradiography was carried out for 24 h.

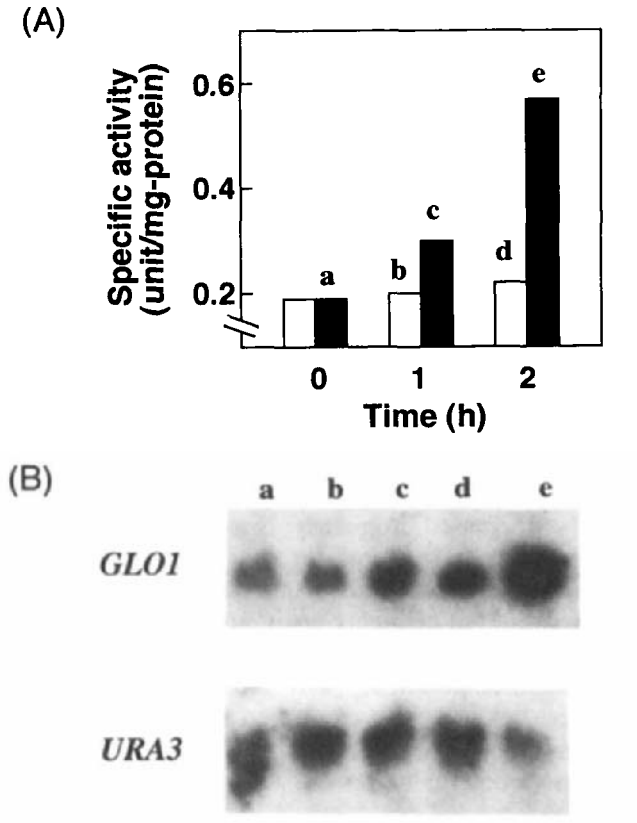


Figure 9 Effect of cell concentration on *GLO1* mRNA levels in *S. cerevisiae*. *S. cerevisiae* cells were cultured after fourfold concentration at 28°C for the indicated periods. (A) Change of specific activity of glyoxalase I during incubation. Black bars indicate samples from the concentrated culture, and white bars indicate the control. (B) Detection of *GLO1* mRNA in the cells. Lanes **a** to **e** correspond to the samples as indicated in panel (A). Ten micrograms of total RNA was applied and the level of *URA3* mRNA was used as an internal control by reprobing the membrane as indicated at the bottom.

showed an approximately 2.5-fold increase compared with that in the control cells (normal cell concentration). The increase in glyoxalase I activity seemed not to be caused by the effect of aeration, because the enzyme activity in the control culture incubated in the same volume did not change. The amount of glyoxalase I mRNA in the yeast cells increased in accord with enzyme activity (Fig. 9) (Inoue *et al.*, 1993e). The activation of glyoxalase I occurred through increased frequency of interaction between cells, because the amount of mRNA of the glyoxalase I gene in flocculent mutant cells (Inoue *et al.*,

1992b) increased compared with the corresponding parental cell or the revertant. Yeast glyoxalase I activity was supposed to be controlled through the phosphorylation/dephosphorylation state of the enzyme (Inoue *et al.*, 1990b). We therefore examined the glyoxalase I-phosphorylating activity in cells exposed to high cell-density environments. However, the glyoxalase I-phosphorylating activity could not be detected in cells from four-fold concentrated cultures, suggesting that phosphorylation of glyoxalase I plays little role in the increases in the glyoxalase I activities observed in cells exposed to high cell-density environments (Inoue *et al.*, 1993e).

The physiological significance of the activation of glyoxalase I in yeast cells under high cell-density environments is obscure at present. One possible explanation is that it increases the intracellular level of *S*-D-lactoylglutathione, thereby controlling the growth and/or proliferation of the cells. *S*-D-lactoylglutathione has several physiological functions (Gillespie, 1979; Thornalley *et al.*, 1987; Kojima *et al.*, 1991), and recently Edwards *et al.* (1993) reported that *S*-D-lactoylglutathione inhibited the growth of human leukemia 60 cells. Increased frequency of cell–cell interaction in a high cell-density culture may be a signal for the yeast cells to terminate their growth, as if they had reached the stationary phase. Alternatively, the restriction (or starvation) of nutrients surrounding the yeast cells might affect the expression of the glyoxalase I gene. However, during the period of the culture concentration experiment (2 h) the nutrients (at least glucose) remaining in the medium were not consumed completely. We are now trying to elucidate the physiological meaning of the alteration of glyoxalase I activity under various environmental stresses with respect to cell growth and aging.

6. *S*-D-LACTOYLGLUTATHIONE

6.1. Physiological Activities

S-D-lactoylglutathione is synthesized from methylglyoxal and glutathione by the action of glyoxalase I. Although methylglyoxal is a natural substance in the cell, it has several undesirable biological activities presumably because of its 2-oxoaldehyde structure. Methylglyoxal can interact with various macromolecules in the cells such as DNA, RNA and proteins to make adducts or reduce their functions. Exogenously added methylglyoxal in the culture medium of microorganisms or tissues inhibits growth at millimolar concentrations, although intracellular concentrations of methylglyoxal are estimated to be 0.1–2 μM (Ohmori *et al.*, 1987b; Thornalley, 1990). *S*-D-lactoylglutathione also induces several biological responses. Although *S*-D-lactoylglutathione seems not to cross the cell membrane freely (Thornal-

ley, 1988; Rae *et al.*, 1991), exogenously added *S*-D-lactoylglutathione arrests the growth of human promyelocytic leukemia 60 cells at micromolar concentrations (Thornalley and Tisdale, 1988; Edwards *et al.*, 1993) and results in the accumulation of cells in G_0–G_1. The ester also potentiates GTP-promoted assembly of microtubules *in vitro* (Gillespie, 1975; Clelland and Thornalley, 1993), influences neutrophil movement and stimulus-induced secretion of granules (Thornalley *et al.*, 1987; Allen and Thornalley, 1993) and inhibits histamine release from leukocytes (Gillespie, 1979). Treatment of haploid cells of *S. cerevisiae* with the mating factor changes the activities of glyoxalase I and glyoxalase II, i.e. it increases glyoxalase I and decreases glyoxalase II activity (Inoue *et al.*, 1989). This produces an apparent increased intracellular concentration of *S*-D-lactoylglutathione. The cell division cycle of *S. cerevisiae* treated with mating factor is arrested in G_1 and the cells undergo morphological change. Our colleagues recently found that *S*-D-lactoylglutathione has anti-inflammatory effects on carrageenan-induced rat edema, and inhibits the melanization of mouse melanoma B16 cell (Kojima *et al.*, 1991).

6.2. Production

6.2.1. *Production by* E. coli *Carrying* P. putida *Glyoxalase I Gene*

We have developed a method for production of *S*-D-lactoylglutathione by microbial systems. Because the ester is a product of the reaction of glyoxalase I, large-scale preparation of the enzyme is one of the major aims. The structural gene for *P. putida* glyoxalase I was cloned and expressed from its own promoter in *E. coli* C600 (Rhee *et al.*, 1987a). Glyoxalase I activity in transformed *E. coli* increased approximately 150-fold to give a specific activity of about 2 μmol/min/mg protein. Enzymatic production of *S*-D-lactoylglutathione was performed using cell extracts of *E. coli* expressing the *P. putida* glyoxalase I gene (Kosugi *et al.*, 1988). Approximately 37 mM (14 g/l) of *S*-D-lactoylglutathione was produced from 50 mM methylglyoxal and 100 mM glutathione at 37°C (pH 7.0) in 1 h. The conversion to glutathione was 37%. *S*-D-lactoylglutathione formed was, however, degraded in the reaction mixture after the reaction reached a maximum. It was probably due to enzymatic breakdown of the product, because the *E. coli* C600 had at least two kinds of glutathione thiolesterases that could be separated on DEAE-Sephadex A-25 column chromatography (Kosugi *et al.*, 1988). Moreover, the hybrid plasmid harboring the *P. putida* glyoxalase I gene was unstable in *E. coli* for unknown reasons (Inoue and Kimura, 1992a). Therefore, obtaining an ample supply of the enzyme without glyoxalase II activity was an important problem to be solved.

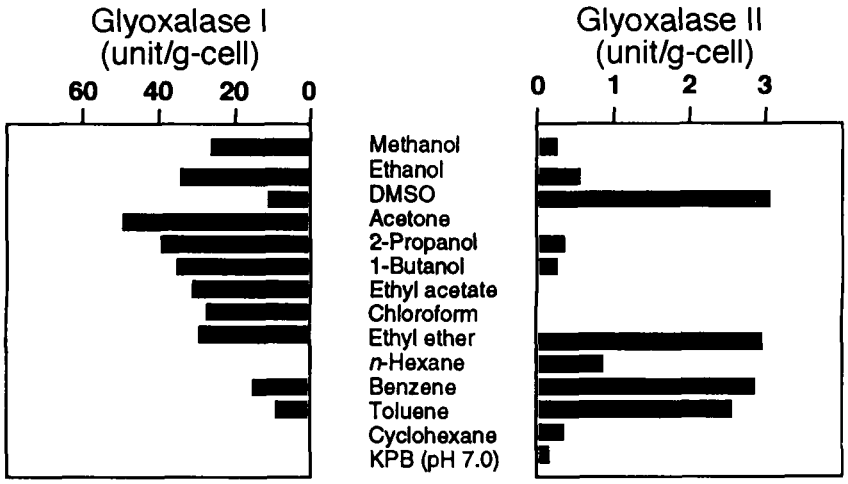


Figure 10 Effect of organic solvents on release of glyoxalase I and glyoxalase II from *H. mrakii*. One gram of the cells were suspended in 2 ml of 10 mM potassium phosphate buffer (pH 7.0) containing 50% organic solvent, and the mixture was incubated at 30°C for 10 h. After the removal of cells by centrifugation, the buffer solution was used as the source of enzyme.

6.2.2. *Production by Glyoxalase I Released by Organic Solvent from* H. mrakii

The glyoxalase system is a major route for detoxification of methylglyoxal in microorganisms, and amplification of glyoxalase I activity in microbial cells makes the cells resistant against toxicity of methylglyoxal (Rhee *et al.*, 1987a; Murata *et al.*, 1988). To obtain a potent producer of glyoxalase I, several yeast strains were screened for resistance against methylglyoxal, and the yeast *H. mrakii* IFO 0895 was selected (Inoue *et al.*, 1991a). *H. mrakii* could grow in a medium containing up to 25 mM methylglyoxal in which any other yeast strains tested could not grow, and glyoxalase I activity in the yeast was remarkably higher (1.5–2.1 μmol/min/mg protein) than in other microorganisms so far investigated (Inoue *et al.*, 1991a). To eliminate glyoxalase II, we treated the cells in a buffer containing several organic solvents and found that both acetone and ethyl acetate could efficiently release glyoxalase I, but not glyoxalase II, into the buffer at 30°C in 10 h (Fig. 10) (Inoue *et al.*, 1992c; Inoue and Kimura, 1992a). The specific activities of glyoxalase I released by acetone and ethyl acetate were 12 and 5.0 μmol/min/mg protein, respectively. By using these enzyme solutions enzymatic production of *S*-D-lactoylglutathione was performed and approximately 82 mM (30 g/l) of *S*-D-lactoylglutathione was produced from 120 mM glutathione (conversion rate, 68.3%).

6.2.3. *Production by Immobilized Glyoxalase I from* H. mrakii

Immobilized enzyme technology has been extensively used in various industrial fields. It is advantageous for the stability of the free enzymes and for the continuous production of the products. Partially purified glyoxalase I from *H. mrakii* was immobilized on DEAE-cellulose and silica gel, and continuous production of *S*-D-lactoylglutathione was examined. Many techniques have been studied for immobilization of the enzyme, but adsorption is the most simple and convenient method. Among the resins and gels tested, DEAE-cellulose and silica gel efficiently adsorbed the enzyme and the adsorbed enzyme was not eluted by a solution containing high concentrations of substrate (hemimercaptal, an equimolar mixture of methylglyoxal and glutathione) at least up to 100 mM. Binding of the enzyme to each carrier was different with respect to optimal pH, temperature and the ratio of methylglyoxal and glutathione in the reaction mixture. By packing the immobilized enzymes into a column, continuous production was performed and approximately 83 mM (30 g/l) *S*-D-lactoylglutathione could be produced from 100 mM glutathione (conversion rate, 83%) with a space velocity of $0.025\ min^{-1}$ (Inoue *et al.*, 1992d). When the concentration of both methylglyoxal and glutathione was fixed at 30 mM, 95% of glutathione could be converted to *S*-D-lactoylglutathione using silica gel immobilized enzyme at a space velocity of $0.03\ min^{-1}$. Because separation of *S*-D-lactoylglutathione from residual glutathione in the reaction mixture is difficult in large-scale production (Uotila, 1973a; Ball and Vander Jagt, 1979), it should be noted that we could perform a high conversion of glutathione to *S*-D-lactoylglutathione continuously. Operational stability of the silica gel immobilized enzyme was, however, not as high as we expected. Fifty per cent of the activity was lost after 6 h operation at a space velocity of $0.025\ min^{-1}$ with 30 mM methylglyoxal and 30 mM glutathione at 37°C. Exposure of the enzyme to high methylglyoxal concentrations was thought to inactivate the enzyme (Inoue *et al.*, 1993d).

6.2.4. *Production by Immobilized Cells of* H. mrakii

The use of immobilized cells is a common alternative to immobilized enzymes. To synthesize *S*-D-lactoylglutathione efficiently with immobilized *H. mrakii* cells, a treatment that increases the permeability of the cell for both substrate and product and simultaneously decreases glyoxalase II activity is preferable. Treatment of intact *H. mrakii* cells with hexamethylenediamine increased the permeability of the cells and decreased the glyoxalase II activity (i.e. degradation of *S*-D-lactoylglutathione was reduced). *H. mrakii* was then immobilized in a κ-carrageenan gel matrix followed by treatment with hexamethylenediamine. The concentration of

hexamethylenediamine was varied in combination with the treatment time; treatment with 80 mM hexamethylenediamine for 10 min most efficiently inactivated glyoxalase II without inactivating glyoxalase I in cells immobilized in the κ-carrageenan gel matrix. Under the optimal conditions (pH 6.0, 30°C, ratio of glutathione and methylglyoxal = 1.0), approximately 80% of glutathione could be continuously converted to *S*-D-lactoylglutathione at a space velocity of 1 h^{-1} for over 1 week (Inoue *et al.*, 1994); thus, operational stability was appreciably improved compared with that of the immobilized enzyme.

7. CONCLUDING REMARKS

We have clarified the metabolic fate of methylglyoxal through systematic studies using several microorganisms (Fig. 1), and we herein name it "*glycolytic methylglyoxal pathway*". Several investigators also termed this metabolism "glycolytic bypass" or "methylglyoxal cycle". There are insufficient data on the route by which glucose is metabolized to pyruvic acid in mutants deficient in the enzymes involved in glycolysis or in the glycolytic methylglyoxal pathway. Therefore, we do not know whether the glycolytic methylglyoxal pathway is an alternative to glycolysis at present. Neither the glyoxalase system (glyoxalase I and glyoxalase II) nor the reduction/oxidation system (methylglyoxal reductase and lactaldehyde dehydrogenase) is energy-generating. Oxidative conversion of methylglyoxal to pyruvic acid is also not energy-generating. These routes, therefore, are not an equivalent to glycolysis with respect to energy conservation.

It has been more than 80 years since methylglyoxal and its metabolic enzymes were discovered; however, the biological functions of methylglyoxal as well as its metabolic enzymes still remain to be discovered. The complexity of the regulation of metabolism of methylglyoxal implies that the glycolytic methylglyoxal pathway is not only a detoxification system in cells, but also may have some significant functions in the characteristic properties of living systems, i.e. growth, proliferation and differentiation.

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Molecular Responses of Microbes to Environmental pH stress

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1. INTRODUCTION

Bacteria display an amazing capacity to survive and grow in extremely hostile environments. Entire group of organisms have adapted their life-styles to prefer these extreme environments (e.g. thermophiles, halophiles, acidophiles, etc.). Even within a given group, a very wide range of environmental parameters may be tolerated. For most, the tolerance can be pushed to maximum limits if the cell is provided sufficient opportunity to sense and adapt to a deteriorating condition. Inducible tolerances to temperature, salt, DNA-damaging agents and oxidative stress are widely known and well studied (Little, 1993; Yura *et al.*, 1993; Farr and Kogoma,

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1991; Bukau, 1993; Csonka and Hanson, 1991). A lesser-known yet extremely important adaptation involves the bacterial response to extremes of pH. As an example, *Escherichia coli* and *Salmonella typhimurium* manage to grow in conditions (minimal medium) ranging between pH 5 and 8.5, which represents more than a 3000-fold range in H^+-ion concentration. These organisms can also survive from pH 4 to pH 9 (a 100 000-fold range) for extended periods of time. However, if the cells are first allowed to adapt to moderate acid or alkaline conditions before testing their limits, one finds they can survive over a 1 000 000-fold range of H^+-ion concentration! This capability can be very important to organisms both in natural or pathogenic situations where pH conditions can fluctuate dramatically (Renberg *et al.*, 1993).

The classic work of Gale and Epps (1942) revealed that many amino acid deaminases and decarboxylases of *E. coli* are induced by high and low pH, respectively. Their paper was one of the first to question how organisms sense and respond to low pH. In the 50 years since that seminal work, most studies on the biological aspects of pH have dealt with the concept of internal pH homeostasis, i.e. how cells maintain an internal pH (pH_i) different from the external environment (pH_o). With a few notable exceptions, little attention was given until recently to studying the genetics of pH regulation.

This article will review what is known about how microorganisms respond to pH stress focusing primarily on molecular aspects of adaptation in bacteria. Because excellent reviews on pH homeostasis have been published elsewhere, this topic will not be addressed in detail here (Booth, 1985; Padan and Schuldiner, 1987). Since the 1980s, many genes regulated by external pH have been identified with various functions. The amino acid decarboxylases, for example, produce alkaline products that help neutralize an acidic environment. However, the role of other pH-regulated genes is less apparent. Some are involved in virulence while others have no known function. Table 1 presents a list of the known pH-regulated genes identified in a variety of prokaryotic and eukaryotic microorganisms.

The mechanisms by which cells sense pH and subsequently alter transcriptional patterns is another important question to address. A mechanism commonly employed by bacteria in responding to environmental stimuli involves two-component signal transduction systems (Miller *et al.*, 1989; Ronson *et al.*, 1987). These systems are usually made up of two proteins, a sensor/transmitter protein and a regulator. The sensor/transmitter is a transmembrane protein with cytoplasmic and extracytoplasmic domains. The extracytoplasmic domain (sensor) senses the environment and transfers the signal through the transmembrane region to the cytoplasmic domain (transmitter). Upon receiving a signal, the transmitter domain uses an intrinsic kinase activity to phosphorylate the amino terminus of the second protein, the transcriptional regulator. Once phosphorylated, the regulator can bind DNA through its carboxyl-terminal region and regulate gene

Table 1 Genes regulated by pH.

Gene/protein	Process/function	Organism	Reference
aceF	Dihydrolipoamide	*E.coli*	Heyde and Portalier (1990) Hickey and Hirshfield (1990)
Acetaldehyde dehydrogenase	Acetate	*Sarcina*	Lower and Zeikus (1991)
aciA, B, J, I	?	*S. typhimurium*	Foster *et al.* (1994)
aniC, I, aciK regulon	Acid, tyrosine/phenylalanine induced	*S. typhimurium*	Foster *et al.* (1994)
Acid-induced proteins observed on gels	?	*A. tumefaciens*	Mantis and Winans (1992)
adh	Alcohol dehydrogenase	*E. coli*	Gale and Epps (1942)
adi	Arginine decarboxylase	*E.coli*	Auger *et al.* (1989)
		P. aeruginosa	Tabor and Tabor (1985)
			Bingham *et al.* (1990)
			Cunin *et al.* (1986)
agr and Agr-regulated genes	Regulation of exoprotein production	*S. aureus*	Regassa and Betley (1992)
alx	? Induced by alkaline pH	*E. coli*	Bingham *et al.* (1990)
aniG	? Co-induced by external acid and mannose	*S. typhimurium*	Aliabadi *et al.* (1988) Foster and Aliabadi (1989)
Arginine deiminase	Arginine catabolism	Streptococci	Cunin *et al.* (1986)
			Burne *et al.* (1991)
aidB	Alkalation-inducible, DNA damage	*E. coli*	Smirnova *et al.* (1994) Landini *et al.* (1994)
iroA	Acid, and iron repressed	*S. typhimurium*	Foster *et al.* (1994)
cadA, cadB	Lysine decarboxylase, lysine/cadaverine transporter	*E. coli,*	Auger and Bennett (1989)
		S. typhimurium	Auger *et al.* (1989)
			Meng and Bennett (1992a,b)
			Tabor and Tabor (1985)
			Watson *et al.* (1992)
			J. W. Foster *et al.* (in preparation)
cta/caa, oxidase	Terminal oxidase	*B. firmis* OF4	Quirk *et al.* (1991, 1993)
cydAB	Cytochrome *d*-type oxidase	*E. coli*	Cotter *et al.* (1990)
cyoABCDE	Cytochrome *o*-type oxidase	*E.coli*	Cotter *et al.* (1990

Table 1—continued

gadS	Glutamate decarboxylase	*E. coli*	Gale (1946)
groEL, dnaK, htpG, htpM, grpE	Stress proteins	*E. coli,* *S. typhimurium*	Abshire and Neidhardt (1993) Foster (1991) Heyde and Portalier (1990) Taglicht *et al.* (1987)
H^+-ATPase	ATP synthesis/hydrolysis	*E. faecalis*	Kobayashi *et al.* (1986)
hrp genes	Pathogenic response in plants	*P. syringae* pv. *phaseolicola,* *E. amylovora*	Rahme *et al.* (1992) Wei *et al.* (1992)
hyd	Hydrogenase-3	*E. coli*	Foster *et al.* (1994)
		S. typhimurium	Rossman *et al.* (1991)
inaA	?Induced by membrane-permeable weak acids	*E. coli*	Slonczewski *et al.* (1987) White *et al.* (1992)
katF (rpoS)	Stationary phase sigma	*E. coli*	Schellhorn and Stones (1992)
lamB	Maltose transport	*E. coli*	Heyde and Portalier (1987)
ldh	Lactate dehydrogenase	*E. coli*	Mat-Jan *et al.* (1989)
lexA	Repressor of DNA repair genes	*E. coli*	Dri and Moreau (1994)
lysU	Lysyl-tRNA synthetase	*E. coli*	Hickey and Hirschfield (1990) Hirschfield *et al.* (1984) Lévêque *et al.* (1991)
lysP (cadR)	Lysine permease	*E. coli*	Steffes *et al.* (1992)
malE	Maltose-binding protein	*E. coli*	Heyde *et al.* (1991)
menCD	Menaquinone synthesis	*B. subtilis*	Hill *et al.* (1990)
Metabolic switch from acidogenic to solventogenic fermentation	Production of butanol	*C. acetobutylicum*	Rodgers (1986)
nhaA	Na^+/H^+ antiporter	*E. coli*	Karpel *et al.* (1991) Rahav-Manor *et al.* (1992)
nmpC	Outer membrane protein	*E. coli*	Coll *et al.* (1994)
nodA, nodF	Nodulation	*R. leguminosarum biovar trifolii*	Richardson *et al.* (1988)
ompF, ompC	Porins	*E. coli,* *S. typhimurium,* *Thiobacillus*	Foster and Hall (1990) Heyde *et al.* (1991) Heyde *et al.* (1988) Heyde and Portalier (1987) Amaro *et al.* (1991)

pag genes	Virulence factors, macrophage survival	*S. typhimurium*	Miller (1991) Aranda *et al.* (1992)
Random *phm::phoA* gene fusions	?Membrane proteins	*E. coli*	Heyde *et al.* (1991)
	Phospholipase C	*S. aureus*	Marques *et al.* (1991)
polA	DNA polymerase I	*E. coli*	Hickey and Hirshfield (1990)
Proteins on 2-D gels observed by various low pH treatments	? Some involved in adaptation	*E. coli*, *S. typhimurium*, *A. hydrophila*	Abshire and Neidhardt (1993) Foster (1993) Foster and Hall (1990) Hassani *et al.* (1991) Heyde and Portalier (1990) Hickey and Hirshfield (1990) K. L. Karem *et al.* (in preparation)
proU	Glycine betaine transport	*S. typhimurium*	Thomas and Booth (1992) Karem and Foster (1993)
psaA	Virulence factor	*Y. pestis*	Lindler *et al.* (1990)
Pyruvate decarboxylase	Ethanol production	*Sarcina*	Lowe and Zeikus (1991)
SOS genes	DNA repair	*E. coli*	Schuldiner *et al.* (1986)
speF	Ornithine decarboxylase	*E. coli*	Kashiwagi *et al.* (1991) Tabor and Tabor (1985)
Sporulation		*Sarcina*	Lowe *et al.* (1989)
sulA	Cell division	*E. coli*	Smirnova *et al.* (1994)
ToxR regulon (*taxA*, *aldA*)	Virulence factors	*V. cholerae*	DiRita (1992) DiRita *et al.* (1991) Mekalanos (1992) Parsot and Mekalanos (1991) Chen and Winans (1991)
vir genes	Bacterial–host interactions	*A. tumefaciens*	Mantis and Winans (1992a,b) Stachel *et al.* (1986) Turk *et al.* (1991) Winans (1990, 1992)
Xylanase		*Thermomyces lanuginosus*	Purkarthofer *et al.* (1993)

expression. The transmitter and receiver domains (the kinase and its target) are highly conserved in all signal transducing systems and are considered to be the core of these systems (for review, see Gross *et al.*, 1989).

Some form of transmembrane signaling must also occur in response to external pH fluctuations in order to alter specific gene expression. There are four obvious potential signals. They are external pH (pH_o), internal pH (pH_i), ΔpH ($pH_o - pH_i$) or proton motive force (Δp). The exact nature of the signal, however, remains a mystery. There is some evidence that all three of these parameters can be used to initiate changes in gene expression. The questions of physiological relevance and signal transduction mechanisms should be kept in mind as we address the molecular aspects of known pH-regulated genes and inducible pH-stress resistance systems.

2. pH-REGULATED GENE EXPRESSION

2.1. pH-Regulated Genes of Known Function

2.1.1. *pH Homeostasis*

Many microorganisms strive to maintain a stable internal pH (e.g. 7.6–7.8 for *E. coli* and *S. typhimurium*) over a wide range of external pH conditions (Booth, 1985; Slonczewski *et al.*, 1981; Padan *et al.*, 1976, 1981; Zilberstein *et al.*, 1984). This phenomenon, termed pH homeostasis, is possible because of the low proton conductance of biological membranes and the presence of proton-driven transporters which either bring protons into the cell at basic external pH or extrude them out of the cell at acidic external pH. Although pH homeostasis does not require induction in all microorganisms (Foster and Hall, 1991; Zilberstein *et al.*, 1982), the expression of some pH homeostasis components is influenced by pH. Several major systems located in the cytoplasmic membrane influence proton circulation and can potentially contribute to pH homeostasis (for reviews, see Padan *et al.*, 1981; Booth, 1985; Padan and Schuldiner, 1992). (1) The K^+/H^+ antiport, K^+ transport and Na^+/H^+ antiport systems couple H^+ movement to the transport of K^+ and Na^+ (Brey *et al.*, 1979; West and Mitchell, 1974). K^+/H^+ antiport is thought to raise the internal pH of the cell in an acidic environment (Slonczewski *et al.*, 1981; Booth, 1985) while Na^+/H^+ antiport will lower the internal pH in an alkaline environment (Ishikawa *et al.*, 1987; Karpel *et al.*, 1988; Gerchman *et al.*, 1993). (2) The F_1F_0 proton-translocating ATPase activity couples H^+ movement with the synthesis and hydrolysis of ATP. (3) Electron transport chains and H^+-ATPase extrude protons and are responsible for the generation of proton motive force. (4) Lastly, there are various

transport systems that couple proton flow with the import of other solutes. Within these major controlling systems of proton flow are several examples of pH or H^+ regulated gene expression.

The *nhaA* locus of *E. coli* encodes a membrane bound Na^+/H^+ antiporter that has been extensively characterized (Goldberg *et al.*, 1987; Karpel *et al.*, 1988, Taglicht *et al.*, 1991). This system is thought to be required for normal pH homeostasis and NaCl tolerance (Padan *et al.*, 1989). It is an electrogenic system that at pH 8.4 will pump 2 H^+ in for every Na^+ pumped out (Taglicht *et al.*, 1993). Mutants lacking this antiporter are unable to grow at alkaline pH_o (Ishikawa *et al.*, 1987). The activity of NhaA changes more than 1000-fold between pH7 to 8. His226 of NhaA is part of the pH sensor that triggers activation of this protein. Transcription of *nhaA* also increases at alkaline pH (>8.0) but in a manner dependent on the presence of Li^+ or Na^+ (Karpel *et al.*, 1991). Increasing pH in the absence of these ions does not induce *nhaA*. However, the higher the pH, the lower is the amount of Na^+ required for induction. The regulation of *nhaA* is dependent on a 28 kDa transcriptional activator NhaR, belonging to the LysR class of regulators (Rahav-Manor *et al.*, 1992). The *nhaR* locus is located immediately downstream of *nhaA*, possibly forming an operon. Activation of *nhaA* is greatly enhanced in the presence of multicopy *nhaR* plasmids in an Na^+-dependent manner, suggesting that NhaR requires Na^+ in its function. NhaR appears to bind to sequences upstream of *nhaA* confirming its role in activating the gene. pH may indirectly affect induction of *nhaA* by influencing intracellular Na^+ concentrations. At alkaline pH the cell cannot maintain as high an Na^+ gradient as it can at acid pH (Pan and Macnab, 1990). Thus, the higher level of intracellular Na^+ could trigger *nhaA* induction. Another locus coding for an antiporter, *nhaB*, has been identified but, unlike *nhaA*, it is independent of pH and *nhaR* control (Pinner *et al.*, 1992; Rahav-Manor *et al.*, 1992). However, a recent report finds that NhaB is essential for regulating intracellular pH under alkaline conditions (Shimamoto *et al.*, 1994). NhaA is expendable at alkaline pH_o, at least under low sodium conditions.

Membrane-associated F_1F_0 ATPases commonly couple proton movement into the cell with ATP synthesis, or extrude protons at the expense of ATP. In *E. coli*, this enzyme is generally thought to be constitutive and unregulated by environmental factors (Jones *et al.*, 1983). On the other hand, *Enterococcus faecalis* possesses an F_1F_0-ATPase that does not function in ATP synthesis, but does extrude protons in response to acidification (Kobayashi *et al.*, 1986; Bender *et al.*, 1986; Bender and Marquis, 1987; Sturr and Marquis, 1990). As the internal pH of the cell decreases, both the synthesis and activity of its proton-translocating ATPase increases, and protons are extruded more aggressively. This compensatory response in ATPase levels restores the internal pH to neutrality. To elucidate how pH regulates ATPase

biosynthesis, two acid-sensitive mutants of *E. faecalis* were isolated (Suzuki *et al.*, 1988). Upon acidification, one mutant synthesized F_0 but not F_1, resulting in a proton leaky F_0 pore-containing membrane. The second mutant produces a defective ATPase that hydrolyzes ATP but fails to translocate protons. While these acid-sensitive mutants provide a genetic link between pH homeostasis and regulation of ATPase synthesis, a closer examination of this system at the molecular genetic level should yield some clear insight into pH signal transduction mechanisms.

Electron transport systems contribute to proton circulation by generating proton motive force. The *cyo* (*cyoABCDE*) and *cyd* (*cydAB*) operons of *E. coli* encode electron transport systems that use oxygen as a terminal electron acceptor. The transcription of these operons is regulated by oxygen availability and by pH. Low oxygen tension induces the high-affinity cytochrome *d* system (*cydAB*) and represses the low-affinity cytochrome oxidase *o* complex (*cyoABCDE*) (Ingledew and Poole, 1984; Cotter *et al.*, 1990; Iuchi *et al.*, 1990; Fu *et al.*, 1991; Iuchi and Lin, 1991; Cotter and Gunsalus, 1992). Two regulators, ArcA and Fnr, mediate repression of the oxidase systems and are involved in the activation and repression of many other genes as a function of oxygen levels (Cotter and Gunsalus, 1992; Cotter *et al.*, 1990; Fu *et al.*, 1991; Gunsalus, 1992; Ingledew and Poole, 1984; Iuchi *et al.*, 1990; Iuchi and Lin, 1991; Gunsalus, 1992; Iuchi *et al.*, 1990). These regulators act independently to repress the *cyo* operon; loss of either regulator results in *cyo* derepression under anaerobic conditions. The involvement of pH in regulating the *cyo* operon has been studied under aerobic and anaerobic conditions and in the presence or absence of Fnr (Cotter *et al.*, 1990). The *cyo* operon is repressed by acid. Under aerobic conditions, an Fnr-independent fourfold repression of *cyo* occurs at pH 6.0 compared with pH 7.0. Anaerobically, expression of *cyo* is completely repressed below pH 7.5 in the presence of Fnr. However, in the absence of Fnr, total repression only occurs below pH 6.0. This suggests that a *cyo* repressing mechanism distinct from Fnr functions below pH 6.0. In contrast to *cyo*, the expression of the *cyd* operon is induced by acid under anaerobic but not aerobic conditions. Anaerobically, *cyd* expression is acid-induced two-fold in the presence of Fnr and undergoes a fourfold induction at pH 5.5 compared with pH 7.5 in the absence of Fnr. Whether ArcA or a separate regulatory system is involved in the pH regulation of these operons remains to be shown.

Bacillus firmus OF4 is an alkaliphile, growing well over a pH range extending from 7.5 to above 10.5. It possesses a branched respiratory chain with at least three terminal oxidases: cytochrome *o*, cytochrome *d* and cytochrome caa_3. The caa_3 oxidase is present at concentrations two- to threefold higher in membranes of cells grown at pH 10.5 than at pH 7.5. The gene encoding this oxidase, *cta*, is induced at alkaline pH (Quirk *et al.*, 1991, 1993). The signal in this case seems to be related to low pmf (proton

motive force) because *cta* is also induced at pH 7.5 if ΔpH is collapsed through the addition of the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). It is hypothesized that there is a unique mechanism for coupling proton movement with the generation of ATP by the F_1F_0 ATP synthase, because at pH 10.5 the low Δpmf is not sufficient to account for the observed phosphorylation potential (Guffanti and Krulwich, 1992). The proposed mechanism involves the direct, intramembranal transfer of protons from respiratory chain complexes to the F_0 sector of the ATP synthase. It was further proposed that an increased level of a respiratory chain complex was required. The caa_3-type oxidase may fulfill this role.

In the filamentous fungus *Aspergillus nidulans*, several enzymes with extracellular exposure are regulated by external pH. For example, under acid conditions, acid phosphatase levels are high whereas alkaline phosphatase levels are low. The reverse is true when the organism is grown under alkaline conditions. Caddick *et al.* (1986) have identified several mutants with altered pH responses. Mutations in five genes, *palA*, *B*, *C*, *E* and *F*, mimic the effect of growth at acidic pH in that they are deficient in alkaline phosphatase whereas mutations in *pacC* mimic the effects of growth at alkaline pH and cause deficiencies in acid phosphatase. A later study also revealed that production of the secondary metabolite, penicillin, is overproduced in *pacC* mutants but is not produced by *pal* mutants (Shah *et al.*, 1991). It is suggested that the *pacC* product is a global regulator whose product directly mediates pH regulation of gene expression. The deduced PacC sequence contains three putative zinc-fingers, supporting a role for PacC as a transcriptional regulator (R. Tilburn and H. Arst, personal communication). Pal A, B, C, E and F are proposed to participate in the synthesis of an effector molecule that may interact with PacC. The *pal* mutations would interfere with the pH sensing system of *Aspergillus* such that the cell "thinks" it is experiencing low external pH and would respond accordingly. The consequences of this would include a deficiency in alkaline phosphatase and an abnormal rise in internal pH as the cells overcompensate. This latter prediction was demonstrated experimentally (Caddick *et al.*, 1986). The opposite scenario is proposed for *pacC* mutants in which cells grown under neutral pH conditions act as though they are experiencing alkaline external pH. This has been confirmed at the molecular level for *ipnA*, encoding isopenicillin N synthetase (Espeso *et al.*, 1993). Alkaline pH_o or *pacC* mutations increase the level of *ipnA* message.

Although essential for the survival of most organisms and despite intense scrutiny, the process of pH homeostasis, for the most part, remains an enigma. As more becomes known about the component parts, details regarding the genetics of this phenomenon will become clearer. Presumably, pH-dependent genetic control of some systems will facilitate the cellular effort to maintain internal pH.

2.1.2. *Decarboxylases, Deaminases and Deiminases*

The genes encoding many decarboxylases and deaminases are believed to be induced by acidic and basic conditions, respectively (Gale and Epps, 1942; Gale, 1946). The best studied are the decarboxylases. However, before discussing these inducible systems, a distinction must be made between inducible and uninducible decarboxylases. Biosynthetic decarboxylases such as arginine encoded by (*speA*) and ornithine encoded by (*speC*) decarboxylases in *E. coli* are constitutively produced in low amounts and are unaffected by pH fluctuations (Tabor and Tabor, 1985). In contrast, biodegradative decarboxylase genes such as those for lysine (*cadA*) and arginine (*adi*) are induced by acid pH. *cadA* requires co-induction by lysine while *adi* has a complex co-inducer requirement (Gale, 1946; Tabor and Tabor, 1985; G. Bennett, personal communications). An inducible ornithine decarboxylase (*speF*) has also been identified (Kashiwagi *et al.*, 1991). The pH inducible enzymes are thought to help organisms survive pH stress by neutralizing their environmental pH or in some way maintaining pHi. For example, the arginine deiminase enzyme complex of *Streptococcus* produces ornithine, ammonia and CO_2. The system is induced by arginine and repressed by low pH (Cassiano-Colón and Marquis, 1988; Burne *et al.*, 1991). Recently, arginine decarboxylase of *E. coli* was shown to be an important defense against severe acid stress, i.e. pH 2.5 (J. Lin et al., 1995).

Perhaps the decarboxylase studied in greatest detail is lysine decarboxylase (Tabor *et al.*, 1980; Auger *et al.*, 1989; Watson *et al.*, 1992; Meng and Bennett, 1992a,b). It is a 715 amino acid protein (78 kDa) which contributes to pH homeostasis by converting exogenously supplied lysine to cadaverine, an alkaline product that, when secreted from the cell, neutralizes an acidic environment (Sabo *et al.*, 1974; Gale, 1946). An antiporter for lysine/cadaverine is encoded by *cadB* (444 amino acids, 49 kDa) while the decarboxylase itself is encoded by *cadA* (Meng and Bennett, 1992a). These loci form an operon located at 93.5 min in *E. coli* (Auger *et al.*, 1989; Meng and Bennett, 1992a) and 55 min in *S. typhimurium* (Foster *et al.*, 1994). A model has been proposed where under high external lysine and H^+ concentrations, the CadB antiporter will import lysine and export cadaverine, the product of lysine decarboxylase. The *cadBA* operon is induced by low pH in the presence of lysine in a manner dependent on an activator protein encoded by *cadC* (Watson *et al.*, 1992). The promoter for *cadBA* (*Pcad*) is located 75 bp upstream of *cadB*, while *cadC* is found 293 bp upstream from *Pcad*. The promoter for *cadBA* has a sigma −70 consensus −10 sequence but a poor −35 region, attributes common to positively activated promoters (Meng and Bennett, 1992a; Watson *et al.*, 1992). Transcriptional activation of *cadBA* is thought to occur through an interaction between CadC and the *Pcad* DNA region. Using point mutation analysis and *lacZ* fusion techniques,

Meng and Bennett (1992b) localized a region required for pH induction of *cadBA*. A 66 bp fragment located upstream (position −97 to −161 bp) of *cadBA* was found to compete for a factor required for *Pcad* activation when placed on a high copy number plasmid. DNA footprinting and complementation studies suggest that this region contains the CadC binding site (Meng and Bennett, 1992b). Sequence analysis of *cadC* revealed an amino terminus showing significant homology to the DNA-binding domain of several bacterial transcriptional activators involved in two-component transduction systems (Watson *et al.*, 1992). Of particular interest is homology to the amino terminus of ToxR from *Vibrio cholerae* (see below). In fact, the model for CadC activation of *cadBA* in response to low pH is based primarily on sequence similarity to ToxR (Watson *et al.*, 1992). CadC, like ToxR, is a transmembrane protein lacking the kinase activity commonly seen in sensory/transmitter proteins of two-component transmembrane signaling systems. The OmpR-like DNA binding domain (amino terminus) would be located in the cytoplasm with one or more regions located on the outer face of the cytoplasmic membrane or in the periplasm. The outer domain(s) would sense environmental changes (external pH) resulting in the ability of the carboxyl terminal end (cytoplasmic) to bind its DNA target site and induce *Pcad* activity (Watson *et al.*, 1992). The transcriptional regulation of *cadC* itself appears to be independent of pH, while the activity of CadC is pH sensitive (Watson *et al.*, 1992; Dell *et al.*, 1994). Although it is essential for pH induction of *Pcad*, CadC is not involved in the regulation of other pH-sensitive loci. This indicates that CadC is not a global regulator of acid-induced genes (Olson, 1993).

Another component involved in the lysine requirement for *cadBA* induction is the lysine transport protein LysP (*cadR*). A role for LysP in the regulation of *cadBA* has been proposed based on the observation that low pH induction of *cadBA* in a *lysP* mutant is lysine-independent (Tabor *et al.*, 1980; Neely *et al.*, 1994). LysP is itself induced by the same conditions as *cadBA* (Steffes *et al.*, 1992). Whether CadC regulates *lysP* or some communication occurs between the two proteins are two questions that need to be addressed. Recent evident showing that overproduction of LysP prevents *cadBA* induction suggests that LysP is a negative regulator of *cadBA* (Dell *et al.*, 1994). Two models are possible. One is that the level of internal or external lysine determines CadC activity and that LysP regulates the concentration of lysine in the two compartments. The second possibility is that LysP itself regulates CadC activity and that lysine regulates the activity of LysP. Data are not available yet that would distinguish between these possibilities. Mutations have been isolated in *cadC* that separate the pH and lysine signals. Some of the mutants retain pH dependence while others retain lysine dependence (Dell *et al.*, 1994). This supports a model in which both pH and lysine signals are mediated by CadC and where CadC probably exists in two different conformations dependent on pH.

The biodegradative arginine decarboxylase (*adi*) of *E. coli* converts arginine to agmatine and has several properties in common with lysine decarboxylase (*cadA*). Both are induced under anaerobic, acidic conditions in complex medium and can increase the surrounding pH by removing acidic carboxyl groups and releasing CO_2 from their substrates. As yet, an *adi* homolog to the CadB antiporter that could exchange arginine with its decarboxylase product has not been found. Both decarboxylases also contain pyridoxal 5′ phosphate as a co-factor. The *adi* gene encodes a 755 amino acid protein of molecular mass 84 420 Da and shares 35% homology with lysine decarboxylase (Stim and Bennett, 1993). However, whereas *cadBA* is regulated by at least one transacting regulator (CadC), no unique regulatory gene products have been shown to interact with *adi*, but recently, Shi and Bennett (1994) have discovered that *adi* may be under positive control by the *cysB* regulator. This raises interesting questions about the role of redox state in *adi* control. In addition, integration host factor (IHF) is required for *adi* expression (K. Stim, M. Rives, B. Waasdorf and G. Bennett, personal communication).

2.1.3. *Fermentation*

Correlations between environmental pH and shifts in fermentation patterns have been known for some time (Clark, 1989; Lowe and Zeikus, 1991; Hartmanis and Gatenbeck, 1984; Gottwald and Gottschalk, 1985; Bobillo and Marshall, 1992). For example, *Clostridium acetobutylicum* switches from "acidogenic" to "solventogenic" fermentation as pH_o reaches 4.3 to 5.0 (Davies and Stephenson, 1941; reviewed in Rodgers, 1986). Where this has been studied in some depth, it is becoming increasingly apparent that the switch involves pH-regulated transcriptional control. At least two genes associated with fermentation in Gram-negative organisms are jointly regulated by pH and anaerobiosis. These are the fermentative lactate dehydrogenase (*ldh*) and the hydrogen-evolving *hyd* loci (Mat-Jan *et al.*, 1989; Foster *et al.*, 1994; Pecher *et al.*, 1983; Schlensog and Böck, 1990). While little is known of *ldh* control, several studies have explored *hyd*. As part of the formate–hydrogen lyase system, hydrogenase-3 is thought to aid in the maintenance of pH_i through the evolution of H_2 gas and the dissimilation of formic acid. The *hyd* locus includes two divergently transcribed operons called *hyc* and *hyp* and requires anaerobiosis, formate and low pH for induction (Böhm *et al.*, 1990). Formate induction of the *E. coli hyd* cluster is regulated by FhlA and sigma-54 (NtrA), the alternative sigma factor used for nitrogen metabolism. Rossmann *et al.* (1991) suggest that pH affects *hyd* expression by regulating formate import and export, perhaps through a pH-sensitive transporter. Recent studies with *S. typhimurium*, however, suggest an additional control that may be influenced by pH. The iron regulatory protein Fur is essential for the expression of

hyd (Foster *et al.*, 1994). Modulating Fe^{2+} levels alone (Fe^{2+} is the co-regulator for Fur) will only partially compensate for pH control, even in the presence of excess formate. Low pH is still required for maximal expression. The fact that *fur* mutants are defective in acid tolerance (see below) suggests that pH may influence the activity of Fur as a regulator of some genes.

2.2. pH-Regulated Genes of Unknown Function

Several studies have sought to explore the complexities of pH-regulated gene expression by searching for pH-regulated genes without regard to function. These screens included the use of reporter gene fusions (e.g. *lacZ*) and two-dimensional SDS-PAGE analysis of low pH-regulated polypeptide synthesis (Foster *et al.*, 1994; Slonczewski *et al.*, 1987; Foster and Hall, 1991; Foster, 1991, 1993; Heyde and Portalier, 1990; Hickey and Hirshfield, 1990). The reporter gene fusion studies have revealed several genes in *E. coli* and *S. typhimurium* regulated by either low pH_o or pH_i. The characterization of these genes will reveal much about the mechanisms of pH-regulated gene expression.

A clever selection strategy involving *lacZ* fusions was used by Slonczewski *et al.* (1987) to identify genes induced by low internal pH. They used benzoate in their medium which, as a weak acid, will penetrate the membrane in its protonated form then deprotonate within the cell causing internal acidification. An internal acid (*inaA*)-induced locus was identified using this approach. The expression of *inaA* occurs in response to several different aromatic weak acids and uncouplers with only a weak response observed to low pH_o, indicating pH_i to be the likely inducing signal. The locus encodes a 168 amino acid polypeptide (White *et al.*, 1992). The role of *inaA* in the physiology of *E. coli* is not yet known; however, it is under control of *marRA*, the multiple drug resistance locus (Rosner and Slonczewski, 1994).

At least 15 pH-regulated genes have been identified using *lacZ* fusions in *S. typhimurium*. Some of these loci (*ompC/F*, *hyd*, *cadA*) have also been described in *E. coli* and their functions are known (Thomas and Booth, 1992; Heyde and Portalier, 1987, 1990; Mat-Jan *et al.*, 1989; Auger *et al.*, 1989). Most of the others are of unknown function. As an example, the *aniC*, *aniI*, *aciK* loci form a regulon under the control of OxrG and are co-induced by low pH, anaerobiosis and tyrosine or phenylalanine (Foster *et al.*, 1994). Another example, *aniG*, will be discussed in more detail below.

Several pH-regulated genes that encode exported or membrane proteins were discovered using *phoA* (alkaline phosphatase) as a reporter gene (Heyde *et al.*, 1991). PhoA displays activity only if exported to the periplasm where it can be folded properly. The transposon Tn*phoA* can, on insertion, create gene fusions that produce chimeric proteins in which the amino portion

of a target gene product is fused to PhoA. If the targeted gene product is an exported or membrane protein and the fusion occurred at a point that will allow PhoA to gain access to the periplasm, the mutant will display alkaline phosphatase activity. Several alkaline pH-induced loci have been identified in *E. coli* using this strategy. Called *phm* (pH modulated), these genes were also regulated by other environmental conditions such as osmolarity and anaerobiosis. While the functions of these genes remain a mystery, their identification illustrates the rather broad impact pH has on gene expression.

2.2.1. *The aniG Locus of* Salmonella

One of the earliest pH-sensitive genetic systems to be identified in *Salmonella* was the *aniG* locus and its negative regulator EarA (Aliabadi *et al.*, 1988, 1988; Foster and Aliabadi, 1989). The functions of the products of "aniG" (actually two genes, see below) are unknown, but the recent discovery that this locus is specific to *Salmonella* suggests it may have a unique role in the physiology of this enteric pathogen (K. Karem and J. Foster, unpublished). The *aniG* locus was first identified through the use of MudJ (*lacZYA*) operon fusions into the *Salmonella* chromosome (Aliabadi *et al.*, 1986). Detailed induction studies revealed an absolute co-requirement for external acid and D-mannose in the regulation of this locus (Foster and Aliabadi, 1989). The *aniG* locus actually comprises two genes designated *amiA* and *amiB* (*a*cid and *m*annose *i*nduction). AmiA is a 40 kDA periplasmic protein whose export and/or processing is apparently facilitated by the *amiB* product (K. L. Karem and J. W. Foster, unpublished).

The *earA* (external acid regulator) locus encodes a 33 kDa negative regulator of *aniG* (Aliabadi *et al.*, 1988; Foster and Aliabadi, 1989). The mechanism of *aniG* regulation by EarA has not been fully elucidated, but there is evidence that pH affects regulation in two ways: first, by altering the affinity of the EarA repressor for the *aniG* operator region; and second, be altering the topology of operator DNA for the repressor (Karem and Foster, 1993). This latter aspect will be addressed below. A second regulatory locus, *earB*, resides upstream of *earA*. However, the role of *earB* in *aniG* control is not well defined.

2.3. pH-Regulated Genes Involved in Pathogenesis

There are a variety of pathogenic situations in which low pH occurs. These acidic conditions can occur in the stomach, parts of the intestine, abscesses, and, for some intracellular parasites, phagosomes and phagolysosomes. The relevance of acid adaptation and acid-regulated gene expression to microbial pathogenesis is becoming more apparent in studies designed to investigate

environmental "virulence signals". The low pH encountered by a variety of pathogens may act as an important signal for the induction of genes required for infection and disease. One virulence system that responds to pH is the *phoP/phoQ* regulon of *S. typhimurium*. *S. typhimurium* is capable of growth within macrophage phagolysosomes and, in fact, uses macrophages as a vehicle for dissemination (Finlay and Falkow, 1989). The *phoP/phoQ* two-component regulatory system is required for resistance to defenses and virulence within host phagocytic cells (Fields *et al.*, 1989). PhoQ is thought to be the environmental sensor of the phagocytic environment which, through PhoP, alters transcription of target genes. These target genes include several associated with survival within the phagocytic environment. Recently, Aranda *et al.* (1992) showed that the *phoP* regulon, comprised of *P*hoP *a*ctivated *g*enes (*pag*), is induced up to 77-fold within the phagosomes of murine bone marrow-derived macrophages, but surprisingly is not induced within cultured epithelial cells. The induction is maximal when the pH of the phagosome drops below 5.0 and can be abolished with the addition of weak bases. *In vitro*, *pag* gene expression also increases as pH drops below 5.2, although to a lesser extent than seen intracellularly (Aranda *et al.*, 1992). This confirms that *pag* gene expression is regulated by low pH. The PhoP/Q system is thought to protect cells from antimicrobial cationic peptides present within macrophage phagolysosomes, but it may also be an important defense against pH stress. *S. typhimurium* is exposed to an intraphagocytic pH of approximately 5.5 for several hours post-invasion in epithelial cells and pH 4.0–4.5 in macrophage phagolysosomes. Because expression of *pag* loci is essential for intramacrophage survival and virulence, it has been suggested that products of the *pag* loci promote intracellular survival at low pH. The fact that mutations in PhoP result in decreased acid resistance supports this hypothesis (Foster and Hall, 1990). During exposure to acidic conditions within the phagosomes, cells may also undergo induction of the acid tolerance response (see below), further aiding survival within acidic phagolysosomes (Aranda *et al.*, 1992).

In addition to the above, *S. typhimurium* exhibits another low-pH-controlled virulence characteristic associated with intracellular parasitism. As *S. typhimurium* invades an epithelial cell, it is engulfed within a membrane-bound vacuole (phagosome) that within 2 h contains lysosomal markers indicating phagosome–lysosome fusion. Once in this vacuole, the organism appears to commandeer components of the microtubule network of the host cell to form long, filamentous structures that often connect different groups of intracellular bacteria within a single epithelial cell. The filaments are synthesized under the direction of *S. typhimurium* genes apparently regulated in response to acidification within the vacuole. Inhibition of vacuole acidification prevents filament formation (Garcia-del Portillo *et al.*, 1993).

The *psaA* locus of *Yersinia pestis*, another facultative intracellular pathogen, encodes the pH 6 antigen, a virulence factor proposed to be a

fimbrial protein (Lindler and Tall, 1993; Lindler *et al.*, 1990). Induction of *psaA* occurs in response to pH conditions below 6.7 and a temperature of 36°C, suggesting that induction may occur in host macrophage phagolysosomes or in extracellular abscesses such as buboes (Lindler *et al.*, 1990). Several lines of evidence support acidic induction of *psaA*. First, induction of *psaA* occurs upon intracellular acidification of a macrophage-like cell. Secondly, treatment of *Y. pestis*-infected macrophage cultures with monensin (an ionophore that disrupts the acidification of intracellular compartments) prevents this induction. Third, the addition of monensin to cultures of *Y. pestis* grown at pH 6.0 *in vitro* has no effect on *psaA* acid induction (Lindler and Tall, 1993; Vodopianov *et al.*, 1990). These data further support the concept that pH is an important virulence stimulus. Mutations in *psaA* result in a 200-fold decrease in virulence compared with wild-type *Y. pestis* when administered intravenously to BALB/c mice. After acidic induction of *psaA* intracellularly, the *psaA* product aggregates into fimbriae organelles (Lindler and Tall, 1993; Vodopianov *et al.*, 1990). Production of these fimbriae may allow the pathogen to interact with uninfected macrophages or other host cells after release of the bacteria from infected cells. Mutations in the *psaB* locus result in lower levels of PsaA fimbriae formation, and sequence analysis suggests that *psaB* is a molecular chaperon protein (Lindler and Tall, 1993). Insertional mutations in another locus, *psaE*, significantly reduce *psaA* expression without eliminating temperature or pH regulation. Unlike *psaA*, expression of *psaE* is independent of pH and temperature. Consequently, one must predict a separate regulatory mechanism that will mediate pH control of *psaA*.

The *Yersinia enterocolitica invA* locus, which encodes the protein invasin, is also regulated by pH but only at 37°C (Pepe *et al.*, 1994). Invasin is a surface protein involved with the invasion process into mammalian cells. Expression of *invA* is elevated at 28°C and reduced at 37°C at neutral pH. However, lowering pH_o to 5.5 triggers high levels of *invA* expression at 37°C. This is significant considering that the organism may induce an acid environment after colonizing the terminal ileum. Metabolism of unabsorbed carbohydrates to volatile fatty acids could dissipate bicarbonate and thereby decrease local pH from 7.8 to $\leqslant$ 6.0 (Sleisenger, 1981). It is also interesting to note that low pH induction of *invA* at 37°C only occurs under anaerobic conditions, another condition present in the intestine (Pepe *et al.*, 1994).

A virulence regulator of *V. cholerae*, *toxR*, also shows aspects of pH activation. ToxR is a transmembrane sensor protein which uses its cytoplasmic domain to bind promoter regions of virulence factor genes. ToxR is a deviation from typical two-component systems because as a single protein it does not require the transmitter and receiver domains typical of two-component systems. The ToxR system further differs in that the amino terminus is cytoplasmic and binds DNA, a role normally carried out by the carboxyl terminal domains of regulatory proteins. ToxR regulates a series

of genes, defined as the ToxR regulon, that include several virulence loci (Parsot and Mekalanos, 1991; Peterson and Mekalanos, 1988). Included are genes encoding cholera toxin (*ctxAB*), TCP pilus production (*tcp*), aldehyde dehydrogenase (*aldA*), accessory colonization factor (*acf*), outer membrane proteins (OmpU and OmpT) and the *tag* loci (identified by *phoA* analysis) (Parsot and Mekalanos, 1991; Peterson and Mekalanos, 1988). This system is complex, involving two other regulators, ToxT and ToxS, and multiple environmental signals (DiRita, 1992). Induction of the ToxR regulon occurs during growth at pH 6.5 but not at pH 8.4. The mechanisms involved in the pH regulation of the ToxR regulon are not yet defined.

Another pH-influenced gene important to virulence is the accessory gene regulator *agr* of *Staphylococcus aureus*. This gene is involved in the production of several exoproteins and cell surface-associated proteins. An Agr^+ cell produces more α-hemolysin, β-hemolysin, toxic shock syndrome toxin and staphylococcal enterotoxin types B, C and D, and less coagulase and protein A than does its *agr* mutant derivative (reviewed in Regassa and Betley, 1992). Thus Agr appears to function both as an activator and repressor. Several reports indicate that expression of *agr* itself is regulated by environmental pH. The *agr* locus actually includes two divergently transcribed genes producing transcripts called RNAII (3.5 kb) and RNAIII (0.5 kb). The RNAII transcript contains four open reading-frames, *agrABCD* (Novick *et al.*, 1989). AgrA and AgrB show sequence homology to members of the two-component signal-transducing systems, which suggests that *agr* may respond to environmental stimuli (Kornblum *et al.*, 1990). However, RNAIII appears to be the component responsible for altering target gene expression. Northern blot analysis of RNAIII message revealed maximal expression at pH 7.0, about one-half as much expression at pH 6.5 and almost no expression at pH 8.0. A similar pattern of expression was noted for target genes of Agr (Regassa and Betley, 1992). Clearly there is a dramatic alkaline repression of the Agr system. But again the mechanism for this control is not well understood.

Plant pathogens such as *Agrobacterium tumefaciens* and *Pseudomonas syringae* also have pH-regulated virulence loci. In *Agrobacterium*, expression of genes located on the Ti plasmid are required for tumor formation and DNA transfer to the host. These Ti virulence genes, *vir*, are transcriptionally regulated by VirA and VirG, a two-component sensor/regulator system which uses a histidine-kinase (VirA) to trigger activation. The activities of these regulators are controlled by environmental conditions such as acidification, temperature, and chemicals such as acetosyringone secreted from plant wounds (Winans, 1992). There appear to be two pH-mediated responses in this organism: the VirA/VirG-independent transcription of *virG*, and the subsequent VirA/VirG-dependent transcription of *vir* operons. In both cases, the genes are induced in response to low pH (Mantis and Winans, 1992; Chen and Winans, 1991; Winans, 1990; Winans *et al.*, 1988; Stachel *et al.*, 1986).

It is not enough simply to increase VirG levels; *vir* operon induction requires high VirG and low pH (Chen and Winans, 1991). VirA is known to be involved in the low pH signaling because deletions of its periplasmic region prevent the ability to respond to low pH while the system remains chemically activated (Melchers *et al.*, 1989).

P. syringae pv. *phaseocola* causes halo blight disease in bean plants. A set of chromosomal genes called *hrp* (harp) controls the ability of this and other phytopathogenic bacteria to cause disease on susceptible plants and to elicit a hypersensitive response on resistant plants (Willis *et al.*, 1990). Most *hrp* genes lie in a 22 kb cluster composed of seven complementation groups. Several of the *hrp* genes (A, B, C, D, E) are induced dramatically by low pH (pH 5.5), a condition applicable to the environment of the leaf apoplast (Rahme *et al.*, 1992; Wei *et al.*, 1992). Thus pH may be a signal that induces the early stages of infection.

It is evident that pH-controlled induction or activation of proteins and genes is a frequent occurrence and one which is intimately associated with the ability of an organism to cause disease. The systems discussed above have generated a wealth of knowledge in terms of physiological signals required for activation of virulence loci. Yet, for the majority of these examples, the molecular mechanisms directly involved remain a mystery.

2.4. Low pH as a Signal for Cell Differentiation

Rhizobium species can fix nitrogen when in the confines of root nodules on host legumes. These unique organs are a result of a developmental program induced by bacteria in a susceptible region of the root. The *nod* genes of *Rhizobium* are responsible for triggering the nodulation program in the plant prior to infection. Richardson *et al.* (1988, 1989) have shown that some of the nodulation genes are acid-repressed. Once nodules begin to develop, the bacteria can infect. Infection culminates with the release of bacteria into certain plant cells where the bacteria proliferate and differentiate into forms called bacteroids. Many of the changes that occur during this process are believed to be induced by the microenvironment within the nodule. These conditions include low oxygen, phosphate starvation and low pH. For example, lipopolysaccharide (LPS) structure is known to change during infection. This alteration can be mimicked *ex planta*, at least in part, by modulating pH to 5 or less (Tao *et al.*, 1992; Kannenberg and Brewin, 1989). Further study may reveal that more features of the bacteroid are controlled by pH.

Another fascinating example of how pH can influence differentiation was discovered with *Leishmania donovani*. This is a parasitic protozoan with a digenetic life-cycle, proliferating as extracellular flagellated promastigotes in the alimentary tract of the insect vector and as obligate intracellular

amastigotes in the phagolysosomal vacuoles of mammalian macrophages (Chang *et al.*, 1985). During their life-cycle the protozoa experience marked changes in environmental pH, from alkaline (pH 7.0–7.5) in the sandfly gut to acid (pH 4.5–5.0) in the phagolysosome. Because the transformation from promastigote to amastigote occurs in the acidic environment of the macrophage phagolysosome, pH was thought to play an important role in triggering this differentiation. This hypothesis was tested by determining the effect of growth medium pH on phenotypic expression in promastigotes (Zilberstein *et al.*, 1991; Zilberstein and Gepstein, 1993). One of the most interesting findings was that switching promastigotes from pH 7.0 to pH 4.5 triggered the expression of an amastigote-specific antigen. Furthermore, the increased level of expression correlated with a morphological change to a rounded, amastigote-like cell. The results certainly support a role for pH in the development of *Leishmania*.

The cellular slime mold *Dictyostelium mucoroides* exhibits clear dimorphism which, depending on environmental conditions, will lead either to the sexual development of macrocysts or the asexual development of sorocarps. During starvation, individual ameboid cells aggregate into a slug-like mass with the aforementioned developmental choice. Factors that influence this choice include light, water and volatile substances (Filosa, 1979). Iijima and Maeda (1990) have determined that pH_i, or something related to pH_i, influences this developmental choice. Proton pump inhibitors such as diethylstilboestrol or dinitrophenol decrease pH_i and convert the developmental mode from macrocyst to sorocarp formation under conditions otherwise favoring macrocyst formation.

The pathogenic and polymorphic fungus *Candida albicans* differentiates between yeast and filamentous forms (Yokoyama and Takeo, 1983). The reciprocal transformations between yeast and filamentous forms correlate with pathogenicity of this fungus. Among the environmental factors that trigger differentiation is pH (Buffo *et al.*, 1984; Pollack and Hashimoto, 1987). Alkalinization of the cytoplasm was correlated with germ tube formation (Stewart *et al.*, 1988, 1989; Kaur and Mishra, 1994). Furthermore, the differentiation from germ tube to yeast growth induced by acid (pH 4) was correlated with the loss of microfilaments from hyphal cells and the deposition of actin at the sites of budding (Yokoyama *et al.*, 1994). Correlation of these events with changes in gene expression has not been made, however.

The developmental process of sporulation in *Bacillus megaterium* also correlates with changes of internal pH (Magill *et al.*, 1994). During sporulation, internal pH of the forespore drops from an initial value of 8.1 to approximately pH 7.0, 90 min before synthesis of dipicolinic acid. The pH_i of the mother cell remains at pH 8.1. It is proposed that acidification of the forespore will regulate the activity of phosphoglycerol mutase, allowing accumulation of 3-phosphoglyceric acid in the developing spore. Other roles

for forespore acidification are also possible. In contrast, during germination, pH_i of the germinating cell increases about 1 pH unit. While all the molecular repercussions of pH_i shifts are not known, it is reasonable to predict multiple effects on gene expression that will prove crucial to differentiation processes.

2.5. Co-inducer Requirements

It should be noted that many genes described as pH-regulated are not expressed in minimal medium and have been shown, or are predicted, to have a co-inducer requirement in addition to pH. Examples include *hyd*, *aniG*, *aciB*, *aniC*, *anil*, and *cad* of *S. typhimurium*. One interesting example is the *ompC* locus in *S. typhimurium* (Foster *et al.*, 1994). This locus is acid-inducible in complex media but not in minimal media, suggesting a co-inducer requirement. However, if the OmpR/EnvZ regulatory system is altered through mutation, pH regulation is now observed in minimal medium but lost in complex medium. This suggests that the co-inducer requirement for *ompC* expression is mediated by the OmpR/EnvZ system and that pH control is separate from, but influenced by, OmpR/EnvZ. This is not unlike the relationship between CadC and LysP in the lysine/low pH co-regulation for *cadA* in *E. coli*. Even in minimal medium, several genes have co-inducer requirements, such as iron for *iroA* and Na^+ for *nhaA*. The common requirement for co-inducers suggests regulatory models in which co-inducer binding sites on transmembrane sensory proteins, or on transport proteins, will only assume their binding conformation when triggered by pH (high or low, depending on the system). This could be the case with aniG, which is induced by acid and external mannose. Alternatively, pH may influence the availability or accumulation of a co-regulator. This may be the case for the *E. coli nhaA* locus and Na^+ or for *iroA* and iron in *S. typhimurium* where alkaline pH diminishes the solubility of iron. Another example can be found in *Rhizobium leguminosarum* where the plant-derived inducer for the *nod* genes, naringenin, will accumulate within the rhizobial membrane at low (pH 5.7) but not high (pH 9.7) pH (Recourt *et al.*, 1989). In this case, naringenin is not ionized and, therefore, membrane permeable at pH 5.7 while the ionized derivative that forms at alkaline pH is membrane impermeable. Thus, inducing concentrations of naringenin occur only at low pH.

It is also curious that many of the identified pH-regulated genes are induced by anaerobiosis; some genes exhibit an absolute requirement for this condition. Reflecting on the example given for *ompC* above, one wonders if the anaerobic requirement could be relieved by eliminating one of the known oxygen regulators, Fnr or Arc. As pointed out for the *cyo* operon as well as for *ompC*, one regulatory system could overshadow the presence of another. Anaerobiosis could also affect the cell's ability to maintain pH_i.

Under anaerobic conditions the magnitude of ΔpH sustainable by bacteria may be insufficient to keep pH_i at 7.5 for *E. coli* grown at acid pH_o (Ugurbil *et al.*, 1978). So, if a gene responds to low pH_i, anaerobiosis may contribute to induction when pH_o is acidic.

2.6. The Influence of DNA Topology on pH-Regulated Gene Expression

Considerable attention has been given recently to environmental influences on DNA topology and the subsequent effects on expression of selected genes. Environmental conditions such as osmolarity, starvation, temperature and, most recently, pH have all been shown to alter DNA topology (Drlica, 1992). In addition, the expression of several environmentally regulated genes is influenced by alterations in DNA topology (Higgins *et al.*, 1990a). Some of these are pH-regulated genes. Examples include *proU* (an osmotically induced gene also regulated by pH), *ompC*, *ompF*, *cad*, *adi* and *aniG* (Karem and Foster, 1993; Shi *et al.*, 1993; Thomas and Booth, 1992; M. Heyde, J. L. Cole and R. Portalier, personal communication). Reporter plasmid DNA supercoiling experiments conducted in *E. coli* and *S. typhimurium* indicate that alkaline growth conditions tend to decrease the plasmid linking number (increase in supercoiling) whereas acid growth increases the linking number (decrease in supercoiling). It has also been noted that genes affected by several different environmental conditions respond to each condition in a manner consistent with the effect of that condition on reporter plasmid supercoiling. For example, both low osmolarity and acid decrease DNA supercoiling and increase expression of *aniG* in *S. typhimurium* (Karem and Foster, 1993). How might changes in the external environment, such as pH, alter DNA topology? Hsieh *et al.* (1991a, b) have shown that osmolarity and anaerobiosis may influence DNA supercoiling by altering ATP/ADP levels. Such changes in ATP/ADP levels could affect DNA gyrase activity *in vivo* with a subsequent dramatic effect on chromosomal supercoiling. Although the effect has not been tested directly for pH, it is reasonable to predict a similar correlation between perturbations in pH and ATP/ADP ratios. For example, changes in external pH, and thus ΔpH, alter the proton motive force, which consequently alters the production of ATP through the Mg^{2+}-dependent H^+-translocating ATPase.

An additional factor that influences DNA topology has recently emerged in the regulation of *cad* and other pH-responsive genes in *E. coli* and *S. typhimurium*. The *hns* locus, formerly *osmZ*, encodes the nucleoid histone-like protein H-NS (Higgins *et al.*, 1990a; Hinton *et al.*, 1992). Histone-like proteins are non-specific DNA-binding proteins associated with bacterial chromosome structure and organization (Drlica and Rouviere-Yaniv, 1987). Of these proteins, H-NS is one of the two most abundant histone-like proteins

in the bacterial cell. Mutations in *hns* are pleiotropic, causing changes in gene expression of several unlinked loci (Higgins *et al.*, 1990a). In *E. coli*, elevated levels of *cad* and *adi* expression at pH 8.0 occur in response to mutations in *hns* (Shi *et al.*, 1993), suggesting a role for *hns* in pH-regulated gene expression. The ability of *hns* to alter gene expression has been demonstrated for several unlinked environmentally induced loci (Hulton *et al.*, 1990; Higgins *et al.*, 1990b; Hinton *et al.*, 1992). The mechanism of regulation may lie in the ability of H-NS to bind DNA globally, thereby altering chromosomal DNA supercoiling. Alternatively, it may act more specifically by binding at a particular locus and influencing the ability of RNA polymerase or regulatory proteins to affect transcription. The mechanism of the H-NS effect on *E. coli cadBA* has not been deciphered. In *S. typhimurium*, *cadBA* regulation is independent of *hns* (J. Foster, I. S. Lee and Y. Park, unpublished), suggesting that chromosomal map location may be important. Other pH-controlled loci exhibiting a regulatory dependence on *hns* include *proU* (Hulton *et al.*, 1990; Higgins *et al.*, 1990a) and *aniG* (Karem and Foster, 1993). However, not all pH-regulated loci are affected by *hns* mutations, suggesting that pH induction and *hns* control are not always linked.

Results with *aniG* and *proU* suggest that genes responsive to multiple environments may have primary and secondary regulatory controls. The primary inducing condition (e.g. low pH for *aniG* and osmolarity for *proU*) is that which produces the greatest level of induction. This primary inducing condition would work through a sensing-regulatory cascade system in which the affinity of a regulatory protein for a target operator is altered. Secondary, or fine-tuning, control by other conditions may occur by altering operator topology, thereby changing its suitability for regulatory protein binding.

3. pH STRESS RESISTANCE

3.1. General Aspects of pH Stress

3.1.1. *Scope*

Despite marked susceptibility to pH conditions outside the growth range, many microbes cope with pH-induced stress in a variety of environmental encounters. For example, acid stress management is important to any microorganism that must survive dramatic pH fluctuations arising as a result of acid rain or through the contamination of natural estuaries by acid mine drainage or other industrial slurries (Foy, 1984; Gyure *et al.*, 1987; Baker *et al.*, 1991). Host–parasite relationships offer their own set of pH barriers that successful pathogens must overcome, including acid conditions in the

stomach, dental caries, abscess and phagolysosome environments (Schachtele and Jensen, 1982; Aranda *et al.*, 1992; Giannella *et al.*, 1973). Food-processing technologies must also take into account the acid-resistant nature of unwanted microorganisms (El-Dahar *et al.*, 1990; Little *et al.*, 1992; Salmond *et al.*, 1984; D'Aoust, 1989). Several investigations have focused on microbial adaptive behavior to assorted pH conditions. The organisms studied include several enterobacteria; *Streptococcus*, *Enterococcus*, *Actinomyces*, *Lactobacillus*, *Rhizobium*, *Thiobacillus*, *Sarcina*, *Staphylococcus*, *Yersinia* and *Listeria* species. Systems examined range from general acid resistance (e.g. El-Dahar *et al.*, 1990; Gorden and Small, 1993; Little *et al.*, 1992) to specific inducible systems (e.g. Lee *et al.*, 1994; Hamilton and Buckley, 1991; Kroll and Patchett, 1992). Throughout the literature, *acid resistance*, *acid tolerance* and *acid habituation* have been used as general terms to describe survival to either low or high pH stress and to describe specific systems. We propose that the term *acid survival mechanisms* be used where possible as a general term to encompass all of the acid protection systems, both constitutive and inducible. The terms *acid tolerance response*, *acid resistance* and *habituation* should be reserved for specific inducible systems. This may not always be possible but would perhaps make the literature less confusing. The reader should also realize that different teams of investigators use different methods for assessing acid resistance. Methodology can differ with respect to media, growth phase, oxygenation, adaptive procedure and challenge pH. Consequently, comparing results between investigators can be difficult.

3.1.2. *Acid Damage and Death*

There are two general strategies that have been used to assay acid-damaged cells: irreversible loss of cell viability and reversible loss of viability on selective media. The former is the basis for studying inducible acid survival strategies and will be examined more closely below. The latter was based on an observation that acid-damaged cells (*E. coli*) exhibit a faster death rate when plated on violet red bile agar (VRBA) than on nutrient agar (Roth and Keenan, 1971). Acid-damaged cells succumb more easily to the bile salts in VRBA than do undamaged cells. Przbylski and Witter (1979) discovered that these acid-injured cells could be recovered as viable colonies on VRBA if they were removed from the acid medium to a neutral pH medium before plating. Recovery occurred within minutes but was not inhibited by protein synthesis inhibitors or metabolic inhibitors such as 2,4-dinitrophenol. Consequently, this is not an inducible recovery system. Its basis remains unknown. However, because the damage induced by Przbylski and Witter (1979) involved acetate at pH 4.2, recovery might simply have been due to diffusion of the acetate out of the cell while suspended in the recovery medium.

One can view potential acid damage to a microorganism as occurring in

two general stages. As pH_o starts to become more acidic, internal pH homeostasis mechanisms are able to maintain pH_i (pH 7.6–7.8 for the Enterobacteriaceae). This will create a large ΔpH. Because the interior pH of the cell remains relatively neutral, it would seem that the earliest site of acid-induced damage should occur at the cell surface, although direct evidence for this is conflicting (Cherrington *et al.*, 1991). As the external pH declines further, pH homeostasis mechanisms eventually fail, proton leakage increases, and pH_i acidifies with ensuing damage to internal macromolecules and acid-sensitive metabolites. So it would seem that membrane composition must play an important role as the first defense against proton infusion. There is some evidence that the fatty acid composition of the membrane can influence proton permeability. In a comparison of obligate *vs.* facultative alkaliphilic species of *Bacillus*, Clejan *et al.* (1986) found that membranes from obligately alkaliphilic species contained more unsaturated and branched-chain fatty acids than did membranes from facultative alkaliphiles. The obligate alkaliphiles appear to experience compromised membrane integrity at near-neutral pH values. Dunkley *et al.* (1991) hypothesize that the combination of a high composition of branched-chain fatty acids and a substantial fraction of unsaturated fatty acids make the membrane weak at neutral pH but may contribute to an unusual energy-coupling mechanism proposed for these organisms at high pH. One could then question whether an environmental pH shift might trigger a change in membrane lipid composition and thus increase acid resistance. In support of this theory, continuous cultures of *E. coli* grown at pH 8.4 do produce membranes with a slightly increased level of unsaturated fatty acids as compared with those grown at pH 6.4 (Arneborg *et al.*, 1993). While the evidence is certainly not conclusive, it does suggest a correlation and may warrant further study at perhaps more acidic pH levels.

Acid death is believed to result from intracellular damage caused by decreasing pH_i rather than extracellular damage caused by acid pH_o. For example, *S. typhimurium* experiences logarithmic death in minimal medium when the external pH falls below 4.0 (Foster, 1991). Loss of viability was subsequently correlated with pH_i falling below 5.5 regardless of the external pH (Foster and Hall, 1991). A similar trend was noted for *E. coli* and *Shigella dysenteriae* (Small *et al.*, 1994) and *Leuconostoc mesenteroides* (McDonald *et al.*, 1990). Damage that occurs once internal pH levels begin to decline has been studied very little; consequently the defining lethal event is not known. DNA damage, in the form of depurination and protein denaturation by altering ionic interactions, is expected. Several studies have attempted to examine acid damage, most of them using short-chain organic acids (e.g. formic or propionic acid) that will cross the membrane in their undissociated form, then dissassociate within the cell into anions and protons both of which exert an inhibitory effect (reviewed in Cherrington *et al.*, 1991). Acidic environments (pH 5 is used most often) increase the effects of these weak

acids by increasing the proportion of undissociated organic acid that can traverse the membrane. Reported effects include inhibition of substrate transport, depression of cytoplasmic pH and inhibition of macromolecular synthesis.

3.2. pH Stress Management Strategies

pH stress management by any organism can be envisioned to include both constitutive and inducible components. Constitutive, or intrinsic, pH stress survival mechanisms include the housekeeping pH homeostasis systems whose activities, rather than synthesis, vary with pH. Also contributing to intrinsic acid or base survival are factors such as membrane structural influences over proton permeability, internal buffering capacity, and the pH stability of essential proteins. Inducible systems of pH stress survival are more poorly defined. Potentially they can include inducible systems that alter proton pumping, decrease membrane permeability, produce chaperones, repair DNA or prevent macromolecular damage. There is evidence that several organisms alter proton pumping through regulated synthesis of their F_1F_0 proton translocating ATPase (see below). DNA repair is suggested for some systems based on the acid sensitivity of DNA repair mutants or induction of SOS repair functions (Goodson and Rowbury, 1991; Foster and Bearson, 1994; Raja *et al.*, 1991a, b). Several reports involving two-dimensional polyacrylamide gel electrophoresis indicate that chaperones such as DnaK and GroEL are induced by low pH stress (Hickey and Hirshfield, 1990; Heyde and Portalier, 1990; Foster, 1991).

It is important to realize when reviewing the field of pH-stress responses that several different approaches have been used to assay resistance to pH stress. The most obvious has been to examine cell viability following an experimenter-imposed acid challenge. There are other assays, used primarily with fermentative organisms, that measure the ability of an organism to handle self-imposed media acidification. Consequently, it is difficult, if not impossible, to compare groups of organisms tested in different manners.

3.2.1. *Salmonella typhimurium*

An inducible acidification tolerance response (ATR) was first identified in *S. typhimurium* by Foster and Hall (1990). The basic discovery was that adapting cells grown in minimal medium at pH 7.7 to pH 5.8 (a mild acid stress) for one generation enabled survival at an extreme acid challenge of pH 3.3. Furthermore, the survival response required protein synthesis and so represents a true inducible system that senses and responds to some consequence of low pH_o. Since that first report, studies have revealed a complex series of overlapping acid protection systems that form the ATR.

There appear to be at least three systems (Lee *et al.*, 1994). Two of them, a log-phase ATR and a stationary phase ATR, are pH-dependent, induced only at low pH. The third system is pH-independent but requires the stationary-phase sigma factor encoded by *rpoS*. It is unclear whether RpoS is involved with a distinct acid tolerance system or rather amplifies the other two systems.

The first pH-dependent system is called log-phase ATR. Log phase ATR has two physiological components designated pre-acid shock and post-acid shock. Acid shock occurs below pH 4.5, a moderately strong acid condition in which cells will not grow. Events related to acid tolerance that occur before a shift to pH 4.5 are referred to as pre-acid shock while those that occur after are called post-acid shock. The key to this system is the ability to synthesize 43 acid shock proteins (ASP), one or more of which is predicted to be essential for acid tolerance during acid challenge at pH 3.3 (Foster, 1991). A shift directly from pH 7.7 to pH 4.0–4.5 allows ASP synthesis and subsequent protection at pH 3.3. But ASP synthesis does not occur at pH 5.8 or below pH 3.9. The latter is due to an acidic internal pH unsuitable for protein synthesis (Foster, 1993). Therefore, one must explain why pH 5.8 adapted cells can survive a direct pH shift to pH 3.3. The answer appears to lie in the synthesis of an enhanced pH homeostasis mechanism induced at pH 5.8 (pre-acid shock event) that will maintain pH_i at a level suitable for protein (and thus ASP) synthesis at pH_o of 3.3 (Foster and Hall, 1991). Since the major Mg^{2+}-dependent proton translocating ATPase was shown to be important to acid tolerance in *Enterococcus* by enhancing pH homeostasis, an ATPase-defective mutant (*atp*) of *S. typhimurium* was examined for acid tolerance (Foster and Hall, 1991). The *atp* mutation eliminated pre-shock adaptation (pH 5.8) but did not have a major effect on post-acid shock (pH 4.3) adaptation. This pattern is consistent with the proposed role for a mild acid-inducible pH homeostasis system that enables ASP synthesis at severe acid pH (pH 3.3) (Foster and Hall, 1991). However, it is not known whether the *atp* operon is induced by mild acid stress. An additional report by Leyer and Johnson (1993) indicates that cell surface hydrophobicity increases in *S. typhimurium* as a result of pH 5.8 pre-acid shock adaptation. This could possibly decrease proton permeability of the membrane, thereby diminishing proton influx during acid stress.

The initial characterization of the log-phase ATR was performed using what is now known to be an *rpoS* mutant of *S. typhimurium*. RpoS is an alternative sigma factor important to stationary phase growth (Lange and Hengge-Aronis, 1991; Siegele and Kolter, 1992). It was discovered, in this strain, that a subset of the ASPs and induction of the ATR itself are transient phenomena. Cells acid-shocked at pH 4.3 for up to 30 min will produce the transient ASPs and survive subsequent pH 3.3 challenge. However, cells acid-shocked for 60 min or more no longer produce the transient ASPs and are acid-sensitive at challenge pH (Foster, 1993). Presumably, one (or more)

of the transient ASPs is essential for effective acid tolerance. Recently, it was discovered that log-phase acid tolerance development is not transient in an *rpoS*$^+$ cell (I. S. Lee and J. Foster, unpublished observation). Acid tolerance levels increase continuously over several hours of acid shock. Recent evidence indicates that RpoS is itself induced by acid shock and initiates synthesis of an additional seven ASPs rather than enabling continued synthesis of the transient ASP subset (Lee *et al.*, 1995). One or more of these seven σ^S-dependent ASPs is thought to be essential for sustained acid tolerance development.

The second pH-dependent system is called stationary phase ATR (Lee *et al.*, 1994). Stationary-phase cells will induce a very effective acid tolerance when they are exposed to a pH below 4.5 for 1 or 2 h. The stationary phase ATR effectively protects cells from pH 3.0 acid challenge for several hours. Its production is *rpoS*-independent and continuous (i.e. not transient as with log-phase ATR). Two-dimensional SDS polyacrylamide gel analysis revealed the production of 15 ASPs, only four of which were previously identified as log-phase ASPs. This, coupled with the fact that several mutations that affect log-phase ATR do not affect stationary phase ATR, suggests that they represent distinct systems of acid protection (I. Lee, J. Lin, H. Hall, B. Bearson and J. Foster, unpublished). In addition to these two pH-dependent ATR systems, there is a pH-independent, RpoS-dependent stationary phase acid resistance mechanism that is observed when comparing log-phase *vs.* stationary-phase cells at pH 7 (Lee *et al.*, 1994). This mechanism may be part of a stationary-phase general stress-resistance phenomenon analogous to that described for *E. coli* (Matin, 1991).

A report by Humphrey *et al.* (1993) notes the presence of a pH-dependent acid habituation system in *S. enteritidis* that is independent of protein synthesis. This appears very different from the two pH-dependent systems reported for *S. typhimurium*, both of which require protein synthesis. However, the study by Humphrey *et al.* essentially used stationary phase cells which would have the RpoS system functional. *S. typhimurium* must be diluted 1000-fold and grown to mid log phase to remove the residual effects of RpoS-dependent acid resistance (Lee *et al.*, 1995). It is possible that the RpoS-dependent system, once in place, still requires activation by low pH before it can function. This would be analogous to what has been observed for *E. coli* RpoH-dependent heat tolerance (Van Bogelen *et al.*, 1987). Overproduction of RpoH, which causes heat shock-independent overexpression of the Htp regulon even at 30°C, is not sufficient to afford heat tolerance without additional heat activation at 42°C. An analogous situation with pH could explain the results of Humphrey *et al.* (1993) and was alluded to for acid habituation in *E. coli* by Raja *et al.* (1991a).

A variety of mutants of *S. typhimurium* have been identified in which acid resistance is affected. Spontaneously acid-resistant mutants that survive long-term pH 3.3 challenge were found to contain mutations in several

biosynthetic genes (e.g. *pyr*, *met*, *pro*, etc.). Many of these mutations occurred in the *icd* locus encoding isocitrate dehydrogenase (Foster and Hall, 1991). The large amount of citrate and isocitrate accumulated by these mutants may increase the internal buffering capacity of *S. typhimurium* and so protect cells during severe acid stress. Other auxotrophic mutations may trigger acid resistance through the starvation-induced general stress resistance system (Matin, 1991) which may be analogous to the RpoS-dependent stationary phase system described above. Procedures designed to detect increased acid sensitivity (i.e. Atr^- phenotype) uncovered several mutants defective in iron metabolism (Foster and Bearson, 1994; Foster *et al.*, 1994; Foster and Hall, 1992; Foster, 1993). Most prominent among these are mutations in the major iron regulatory locus, *fur*. These mutants are defective in both the pre- and post-acid shock stages of the ATR, do not produce the ATR-specific pH homeostasis system and show altered expression of several ASPs including six of the 14 transiently expressed ASPs (Foster, 1993; Foster and Hall, 1992). None of these *fur* mutations affects stationary-phase ATR, however. A recent study of the role of iron in log-phase ATR has revealed that iron is required for an efficient response and that either over- or under-expression of Fur will compromise ATR development (H. Hall and J. Foster, unpublished observation). In addition, alterations in iron availability alone will not trigger the ATR. The cell must also experience a low adaptive pH (either pH 5.8 or 4.3). Although the precise role of iron in the ATR remains an enigma, iron may affect ATR development as a co-regulator with Fur and/or as a co-factor necessary for the function of one or more ATR proteins.

Other mutations that prevent ATR development include those in *polA*, suggesting a role for DNA repair, and unknown genes designated *atrB*, *atrG*, *atrH*, *atrI* and *atrJ* (Foster and Bearson, 1994; I. S. Lee and J. W. Foster, unpublished observation). AtrB is a member of a regulon comprising 10 genes under negative control by the regulator AtbR (Foster and Bearson, 1994). Whereas insertion mutations in *atrB* cause an Atr^- acid-sensitive phenotype, insertions into the regulator *atbR* produce a constitutively acid resistant phenotype that can suppress the Atr^- phenotype of other acid-sensitive mutants including those defective in *fur*. It is interesting that in an $atbR^+$ background, the AtrB protein can be observed in $rpoS^-$ but not $rpoS^+$ strains of *S. typhimurium* (J. Lin and J. Foster, unpublished observation). This finding may provide a clue to the relationship between RpoS and log-phase ATR as described above. It is also curious that mutations in *atrB*, *fur*, *atrG* and *polA* do not affect acid tolerance in strains with a strong $rpoS^+$ allele. Combinations of these mutations will, however, confer acid sensitivity on $rpoS^+$ strains (M. Wilmes-Riesenberg, B. Bearson, J. Foster and R. Curtiss, unpublished results).

Acid tolerance may also affect the virulence of *S. typhimurium*. Mutants defective in the *rpoS* locus are avirulent, presumably because of decreased

stress resistance (Fang *et al.*, 1992). One of the conditions affected is the low pH-induced ATR. Several proteins produced by *S. typhimurium* while harbored within macrophage phagolysosomes are known to be acid induced and may contribute to survival within this host environment (Abshire and Neidhardt, 1993; Aranda *et al.*, 1992). Finally, recent work by Wilmes-Riesenberg and Curtiss (unpublished observation) has revealed a clear correlation between acid resistance and virulence capability in *S. typhimurium*. So it seems certain that the ability to survive acid conditions will prove to be an important virulence determinant for this and other pathogens.

3.2.2. *Escherichia coli and Shigella flexneri*

Using *E. coli*, Goodson and Rowbury (1989a, b, c) were able to enhance resistance to extremes of acid and alkali by pre-treating cells with non-lethal acclimating doses of HCl and NaOH. The adaptive phenomena were termed "habituation to acid" for initial treatment at pH 5.0 with challenge at pH 3, and "habituation to alkali" for treatment at pH 9.0 with challenge at pH 11. Specifically, acid habituation resulted in cells grown in nutrient broth at pH 5.0, challenged at pH 3.0 or pH 3.5, and subcultured for viability on pH 7.0 nutrient agar. A three- to 100-fold increase in acid resistance was detected in the acid habituated cells. Acid habituation, which also occurs in minimal media, involves protein synthesis-dependent and -independent steps (Raja *et al.*, 1991a; Rowbury and Goodson, 1993). DNA damage and its repair are key features of acid habituation (Goodson and Rowbury, 1991; Raja *et al.*, 1991a, b). A role for phosphate and the phosphate-specific PhoE porin was implicated by showing that phosphate ions inhibited acid habituation and finding that *phoE* mutants were acid resistant (Rowbury *et al.*, 1992). It is proposed that the PhoE porin provides a conduit for H^+ influx allowing acidification of the periplasm and stimulation of a transmembrane sensory protein that would signal induction of acid habituation. A high level of phosphate (3 mM) in the medium is proposed to block access of H^+ to the PhoE pore and thereby interfere with signal transmission. It should be noted that the acid tolerance response system of *S. typhimurium* described above is not beholden to the phosphate control mechanism since it is performed at very high phosphate levels (75 mM). Another apparent difference between the results with *S. typhimurium* and the acid habituation system of *E. coli* is the failure of *fur* and *atp* mutants to affect the latter (Rowbury and Goodson, 1993). This could be explained by several factors including the RpoS status of the *E. coli* strains used and the fact that the dilution of overnight cultures (20–50 fold) may not have been sufficient to remove residual RpoS in the *E. coli* study.

In addition to alkaline habituation, Rowbury *et al.* (1993a) report that a shift from neutral to alkaline pH (9.0) induces acid sensitivity (pH 3.0) in

E. coli. This finding suggests that the low and high pH adaptive systems are distinct and that the induction of one leaves the cell more vulnerable to the opposite stress. As with acid habituation, this phenomenon also involves protein synthesis-dependent and -independent components (Rowbury *et al.*, 1993b). Alkaline induction of acid sensitivity is most pronounced in a *phoE* mutant which, as noted above, is considered constitutively resistant to pH 3.0. This apparent paradox between the degree of acid sensitivity induction, acid habituation and the role of PhoE remains unresolved.

A separate series of studies (Gorden and Small, 1993; Small *et al.*, 1994) with *E. coli* implicates aspects of stationary phase physiology and anaerobiosis in what is referred to as acid and base resistance responses of *E. coli* and *Shigella flexneri* (the etiologic agent of bacterial dysentery). These adaptive responses appear distinct from the acid and base habituations described above and were initially thought to resemble the acid tolerance responses described for *S. typhimurium*. However, a comparison of ATR of *E. coli* and *S. flexneri* conducted in minimal media revealed that *Shigella* had neither log phase nor stationary phase ATR systems and that *E. coli*, although it has a log phase system, was constitutively acid tolerant in stationary phase (I. S. Lee and J. W. Foster, unpublished). Although the acid tolerance responses for these organisms appear different, both *E. coli* and *S. flexneri* are more acid resistant than is *S. typhimurium* when resistance is measured at pH 2.5 in complex medium (Gorden and Small, 1993).

Recent studies suggest that the acid resistance defined by Gorden and Small (1993) and Small *et al.* (1994) requires components of complex medium either for induction or function (Lin *et al.*, 1995). One acid survival mechanism requires glutamate during acid challenge, another requires arginine. Presumably, degradation of these compounds produces internal buffers that bolster internal pH.

Small *et al.* (1994) have found the alternative sigma factor, RpoS, to play an important role in acid resistance for both *E. coli* and *S. flexneri*. It is interesting that the *rpoS* alleles were interchangeable between *E. coli* and *S. flexneri* but the *rpoS* allele from *S. flexneri* could not increase acid resistance of *S. typhimurium*. This suggests that some fundamental differences exist between these organisms with respect to components of acid tolerance (Small *et al.*, 1994). In this same study, RpoS was shown to contribute to pH homeostasis of stationary phase *E. coli* at external pH levels below 4.0. As noted above for *S. typhimurium*, these organisms have several systems of acid tolerance, including pH-dependent log-phase and stationary-phase systems, both of which function in the absence of RpoS, although at a greatly reduced level. In addition, anaerobiosis is capable of suppressing the effect of *rpoS* mutations on acid tolerance, adding another layer of complexity to the low-pH response (Small *et al.*, 1994).

As RpoS appears to influence significantly acid resistance both in *E. coli*

and *S. typhimurium*, one must wonder whether environmental pH could influence the expression of *rpoS*. Schellhorn and Stones (1992) have shown that weak acids such as acetate could induce *rpoS* even in exponential-phase cells. The effect was at both the transcriptional and translational levels. It seems possible that acidic pH_i may be responsible for *rpoS* induction because non-metabolizable weak acids such as benzoate and propionate also effect induction.

3.2.3. *Helicobacter pylori*

H. pylori plays an etiological role in a variety of gastroduodenal diseases including gastritis, peptic ulcer disease, nonuclear dyspepsia and gastric carcinoma (Buck, 1990). Acid resistance must play an important role in the pathogenesis of this organism because the human stomach and duodenum are the usual sites of colonization. A remarkable urease produced by *H. pylori* is proposed to combat the catastrophic acidity (<pH 2.5) present in the stomach (Marshall *et al.*, 1990; Pérez-Pérez *et al.*, 1992). The urease activity does not neutralize the external environment at pH values ≤ 2, yet the organism still survives. This suggests that the production of ammonia and bicarbonate helps neutralize internal pH even in a very acidic external environment and so helps *H. pylori* cells establish gastric infection. Whether or not the two structural genes for urease might be regulated by low pH is not known. One must also consider that additional inducible acid tolerance systems may exist in this organism. Recent results using a modified brucella both suggest that *H. pylori* is actually a facultative acidophile growing better at pH 4.5 than pH 7.0 (Kangatharalingam and Amy, 1994).

3.2.4. *Lactobacillus and Leuconostoc*

Another way to assess acid resistance is to monitor the degree to which an organism can survive acidifying its own environment as a result of fermentation. This is called the limiting lower external pH at which growth terminates (pH_{let}). For example, in the natural fermentation of vegetables (e.g. sauerkraut), a predictable succession of microorganisms is observed, with the growth of one organism giving way to another, more acid-resistant organism. *Leuconostoc mesenteroides* will grow early in the fermentation but as the pH of the medium declines as a result of the production of organic acids, its growth ceases and *Lactobacillus plantarum* will terminate the fermentation (Pederson and Albury, 1961). In a glucose medium, the pH_{let} values of *L. mesenteroides* and *L. plantarum* are 4.38 and 3.81, respectively. With these two organisms, acid resistance correlates with different capacities for tolerating low pH_i (McDonald *et al.*, 1990). As noted above, undissociated organic acids (e.g. lactic acid) can cross the membrane and

dissociate intracellularly, thereby acidifying internal pH. Fermentative microorganisms exhibit a greater range of internal pH values than respiratory microorganisms (Booth, 1985). This ability presumably diminishes the energy cost of maintaining pH homeostasis by energy-consuming proton pumps (Goodwin and Zeikus, 1987). McDonald *et al.* (1990) discovered growth of *L. mesenteroides* stopped when pH_i reached 5.4 to 5.7 but *L. plantarum* continued to grow until a pH_i of 4.6 to 4.8 was reached. While the pH_{let} values differ depending on the growth medium, the pH_i values at which growth ceases do not. A role for malo-lactic fermentation (MLF, L-malic acid decarboxylation) in the acid resistance of *L. plantarum* has been proposed. The presence of L-malic acid can protect cells in buffers at pH 3.0 presumably through degradation by malo-lactic enzyme (Garcia *et al.*, 1992). Internal pH will increase because one H^+ is consumed per molecule of L-malic acid degraded. This will generate a ΔpH that can alleviate acid damage. In addition, this organism can switch from lactic acid fermentation to acetoin (a neutral product) production at low pH values, essentially preventing self-destruction (Tsau *et al.*, 1992). Whether inducible systems are involved with acid resistance in these organisms is unknown.

3.2.5. *Streptococcus mutans*

Tolerance of acid environments is of major importance in the ecology of dental plaque and also in the pathogenesis of dental caries, which involves acid dissolution of tooth mineral. An important feature for organisms in this environment is to grow and metabolize carbohydrates at low pH values (Bowden and Hamilton, 1987). Consequently, the approach originally used to characterize acid tolerance in these organisms involved testing the minimum pH at which glycolysis could still occur (Bender *et al.*, 1985). One way in which this was measured involved a suspension pH drop method using dense cell preparations. The suspension pH drop method uses excess carbohydrate and measures the lowest pH where glycolysis ceases due to acidification by glycolytic end products. Organisms associated with dental caries, listed in order of decreasing acid resistance, include *Lactobacillus casei*, *Streptococcus mutans*, *Streptococcus sanguis* and *Actinomyces viscous*. Bender and Marquis (1987) suggest the increased acid resistance of *Lactobacillus casei* relative to *Actinomyces viscous* is due to the level of proton-translocating ATPase associated with the membranes (3.29 *vs.* 0.06 U/mg protein, respectively).

Adaptation to low pH by *S. mutans* was shown in that cells prepared from pH 5.0 cultures exhibited a lower pH drop than did cells prepared from pH 7.0 cultures. This is due to a shift in the pH optima of glucose uptake and glycolysis, as well as to an increase in the F_1F_0 H^+-translocating ATPase (Hamilton and Buckley, 1991; Belli and Marquis, 1991). The pH 5 adapted

cells were able to maintain a pH gradient at lower pH values than were pH 7.5 adapted cells (Hamilton and Buckley, 1991). In addition, pH 5.5 grown cells exhibit reduced proton permeability relative to cells grown at pH 7.5 (Hamilton and Buckley, 1991). The combination of decreased membrane permeability and increased ability to expel protons essentially enhances internal pH homeostasis and enhances resistance to acid killing (Belli and Marquis, 1991). Mutants with diminished acid tolerance have been isolated based on an inability to grow on trypticase soy agar containing 50 mM sodium acetate at pH 4.4 (Yamashita *et al.*, 1993). One such insertion was found in the gene for diacylglycerol kinase which generates phosphatidic acid (Walsh *et al.*, 1990). It is suspected that loss of this enzyme interferes with signal transduction of extracellular environmental signals due possibly to abnormal membrane function (Yamashita *et al.*, 1993). It is not known whether this enzyme constitutes part of the inducible or constitutive acid tolerance mechanisms for *S. mutans*.

A related organism, *S. rattus*, also increases ATPase levels in response to acidification. In addition, it copes with acid stress by altering its fermentation end-products from a mixture of formate, acetate and ethanol at pH_o 7.0 to mostly lactate at pH_o 5.0 (Miyagi *et al.*, 1994). At a pH_o of 5.0, the lower pKa of lactate (pKa 3.86) compared with acetate (pKa 4.75) effectively lowers the amount of lipophilic undissociated weak acid available to permeate the membrane and cause intracellular toxic effects.

3.2.6. *Rhizobium*

Low soil pH restricts legume productivity in many parts of the world. Thus, the effects of low pH on the growth and survival of root nodule bacteria are of global importance. Different species of root nodule-forming *Rhizobium* display varying degrees of acid resistance as measured by their ability to grow (not just survive) at low pH (Graham *et al.*, 1994; Glenn and Dilworth, 1994). *Rhizobium leguminosarum* is moderately acid-resistant, capable of growth down to pH 4.0–4.5. *R. meliloti*, however, is more acid-sensitive, growing only down to pH 5.5. Studies designed to dissect the basis of acid resistance in these organisms have revealed that acid resistant strains are better able to maintain internal pH when grown in acid conditions (O'Hara *et al.*, 1989). Genes associated with acid resistance in *R. leguminosarum* are located on the second largest of four megaplasmids (Chen *et al.*, 1993a). Loss of this plasmid was shown to alter cell surface LPS structure, interfere with pH homeostasis at low pH and confer an acid-sensitive growth phenotype. In a separate study, two loci (either chromosomal or plasmid) associated with acid resistance were identified using Tn5 insertional mutagenesis (Chen *et al.*, 1993b). The mutations interfered with membrane permeability, but not proton extrusion, resulting

in diminished pH homeostasis capability leading to the acid-sensitive phenotype. Other work with *R. meliloti* has shown that this organism will respond to increasing concentrations of calcium in the growth medium by growing at progressively lower pH values (Tiwari *et al.*, 1992). Five Tn5 insertion mutants with increased acid sensitivity have been isolated and grouped into two classes: those that are calcium repairable and those that are not. How calcium participates in acid resistance and whether any of the systems are inducible are questions that have not been examined.

Rhizobium leguminosarum and *Bradyrhizobium japonicum* also have a demonstrable adaptive acid tolerance response induced by growth at pH 5.0 (O'Hara and Glenn, 1994). Low pH-induced acid tolerance is greatest in stationary phase cells although significant induction also occurs in log phase cells. Induction of acid tolerance requires protein synthesis and appears to be similar to the ATR systems described for *S. typhimurium*.

3.3. Stress Cross-protection

The ability of one environmental stress to produce resistance to one or more other stresses is called stress cross-protection. It is well known, for example, that stationary phase and starvation can elicit protection against a variety of environmental stresses, including pH (Matin, 1991). But what of other stresses? Many of the proteins identified as inducible by pH stress are also induced in response to other stresses (Heyde and Portalier, 1990; Hickey and Hirschfield, 1990; Foster and Hall, 1990; Foster, 1991, 1993; Amaro *et al.*, 1991; Taglicht *et al.*, 1987). The cross-induction of heat shock proteins is conspicuous throughout these studies. Can any of these other stresses also induce acid or base resistance? *S. typhimurium* does not appear to increase its acid resistance in response to oxidative or heat stresses but acid adaptation will increase heat and salt tolerances (Leyer and Johnson, 1993; Lee *et al.*, 1995). Humphrey *et al.* (1993) have found that *Salmonella enteritidis* transferred to alkaline conditions will not only induce base resistance but heat resistance as well. In *E. coli*, habituation to alkali will increase ultraviolet light resistance (Goodson and Rowbury, 1990) and induce the SOS response (Schuldiner *et al.*, 1986). Acid-shocked *Listeria monocytogenes* also experiences significant thermotolerance (Farber and Pagotto, 1992). One mutant of *S. mutans* with diminished acid resistance is also more sensitive to osmotic and heat stress. Clearly there is redundancy between the regulatory circuits of various stress-inducible modulons. What is unclear is the extent of which the actual resistance mechanisms are distinct. For example, low pH adaptation induces acid and heat tolerance. Are some components of pH-induced acid tolerance also required for heat-induced heat tolerance, as might be suggested by the cross-protection, or is the acid-induced heat tolerance mechanism totally distinct from that of heat-

induced heat tolerance? These are questions that cannot be addressed until more details are known about each system.

4. CONCLUDING REMARKS

We have attempted in this review to summarize the current status of microbial pH stress responses. What has emerged is the realization that pH stress responses are found throughout the microbial world and that common themes exist. The requirement for co-inducer molecules is frequent among pH-regulated genes. Most pH-stress protection systems include a mechanism for sustaining cytoplasmic pH and many inducible systems offer cross-protection to other stresses. The future challenges of this area are to further decipher the various molecular mechanisms used by microorganisms to resist pH stress and to control, transcriptionally or translationally, pH-regulated genes. Knowledge of pH-controlled gene expression will lead to improved biotechnologies necessary for producing recombinant DNA products under special conditions and for the delivery of live recombinant vaccines. For example, placing a recombinant vaccine gene under the control of a low pH-regulated promoter in an attenuated strain of *S. typhimurium* may allow increased expression of that gene and thus engender a more vigorous immune response as *S. typhimurium* enters the reticuloendothelial system. Studies of the molecular responses to pH stress may provide insight into the pathogenic processes and ultimately lead to better vaccine development or antimicrobial targets. Knowledge of pH-stress survival mechanisms can also lead to the construction of hardier strains of root nodule bacteria that will better survive soil acidity and improve crop yields. It is clear that the new insights provided by recent advances in molecular genetics have rekindled interest in this important aspect of communication between microorganisms and their environment.

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Osmoadaptation in Bacteria

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1. INTRODUCTION

In its early stages, life on earth was dominated for a considerable length of time by microorganisms, and what we now call "extreme" (from an anthropocentric point of view), such as extremely hot, acid, alkaline or saline environments, may have at some time and in certain regions resembled the norm. Osmotic stress is one such restrictive environmental factor, which is caused by large concentrations of either salts or non-ionic solutes in the surrounding medium with the resulting deficit of water. Given the abundance of sea water on earth and bearing in mind that life originated from the sea, it is not surprising that the story of osmotic adaptation has largely focused on halophilic and halotolerant organisms (Kushner, 1978; Larsen, 1986;

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Oren, 1994). Although conspicuous natural environments of elevated salinity such as salt lakes are relatively rare nowadays, on a geological time scale the closing off and slow evaporation of marine basins was a relatively common event, providing ample time for the evolution of halophilic microbes. Evidence of this can be found in the abundance of global rock salt deposits, such as those in Northern Europe originating in the Zechstein period (Petrascheck & Petrascheck, 1950; Hardie & Eugster, 1970). In addition to salt-lake environments, all kinds of surfaces exposed to low humidity face the problem of elevated salinity, e.g. animal skin and plant cuticula in hot climates of high evaporation, the skin of rock and soil as well as the faces of buildings (under a regime of alternate wetting and drying).

Osmotic adaptation, however, is not confined to ionic environments, but can also be found in concentrated non-ionic solutions such as sugary plant saps, honey, etc. As only adapted organisms are able to thrive in both sugar and salt, it is no coincidence that these substances have been used since ancient times for the preservation of food. The typical spoilage organisms to be observed on sugary products such as jam are eukaryotic osmophilic/osmotolerant yeasts and fungi (not to be covered in this review), while within the bacterial kingdom *Zymomonas mobilis*, the fermenting bacterium isolated from agave sap, is one of the few truly osmophilic/osmotolerant (but salt-sensitive) representatives that has been studied in detail. Scientific interest in osmotic adaptation is closely linked to research into survival strategies in a dry environment, covering a wide range of topical areas such as extremophilic enzymes, preservation of living cells and anhydrobiosis, to name just a few of biotechnological interest (Galinski and Tindall, 1992).

The term "osmoadaptation" rather than "osmoregulation" has been chosen for this review, because—following a definition by Reed (1984)—the latter is more appropriately applied to immediate regulatory responses involving the action of a sensor and/or trigger which transforms environmental changes into physiological signals. While the term osmoacclimation refers specifically to the observable phenotypic responses (i.e. changes in membrane composition, cytoplasmic solute concentration, etc.), osmoadaptation has the broadest meaning, covering both physiological and genetic manifestation of adaptation to a low water environment. It is in the field of "physiological adaptation" (i.e. "acclimation") where we find an abundance of information and where this review will start. It will then continue with the molecular level of osmoadaptation, elucidating present knowledge of regulatory aspects, and provide first insights into compatible solute function in a changing osmotic environment. For previous descriptions covering various aspects of the subject from different angles, the reader is referred to reviews by Yancey *et al.* (1982), Imhoff (1986), Reed and Stewart (1988), Brown (1990), Csonka and Hanson (1991) and Kinne (1993).

2. THE CHALLENGE OF LOW WATER ACTIVITY

In general, living organisms are adapted to function in a rather limited set of physiological conditions, and significant deviations will immediately trigger stress mechanisms (Somero, 1986). One such environmental factor—which has often been overlooked—is humidity or water concentration. An environment of high solute concentration is always low in water activity, and as the cytoplasmic membrane is freely permeable to water this situation will lead to subsequent dehydration and cessation of growth unless the organism has the means to adapt and respond to such a low water environment. A most remarkable paper by Cayley *et al.* (1991) has demonstrated, with *Escherichia coli* as a model organism, how osmotic changes in the environment affect cytoplasmic volume and growth rate. Surprisingly, the amount of *bound* water in the cytoplasm was relatively independent of the osmolarity (0.4 ml/g dry weight), approximating 20% of total water in fully hydrated cells. Therefore, the immediate effect of high osmolarity was shown to be a reduction in the volume of *free* water. From their data they deduced a linear relationship between cytoplasmic volume and growth rate (Cayley *et al.*, 1991). An extrapolation yielded an estimate of the minimum volume of cytoplasmic water required for growth of approx. 0.5 ml/g dry weight. This leads us to the startling observation that (in a plasmolysis experiment) growth ceases when free water approaches zero. However, it should be noted in this context that Gram-positive bacteria usually have a much higher cytoplasmic turgor and can tolerate higher osmolarities even in the absence of adaptational strategies.

Mechanisms for survival and growth in a high-osmolarity environment must therefore aim at maintaining osmotic equilibrium across the membrane. In principle, two strategies have been adopted to cope with this situation: the salt-in-cytoplasm type and the organic-osmolyte type (Galinski and Trüper, 1994). One way of adapting the osmotic strength of the cytoplasm is to allow influx of salt or solutes (Fig. 1A). The most conspicuous representatives of organisms adopting this strategy are the halobacteria, and a range of anaerobic eubacteria (Zhilina and Zavarzin, 1990; Oren, 1991; Caumette *et al.*, 1991). An alternative strategy of adaptation involves the exclusion of salt or solutes with the resulting production of organic osmolytes, so-called compatible solutes, which allow the cell to maintain a "normal" cytoplasmic environment (Fig. 1B). In the case where organic osmolytes are present in the surrounding medium, uptake is usually preferred over synthesis *de novo* (Fig. 1C). However, even organisms unable to produce their own compatible solutes (e.g. normal non-halophilic bacteria) may acquire a certain range of osmotolerance by the accumulation of external osmolytes.

It is obvious from the above that a major dividing line exists between organisms of type A (the salt-in-cytoplasm type) and the organic-osmolyte

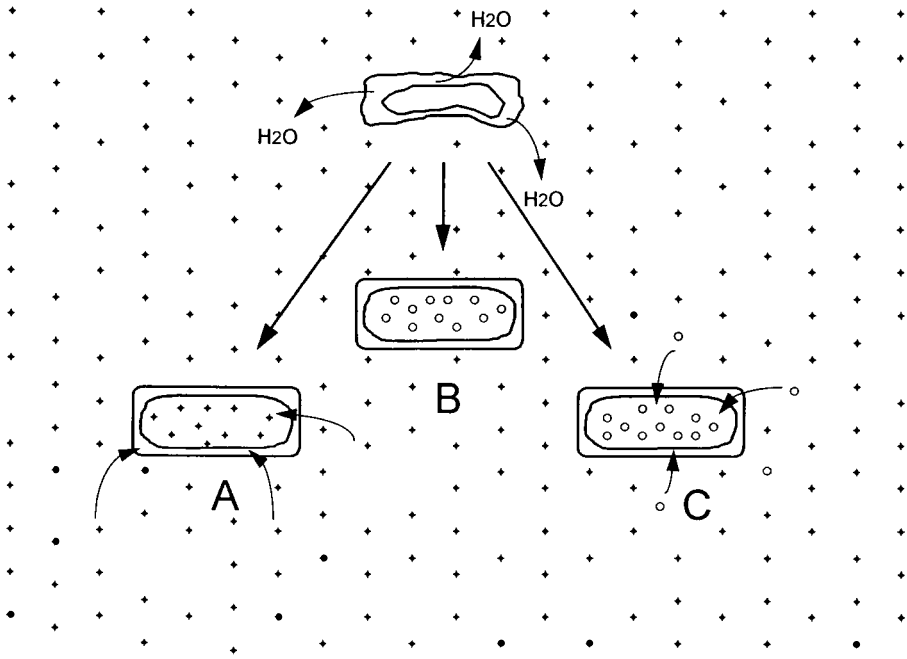


Figure 1 Three scenarios following osmotic up-shock. On exposure to a low-water environment (i.e. high concentrations of salt or solute) bacteria will primarily experience rapid efflux of water, collapse of turgor and shrinking of volume. These events lead to an increase in cytoplasmic ionic strength and molecular crowding, which will in turn terminate growth unless the organisms have the means to balance the osmotic gradient: (A) external solutes enter the cell, a strategy that requires adjustment of all cytoplasmic components; (B) external solutes are excluded and intrinsic compatible solutes are synthesized; (C) external solutes are excluded, but some components are accumulated from the medium and used as compatible solutes.

types (B, C). Whereas the former type of adaptation requires extensive adjustment of cellular components to the new environment (salts or solutes), the latter types keep the cytoplasm largely free of salt and simply adjust their cytoplasmic water potential by the production and/or accumulation of a narrow range of solutes which, by name, are compatible with cellular functions. This difference is clearly shown by the salt response of cytoplasmic enzymes, which are either salt-dependent or rapidly inactivated by increasing salt concentrations (Oren and Gurevich, 1993). Adaptation to a saline cytoplasm usually covers only a relatively narrow range of physiological conditions, whereas compatible solute producers have developed a more flexible type of adaptation.

It is also apparent that a seemingly salt-sensitive organism may display this phenotypic response only because of the lack of suitable solutes. Hence the composition of the growth medium has a great influence on the individual salt tolerance of bacteria. This is especially pronounced with media containing complex components that may serve as a source for compatible solutes. It should, however, be noted that osmoadaptation at higher levels is determined not only by the composition of the cytoplasm. It also requires major changes in the composition of the membrane. The external phase of the cytoplasmic membrane as well as the periplasmic space are always in contact with a saline environment and require the necessary adjustments.

3. SALT IN CYTOPLASM: THE HALOBACTERIAL SOLUTION

3.1. Halobacteria

The most conspicuous representatives of halophilic bacteria, the halobacteria, belong to the Archaea and have attracted considerable attention due to several structural characteristics. A typical halobacterial environment contains NaCl concentrations ranging from 3 mol/l to saturation. Despite these enormous concentrations, halobacteria keep their cytoplasm relatively free of sodium (Na^+), accumulating instead considerable amounts of potassium (K^+). This is achieved by a potassium uptake system together with an effective sodium export system, which is driven by a proton gradient. This means that part of the proton motive force (geared for ATP synthesis) is diverted to maintain a potassium gradient in the order of 100:1 (3 M internal to 0.03 M external). This expenditure of energy, however, is not lost, but partly drives, for example, the uptake of sodium glutamate or maintains ATP synthesis even in the absence of light or oxidizable substrates (emergency energy supply) (Larsen, 1973; Wagner, 1979). As a consequence, the entire cytoplasm is exposed to high ionic strength and requires structural adaptations. On the enzymatic level, this phenomenon, termed "the halobacteria's confusion to biology" by Larsen (1973), has been extensively studied and the reader is referred to reviews by Eisenberg and Wachtel (1987) and Eisenberg *et al.* (1992). A common characteristic and the most prominent feature of not only halophile enzymes but also co-factors, ribosomes and subsequently whole cells seems to be an additional negative charge (10–14 mol%). The well-studied halophilic malate dehydrogenase from *Halobacterium marismortui*, for example, has an excess of 20 mol% acidic over basic amino acid residues as compared with only 6 mol% in the non-halophilic enzyme (Mevarech *et al.*, 1977). This has been explained by the need to strongly attract a hydration shell in a surrounding environment of low water activity. Owing to the general instability of halophilic enzymes at low salt concentrations

(albeit with exceptions), purification procedures using methods designed for low salt experimental conditions such as ion exchange chromatography, electrophoresis, etc. are severely hampered. This basic methodological difficulty demanding novel procedures (Eisenberg *et al.*, 1992) is the reason why the number of halophilic enzymes studied in pure form is so limited. One of the few exceptions is halophilic malate dehydrogenase (hMDH) of *Halobacterium marismortui*, which was purified by Mevarech *et al.* (1977) and subsequently subjected to stabilization studies (Mevarech and Neumann, 1977; Hecht and Jänicke, 1989; Hecht *et al.*, 1989).

The interactions between proteins and high-salt environments are still poorly understood, mainly because, in contrast to structure resolution of crystals, studies investigating the interaction with solvents and solutes (i.e. protein complexes in solution) have to cope with much lower resolution. Experimental approaches for such studies include density ultracentrifugation, light scattering and small-angle X-ray and neutron scattering. For a review on thermodynamic approaches to macromolecular interaction with solvent components, the reader may refer to Eisenberg (1990). Such elaborate investigations have revealed that native halophilic MDH binds extraordinary amounts of water and salt (0.8–1.0 g water and approximately 0.3 g salt/g protein as compared with 0.2–0.3 g water and approximately 0.01 g salt/g protein of a non-halophilic globular protein) (Zaccai *et al.*, 1986, 1989). However, as the denatured protein does not display unusual hydration properties, a model for the stabilization of halophilic proteins proposed by Zaccai *et al.* (1989) is based on the assumption that the bound water and salt molecules are not associated separately but as hydrated salt ions. The enzyme's tertiary or quaternary structure is therefore important to coordinate hydrated salt at a local concentration higher than in the solvent. A tentative model proposed for hMDH (based on X-ray and neutron scattering studies) sees the protein with a core similar to that of the non-halophilic counterpart, but with loops (containing anionic amino acid residues) extending outwards, interacting with water and providing a large interface with the solvent (Zaccai *et al.*, 1986).

If the exceptional water- and salt-binding properties of halophilic enzymes are indeed based on their unique three-dimensional structure, it should not be surprising that they lose this ability upon denaturation. The observed predominance of negative charge on the enzyme's surface will then in turn lead to a weakening of the conformation due to repulsion when shielding cations are removed (below 0.5 M), hence the dependence on salt. This is convincingly demonstrated by the fact that in some instances decreasing the pH (thus shielding the carboxyl groups) will effectively increase enzymatic activity and stability at low salt concentrations (Eisenberg *et al.*, 1992). In addition, salting-out salts such as potassium phosphate seemingly counteract destabilization at low salinity, albeit at higher concentrations than needed for normal enzymes. The overall effect of salt is, therefore, structure

stabilization by means of tightening the folded conformation and strengthening hydrophobic interactions. Interestingly, this stabilizing effect of potassium salts is (at least partly) also achieved by the addition of sugars, glycerol and other compatible solutes.

3.2. Anaerobic Eubacteria

Among anaerobic halophilic eubacteria, attempts to find organic osmolytes have so far been unsuccessful; instead, high cytoplasmic amounts of potassium and sodium chloride were detected and proteins displayed an excess of acidic amino acids (Oren, 1985). As all enzymes investigated also showed maximal activity at high salt concentrations (Rengpipat *et al.*, 1988; Oren and Gurevich, 1993) there seems to be sufficient evidence that this eubacterial physiological group is adapted to an ionic cytoplasm. The salt-in-cytoplasm type of osmoadaptation is therefore apparently not confined to archaebacterial representatives, although NaCl rather than KCl seems to be the dominant cytoplasmic solute in these eubacteria. However, as the addition of glycine betaine to the growth medium has in some cases led to a marked stimulation of growth, at least partial accumulation of compatible solutes cannot at present be excluded (J. Overmann, personal communication). In view of the little information available to date, osmoadaptation of halophilic anaerobic eubacteria clearly represents an area in need of intensive investigation.

4. ORGANIC OSMOLYTES: THE MOST COMMON SOLUTION

4.1. Low-level Response (Non-halophiles)

4.1.1. *Potassium and Glutamate*

Model organisms and representatives of non-halophiles with limited osmoadaptation (tolerating 0.5 M NaCl) are *E. coli*, *Salmonella typhimurium* and *Rhizobium meliloti*. Here, increased levels of K^+ have been observed as a primary response phenomenon in connection with a salt-stress situation (reviewed by Epstein, 1986). Epstein and Schultz (1965) were able to demonstrate a fairly linear increase in cytoplasmic K^+ from 0.2 M to 0.5 M in *E. coli* with increasing salinity of the medium (up to 0.5 M NaCl equivalent to approximately 1 osmolal). It was, therefore, assumed that potassium accumulation is largely responsible for osmotic balance across the membrane, and, in combination with a corresponding anion, can account for osmotic equilibrium. Other authors reported, for similar conditions, changes in

potassium levels from 0.45 M to 1.05 M, followed by a subsequent decrease to 0.7 M (Dinnbier *et al.*, 1988) and from 0.23 M to 0.8 M (Cayley *et al.*, 1991). It must, however, be noted that volumes vary dramatically in response to osmotic shock and that consequently concentration data largely depend on accurate volume determinations. Based on protein or dry weight, the data proved more consistent and revealed a potassium increase of 0.83 μmol/mg protein (Dinnbier *et al.*, 1988) and 0.47 μmol/mg dry weight (Cayley *et al.*, 1991). A high pre-stress background of potassium of similar size (0.48 μmol/mg) was shown to be associated with macromolecules, and the cytoplasm therefore behaves like a concentrated solution of potassium salts of polyanions, in which additional solutes balance increasing osmolarity of the medium (Cayley *et al.*, 1991).

As a charge counterbalance for K^+ influx, organic anions such as glutamate or, to a minor extent, γ-glutamylglutamine and glutathione (McLaggan *et al.*, 1990) are synthesized. The osmotic regulation of glutamate levels has been well documented, but an in-depth analysis at the molecular level is still pending. Because of the central role of glutamate in bacterial metabolism, it will probably be difficult to resolve. As the amount of glutamate synthesized in many cases does not seem to match the potassium accumulated, alternative or additional measures to maintain charge balance, such as the expulsion of cationic polyamines (e.g. putrescine^{2+}) and Na^+, have been suggested (Munro *et al.*, 1972; Gunther and Peter, 1979; Dinnbier *et al.*, 1988). However, a thorough quantitative analysis by Cayley *et al.* (1991) revealed that the potassium increase in the order of 0.47 μmol/mg dry weight was almost completely balanced by organic anions, comprising synthesized glutamate and accumulated buffer components (3-morpholino-1-propanesulfonic acid, MOPS), both of which contributed to the cytoplasmic anion pool. This increase in potassium glutamate to much higher cytoplasmic values (e.g. from approximately 30 mM to 300 mM when the external osmolality was raised to that of sea water) appears to be a general phenomenon (Tempest *et al.*, 1970). The upper limit for glutamate levels seems to be around 400 mM as calculated on total cytoplasmic water, at least for Gram-negative bacteria (Dinnbier *et al.*, 1988; Welsh *et al.*, 1991), and is often reached only transiently before the onset of subsequent adaptational mechanisms.

4.1.2. *Sugars*

Larsen *et al.* (1987) observed by means of nuclear magnetic resonance (NMR) spectroscopy that the non-halophilic *E. coli*, when stressed osmotically in a glucose mineral medium, accumulated, in addition to potassium glutamate, trehalose in significant amounts (approximately 200 mM). This observation was subsequently investigated in greater depth by Dinnbier *et al.* (1988) and has led to the concept of a sequence of events following osmotic upshift in *E. coli* K12 (Fig. 2). The authors were able to show that, under the given

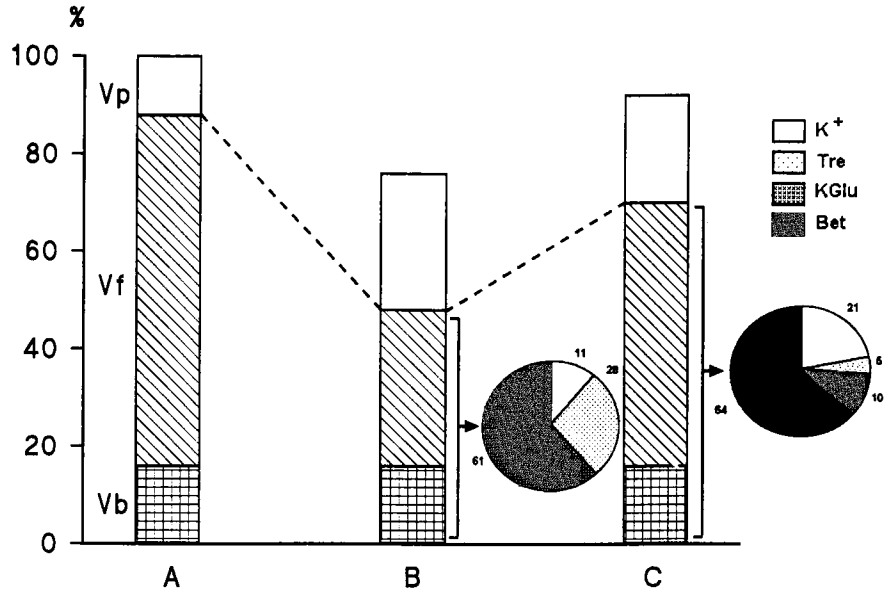


Figure 2 Schematic presentation of response of non-halophilic bacteria (e.g. *E. coli*) to increased salinity (0.5 M NaCl). Bar charts represent relative cell volume for cells grown without salt (A), under salt stress (0.5 M NaCl = approximately 1 osmolal) (B), and salt-stressed in the presence of 1 mM glycine betaine (C). Pie charts display the relative proportion of cytoplasmic osmolytes on an osmolal basis (in total, approximately 1 osmol/kg water). Normal cells contain very little glutamate (approximately 30 mM) but have large amounts of potassium associated with macromolecular polyanions (200 mM in total, equivalent to 1 M if calculated on the basis of bound water). It is clearly seen how osmotic stress affects cytoplasmic volume (Vf + Vb) and how supply of glycine betaine replaces cytoplasmic potassium glutamate and trehalose and also partially restores cell volume. Based on data of Larsen *et al.* (1987), Dinnbier *et al.* (1988) and Cayley *et al.* (1992). Vp, periplasmic volume; Vf, proportion of free water of cytoplasmic volume; Vb, proportion of bound water of cytoplasmic volume; K^+, potassium; Tre, trehalose; KGlu, potassium glutamate and other potassium salts of organic anions, e.g. 3-morpholino-1-propanesulfonic acid (MOPS); Bet, glycine betaine.

experimental set-up, the primary response of rapid potassium uptake (and concomitant synthesis of glutamate) is followed by a replacement of potassium glutamate with other *more compatible* solutes, namely trehalose (in the absence of other alternatives). However, this view has (with regard to the role of trehalose) been challenged by Welsh *et al.* (1991), who monitored the sequence of events in continuous culture of *E. coli* NCIB 9484 (a wild-type strain). They too observed a rapid accumulation of potassium and concomitant synthesis of glutamate, followed by a somewhat delayed

synthesis of trehalose. More importantly, however, these authors reported in the case of both nitrogen- and carbon-limited cultures that the intracellular potassium concentration stayed at elevated levels (2.0 μmol/mg protein), while the time course of the glutamate concentration depended on the experimental conditions (constant in carbon-limited cultures at 1.1 μmol/mg protein, and declining under nitrogen limitation). Therefore, only under nitrogen limitation did the accumulating trehalose appear to replace glutamate (but not potassium). The authors concluded that growth rate and phase of growth may markedly influence the relation of potassium, glutamate and trehalose, hence their interdependence may be difficult to judge.

This view agrees with that of others who regard trehalose as a general stress metabolite, which is synthesized under various adverse growth conditions (Van Laere, 1989; Wiemken, 1990). Supporting evidence is given by the fact that trehalose synthesis is under the control of *rpo*^{s}, a gene encoding sigma factor σ^{s}, which is responsible for the expression of a large number of genes induced upon entry into stationary phase (Hengge-Aronis, 1993). Although, in the light of the above, a dedicated and exclusive role of trehalose in osmoadaptation seems unlikely, it certainly participates in some way to alleviate osmotic stress as mutants defective in trehalose synthesis reveal a phenotype of impaired osmotolerance (Strøm *et al.*, 1986).

Interestingly, the occurrence of sucrose and/or trehalose as the major organic osmolytes is also often connected with marine organisms growing at 3% NaCl (0.5 M) or slightly above that salinity, which is comparable to the environmental range of salt-stressed non-halophiles. Examples include a wide range of cyanobacteria (Reed *et al.*, 1984a; Mackay *et al.*, 1984) and anoxigenic phototrophic species of the genera *Thiocapsa*, *Thiocystis*, *Amoebobacter*, *Chromatium* and *Chlorobium* (Welsh and Herbert, 1993). It is therefore probably justified to conclude that these disaccharides alone have only very limited potential as osmoadaptors. They are, however, often found in really halophilic bacteria as minor components (<500 mM) in combination with other more potent compatible solutes such as betaine or ectoine (Severin *et al.*, 1992). Here, too, one may speculate that trehalose has no primary osmotic function and that the phenomenon of trehalose accumulation is caused by "bottle necks" within the cell's nitrogen metabolism, either due to nitrogen limitations or secondary stress effects, which in turn influence the cell's nitrogen metabolism (Galinski and Herzog, 1990). This view does not, of course, exclude a cross-connection of several stress factors involving multi-faceted response mechanisms. Trehalose and sucrose are known to stabilize membranes, proteins and whole cells, even against total dehydration (Crowe *et al.*, 1987; Carpenter and Crowe, 1989; Louis *et al.*, 1994). Considering the fact that progressing dehydration is indeed often connected with increasing salinity, sugars such as trehalose and sucrose may be primarily meant to secure survival under adverse living conditions, possibly anticipated by an osmo/salt sensor.

It is worthy of note that a novel disaccharide mannosucrose (β-fructofuranosyl-α-mannopyranoside) has been detected in *Agrobacterium tumefaciens* biotype I (salt tolerant up to 2%), where it seems to be involved in osmoregulation (Smith *et al.*, 1990). The choice of this unusual sugar has been explained in terms of the ecology of the organism, which must avoid both sucrose and trehalose, the former because it functions as an attractant towards the rhizosphere, and the latter because it is toxic to many angiospermic plants (Smith *et al.*, 1990). Here, too, the level of acquired salt tolerance proved very low (up to 0.5 M NaCl), and the sugar is rapidly exchanged by other solutes such as betaine, if available.

4.1.3. *Periplasm*

The cytoplasm of bacteria, like that of other cells, contains essential constituents with a minimum total concentration of about 300 mosM (the universal cytoplasm concept; Somero, 1986). At low salinity, the periplasmic space is almost iso-osmotic with the cytoplasm (ca. 300 mosM), which means that turgor—usually around 3 bar (300 kPa) in Gram-negative organisms—is maintained across the outer membrane (reviewed by Benz, 1994). This is achieved by the presence of anionic membrane-derived oligosaccharides (MDOs) in the case of *E. coli* and the analogous cyclic glucans in members of the family Rhizobiaceae and of the *Agrobacterium* group comprising a class of periplasmic glucans widely distributed among Gram-negative bacteria (Schulman and Kennedy, 1979; Miller *et al.*, 1986). This osmotic role for periplasmic oligosaccharides under normal growth conditions has fostered speculations as to the osmotic status of the periplasm at elevated salinities.

Salt-dependent alterations of *E. coli* K12 outer membrane porins and of periplasmic membrane-derived oligosaccharides have been reported (Kennedy, 1982; Fiedler and Rotering, 1988). The two major proteins OmpC and OmpF are osmoregulated (ratio of OmpC/OmpF increasing with salinity), but at high osmolarity only OmpC is relevant. The synthesis of these outer membrane porins is controlled by a two-component regulatory system involving EnvZ (located in the cytoplasmic membrane) and OmpR (a DNA-binding protein), both products of the *ompB* locus (Hall and Silhavy, 1981a, b). The mechanism of osmosensing is not yet known and an involvement of MDOs has been discussed, either as a direct osmotic signal (Fiedler and Rotering, 1988) or as a means to provide a minimal ionic strength needed for porin regulation in media of very low osmolarity (Geiger *et al.*, 1992). Although expression of porins is clearly influenced by osmolarity (in a reciprocal fashion), other factors such as carbon source, pH, oxygen and temperature also influence the expression of the *ompC* and *ompF* genes (for reviews, see Graeme-Cook *et al.*, 1989; Mizuno and Mizushima, 1990).

In addition, the outer membrane general diffusion pores have exclusion limits of 600 Da and higher and are considered relatively unselective even for charged small molecules such as inorganic ions (reviewed by Benz, 1994). We have therefore very little evidence to support the view that the outer membrane should exclude small ionic solutes such as NaCl from the surrounding medium.

As regards the role of MDOs in osmoadaptation, it must be stressed that their synthesis is *inversely* related to osmolality, which means that their concentration is about 10-times higher (up to 5% of dry weight) in a medium of low osmolarity (Miller *et al.*, 1986). This excludes an active implication in osmotic adjustment (buffering capacity) and the periplasmic space should, therefore, be seen as a compartment similar to the surrounding medium as far as osmotic pressure and ionic charge are concerned. Consequently, the cytoplasmic membrane forms the main permeability barrier for external solutes, be they salts or small organic osmolytes.

4.1.4. *External Solutes*

The upper limit of salt-tolerance of *E. coli* in mineral medium (close to 0.5 M NaCl) can be extended towards higher salinities (0.85 M = 5% NaCl) with concomitant improvement of growth rates by the addition of external osmolytes to the medium. This salt protection was first observed by Larsen *et al.* (1987), who were able to show that betaine (like other solutes) was accumulated inside the cells, enhancing growth and salt tolerance of *E. coli*. In a recent paper, again on *E. coli*, Cayley *et al.* (1992) demonstrated that high levels of cytoplasmic potassium ions, trehalose and glutamate are rapidly replaced by potent organic osmolytes such as betaine and that the original cell volume is partially restored in this process (Fig. 2). It is important to note that the characterization of an organism's salt tolerance therefore largely depends on the availability of protecting compounds and the presence of suitable uptake systems. These inter-relations have pronounced consequences not only for our understanding of microbial interaction in saline ecosystems, including survival, but also for the enrichment and isolation of organisms from such habitats (Gauthier and Le Rudulier, 1990; Ghoul *et al.*, 1990; Marthi and Lighthart, 1990).

As media supplements such as yeast extract contain considerable amounts of protecting solutes (often betaine) (Fig. 3) or precursors thereof, non-halophiles usually display a wider salt tolerance in complex media. *E. coli* K12, for example, is easily grown on a yeast extract medium with 5% NaCl, but barely tolerates 3% NaCl in the absence of complex supplements. An organism's salt tolerance and/or osmotolerance is therefore not a species-specific parameter but rather a very variable medium-dependent characteristic. As illustrated in Fig. 3, even small amounts of glycine betaine are

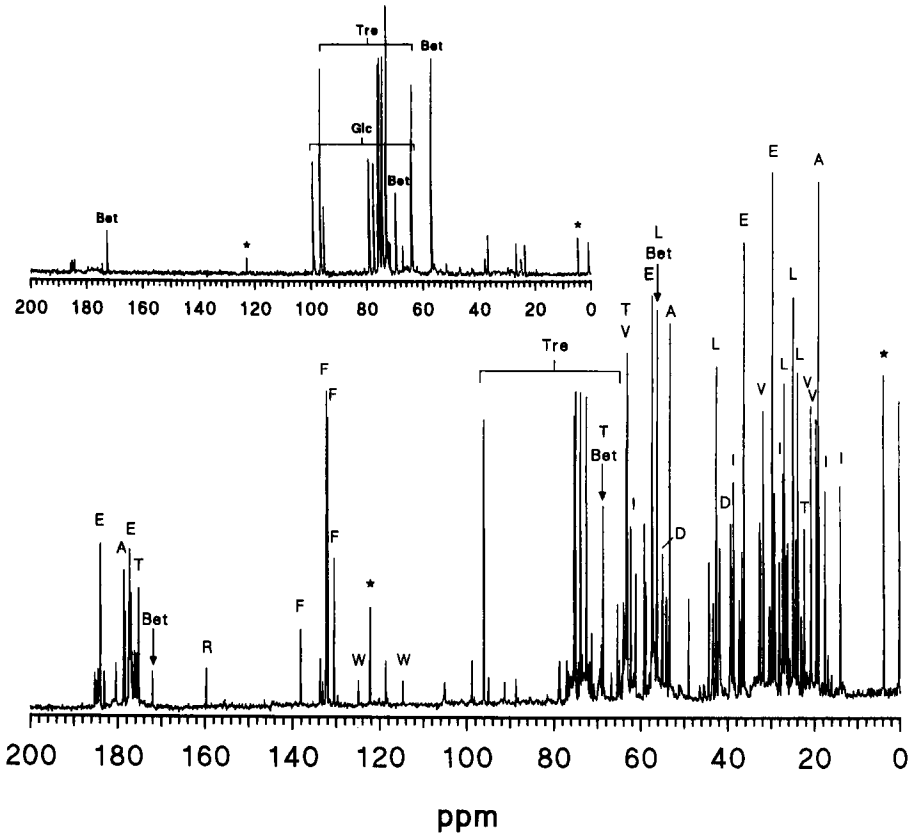


Figure 3 NMR spectra of Difco yeast extract (large spectrum) and of a crude cell extract of *E. coli* grown at 5% NaCl in the presence of yeast extract (2 g/l) (small spectrum). It is clearly seen that glycine betaine, which constitutes only a minor component of yeast extract (1–3%), is taken up and used as osmolyte in a salt-stress situation. The high proportion of glucose results from the action of a periplasmic trehalase (cf. chapter 3). Letters represent international codes for amino acids; Bet, betaine; Glc, glucose; T, trehalose.

specifically accumulated from an abundance of organic carbon and used as salt-stress osmolytes.

The information available on growth improvement of non-halophiles by the addition of solutes reveals a whole range of compounds of similar salt-protecting effects. These include betaines, amino acids and derivatives as well as sulfonium compounds, either synthesized by bacterial primary producers or gained from animal or plant resources. The situation is even more complicated by the fact that bacteria are able to convert suitable

precursors into compatible solutes. As uptake and/or conversion of organic osmolytes is also an important process for the adaptation of *truly* halophilic species, the whole spectrum of potential osmolytes and structural implications for osmoadaptation and for the understanding of the function of compatible solutes will be discussed in later sections.

4.2. High-level Response (Above 5% NaCl)

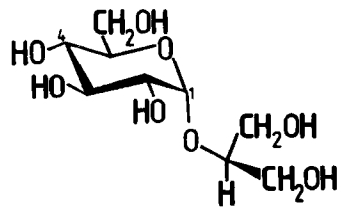
4.2.1. *Membrane Adjustment*

A saline environment well above marine concentrations (5% and higher) clearly presents an obstacle for non-halophiles and provides an ecological niche for halophilic/halotolerant microorganisms. Even though the cytoplasmic interior of a compatible-solute user (Fig. 1B,C) is protected from salt, the outer surface of the cytoplasmic membrane (as well as periplasmic space and outer membrane) is permanently exposed to high salt concentrations. One would therefore expect adaptive changes in response to varying salinities. Alterations of membrane composition have been extensively studied in a range of halophilic eubacteria (Vreeland *et al.*, 1984; Adams *et al.*, 1989; Thiemann and Imhoff, 1991; Imhoff and Thiemann, 1991; Adams and Russell, 1992; Ritter and Yopp, 1993). As a rule, the proportion of anionic phospholipids (often phosphatidylglycerol and/or glycolipids) increases with salinity at the expense of neutral zwitterionic phospholipids. This structural modification adds additional surface charge to the membrane, and as such parallels the mode of adaptation described for "halophilic" enzymes. Excess negative charge probably helps to maintain the hydration of the interface, and has a pronounced effect on lipid phase behavior (Russell, 1989; Sutton *et al.*, 1991). The potential to adjust the membrane according to the external salinity seems to be one prerequisite that probably distinguishes slightly adaptable non-halophiles from truly halophilic/halotolerant bacteria. It has often been the subject of speculation whether the exclusion of salt and the maintenance of a steep ionic gradient is achieved by active (energy-dependent) transport mechanisms or by a tightening of the permeability barrier of the membrane. As far as I know, this question has not been pursued in depth with halophilic eubacteria of the organic-osmolyte type. Our own continuous culture experiments with *Halomonas elongata* (J. Severin and E. A. Galinski, unpublished) strongly indicate that the maintenance energy is relatively independent of the salinity of the growth medium, providing evidence for the concept of an ion-tight membrane. Thorough investigations into the maintenance and regulation of ion permeability in so-called "salt-free" halophilic bacteria of the osmolyte type are necessary.

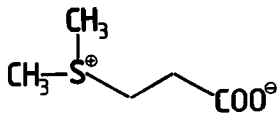
4.2.2. *Compatible Solutes*

(a) *Diversity of compounds.* Following earlier inconclusive reports and ambiguities as to the composition of the cytoplasm of halophilic eubacteria (Measures, 1975; Koujima *et al.*, 1978; Chan *et al.*, 1979; Vreeland, 1987; Hart and Vreeland, 1988), extensive use of natural abundance ^{13}C NMR techniques has finally revealed a whole range of compounds synthesized by these organisms (Fig. 4). Almost all halophilic strains available from culture collections (and, in addition, approximately 200 of our own isolates from various saline environments) have been investigated for their ability to synthesize compatible solutes. Compatible solute screening included cyanobacteria (Borowitzka *et al.*, 1980; Reed *et al.*, 1984a; Mackay *et al.*, 1984), anoxygenic phototrophic bacteria (Galinski and Trüper, 1982; Galinski *et al.*, 1985; Galinski, 1986; Trüper and Galinski, 1986), aerobic chemoheterotrophic Proteobacteria of the α- and γ-subdivisions, actinomycetes, Gram-positive cocci, bacilli and related species such as *Staphylococcus* and *Salinicoccus* species (Wohlfarth *et al.*, 1990; Müller, 1991; Severin *et al.*, 1992; del Moral *et al.*, 1994) as well as moderately halotolerant species of the genera *Brevibacterium* and *Corynebacterium* (Gouesbet *et al.*, 1992; Bernard *et al.*, 1993; Frings *et al.*, 1993). Although this survey is fairly comprehensive, at least within the field of chemoheterotrophic eubacteria, one should keep in mind that only three eubacterial divisions have been covered, i.e. Cyanobacteria, Proteobacteria and Firmicutes, and that the field is still open for further discoveries of novel compatible solutes. This search for nature's osmolytes has also been supplemented by work with archaebacterial methanogens, which in turn has shown that this strategy of osmoadaptation has been developed in both bacterial domains (Robertson *et al.*, 1990; Lai *et al.*, 1991). From our present state of knowledge, the following conclusions can be drawn.

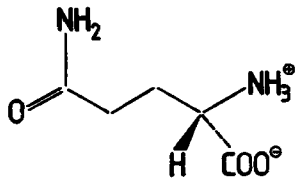
1. Biosynthesis *de novo* of polyols such as glycerol, arabitol and inositol, which are often found in halophilic/osmophilic fungi, algae and salt-tolerant plants (Wegmann, 1971; Ben-Amotz and Avron, 1973; Storey and Wyn Jones, 1977; Hocking and Norton, 1983), has so far not been detected in halophilic bacteria.
2. Charged amino acids such as glutamate, β-glutamate, glutamate betaine and others are never accumulated to very high concentrations (above approximately 400 mM).
3. All compatible solutes synthesized and employed at concentrations of approximately 500 mM and higher are polar, highly soluble molecules which carry no net charge. They fall into the following classes of compounds (Fig. 4):



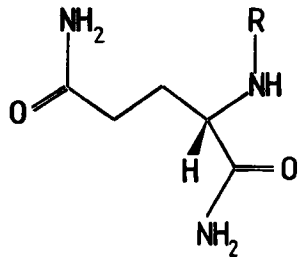
Glucosylglycerol



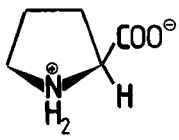
Dimethylsulfoniopropionate



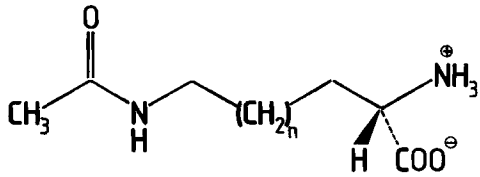
Glutamine



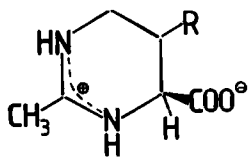
Glutamine amides



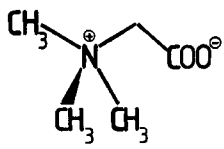
Proline



N-acetylated diaminoacids



Ectoines



Glycine betaine

Figure 4 Important organic osmolytes (compatible solutes) synthesized by halophilic and/or halotolerant bacteria: glucosylglycerol; dimethylsulfoniopropionate (DMSP, propiothetine); the amino acids proline and glutamine; glutamine amide derivatives such as *Nα*-carbamoylglutamine-1-amide (R = $-CONH_2$) or *Nα*-acetylglutaminylglutamine amide (R = -*Nα*-acetylglutaminyl); *N*-acetylated diamino acids such as *Nδ*-acetylornithine (n = 1) and *Nε*-actyllysine (n = 2); ectoine (R = H) and hydroxyectoine (R = OH); glycine betaine; *β*-forms of amino acids such as *β*-glutamine and *Nε*-acetyl-*β*-lysine are found in methanogenic Archaea.

—sugar polyol derivatives (glucosyglycerol);
—zwitterionic trimethyl ammonium and dimethylsulfonium compounds (betaines, thetines);
—natural amino acids (proline, glutamine);
—glutamine amide derivatives (*Nα*-carbamoylglutamine amide; *Nα*-acetyl-glutaminyglutamine amide);
—*N*-acetylated diamino acids (*Nδ*-acetylornithine, *Nε*-acetyllysine);
—ectoines (ectoine, β-hydroxyectoine).

Their occurrence among halophilic/halotolerant producer strains is summarized in Table 1.

Glucosylglycerol (O-α-*D*-glucopyranosyl-(1→2)-glycerol). This glycerol glucoside, which is structurally related to floridosides (O-α-*D*-galactopyranosyl-(1→2)-glycerol) and isofloridosides (O-α-*D*-galactopyranosyl-(1→1)-glycerol) of certain red algae and chrysophyceae (Impellizzeri *et al.*, 1975; Kauss, 1979), seemed until recently typical for a wide range of moderately halophilic cyanobacteria (Borowitzka *et al.*, 1980; Mackay *et al.*, 1983, 1984; Reed *et al.*, 1984a). Here it was shown that the choice of organic osmolyte employed is closely correlated with the upper salinity level for growth, dividing cyanobacteria into groups of intermediate and high salt tolerance, with glucosylglycerol as the typical solute for strains of intermediate salt tolerance (Mackay *et al.*, 1984; Reed *et al.*, 1984a,b).

Glucosylglycerol was subsequently also found in a purple bacterium (*Rhodobacter sulfidophilus*) (Galinski, 1986; Severin *et al.*, 1992) and, most recently, in *Pseudomonas mendocina* and *P. pseudoalkaligenes* (Pocard *et al.*, 1994). This first description of glucosylglycerol in a chemoheterotrophic eubacterial genus, which is easily grown by conventional fermentation, will most certainly have a large impact on the future availability of this compound.

Dimethylsulfonioproprionate (DMSP). Apart from their well-documented occurrence in certain higher plants (Rhodes and Hanson, 1993; Paquet *et al.*, 1994), the importance of methylated sulfur compounds such as DMSP as osmotic solutes has so far been shown only for a limited number of cyanobacteria (Visscher and van Gemerden, 1991) and marine algae (Blunden and Gordon, 1986; Blunden *et al.*, 1992). Structurally, the thetines are comparable to *N*-methylated ammonium compounds such as glycine betaine; they are, however, less stable against dehydration and the characteristic smell of rotten seaweed is partly caused by dimethylsulfide, a degradation product of DMSP. Assuming that DMSP acts as a compatible solute in marine algae, it has been proposed that it substitutes for betaine under nitrogen-limiting conditions (Turner *et al.*, 1988). This compound, as well as its shorter homologue (dimethylsulfonioacetate), are readily

Table 1 Occurrence of compatible solutes in different groups of halophilic/halotolerant bacteria.

Solute	Organism	Reference
Glucosylglycerol	Cyanobacteria	Reed *et al.* (1984a)
	Rhodobacter sulfidophilus	Galinski (1986)
	Pseudomonas mendocina	Pocard *et al.* (1994)
Dimethylsulfonio-propionate (DMSP)	Marine cyanobacteria	Visscher and van Gemerden (1991)
Amino acids		
Proline	Some bacilli and related species	
	Planococcus citreus	
	*Staphylococcus epidermidis**	
	Salinicoccus sp.*	Severin *et al.* (1992)
Glutamine	Corynebacteria	Frings *et al.* (1993)
β-Glutamine	*Methanohalophilus* sp.	Lai *et al.* (1991)
Glutamine amides		
CGA	*Ectothiorhodospira marismortui*	Galinski and Oren (1991)
AGGA	*Chromatium, Thiocapsa* sp.	Welsh and Herbert (1993)
	Rhodopseudomonas sp.	Severin *et al.* (1992)
	Azospirillum brasilense	Severin *et al.* (1992)
	Rhizobium meliloti	Smith and Smith (1989)
	Pseudomonas aeruginosa	D'Souza-Ault *et al.* (1993)

Acetylated DAA		
*N*δAcetyl-ornithine	Strain M96/12b	Wohlfarth *et al.* (1993)
	Sporosarcina halophila	Severin *et al.* (1992)
	Bacilli and other related organisms	Severin *et al.* (1992)
*N*ϵAcetyl-lysine	*Sporosarcina halophila*	
	Planococcus citreus	Severin *et al.* (1992)
	Some bacilli	del Moral *et al.* (1994)
*N*ϵAcetyl-β-lysine	Methanogenic bacteria	Sowers *et al.* (1991)
Ectoines	All heterotrophic Proteobacteria of the γ-subdivision	
	Rhodobacter sulfidophilus	
	Nocardiopsis sp.	
	Brevibacteria	
	Micrococcus halobius	
	Micrococcus varians ssp. halophilus	
	Many bacilli	
	Marinococcus sp.	
	Sporosarcina halophila	Galinski (1986)
		Wohlfarth *et al.* (1990)
		Müller (1991)
		Severin *et al.* (1992)
		Frings *et al.* (1993)
Betaines		
Glycine betaine	Cyanobacteria	Mackay *et al.* (1984)
	Anoxigenic phototrophic bacteria	Severin *et al.* (1992)
	Methanogenic bacteria	Robertson *et al.* (1990)
	Actinopolyspora halophila	Severin *et al.* (1992)
Dimethylglycine	*Methanohalophilus* sp.	Menaia *et al.* (1993)

CGA, *N*α-carbamoylglutamine amide; AGGA, *N*α-acetylglutaminylglutamine amide; DAA, diamino acids.

*No growth on synthetic media, complex supplements required.

taken up by *E. coli*, *S. typhimurium* and others and are apparently interchangeable with betaine as an osmolyte (Paquet *et al.*, 1994; Diaz *et al.*, 1992). However, the synthesis of *S*-methylated zwitterions for osmotic purposes has not been demonstrated in heterotrophic bacteria. It still remains to be shown if heterotrophic bacteria, given the right environmental conditions, may also prove to be potential producers of methylated sulfonio compounds.

Natural amino acids (proline and glutamine). Apart from glucosylglycerol and DMSP, all other compatible solutes synthesized *de novo* are amino acids or amino acid derivatives. Among prokaryotes, proline was originally considered the typical osmolyte of halophilic *Bacillus* species. This view was primarily based on investigations into *Bacillus subtilis* and closely related species (Measures, 1975). A thorough screening of a whole range of halophilic/halotolerant bacilli and related species, however, has revealed that the majority of species produce ectoine, either alone or in combination with proline and/or acetylated diamino acids (Müller, 1991; Severin, *et al.*, 1992; del Moral *et al.*, 1994).

The observation that other solutes such as betaine or ectoine are usually preferred over proline (if there is a choice) appears to support the view that proline is a solute of lesser compatibility, at least at very high salt concentrations. *B. subtilis* and *Planococcus citreus*, therefore, seem to belong to a minority of proline-osmoregulated bacillus-type organisms which are unable to synthesize other osmolytes. Further proline producers are found among *Staphylococcus* and *Salinicoccus* species, which usually require complex media components for growth. It should also be pointed out that the compatible solute spectrum can vary remarkably with media composition and growth conditions, especially among proline producers. Strain M96/12b, for example, upon entering the stationary phase, converts proline into another compatible solute, *N*δ-acetylornithine (Wohlfarth *et al.*, 1993).

The homologous pipecolic acid has been described as an osmolyte in *Brevibacterium ammoniagenes* (renamed *Corynebacterium glutamicum*) (Gouesbet *et al.*, 1992). This observation, however, was not confirmed by the work of Frings *et al.* (1993), and it still has to be shown if synthesis of pipecolic acid *de novo* is genuine or if this compound is primarily accumulated from the medium.

The amino acid α-glutamine has a relatively low solubility (approx. 0.3 M) compared with other solutes (e.g. proline, 14 mol/kg). Because of this, high cytoplasmic concentrations reported in moderately halotolerant members of the genus *Corynebacterium* (Frings *et al.*, 1993) come close to saturation. However, this does not take into account the possible influence of the cytoplasmic environment on the solubility of this amino acid *in vivo*. It is nevertheless tempting to speculate that physical

properties of solutes may determine their osmotic limitations. The β-form of glutamine, which has so far not been detected in eubacterial halophiles, reaches cytoplasmic concentrations well above 0.5 M in halophilic methanogenic archaebacteria (Robertson *et al.*, 1990; Lai *et al.*, 1991; Roberts *et al.*, 1992), and it is this higher solubility (as compared with the α-isomer) which probably makes this compound a superior compatible solute. However, the fact that only glutamine (and occasionally alanine) but none of the more soluble amino acids (for example, serine with a solubility of 2.4 mol/kg) seems to have osmotic functions, suggests that solubility alone cannot account for a solute's compatibility.

***N*-derivatized glutamine amides.** Glutamine derivatives containing glutamine-1-amide as a characteristic structural element are typically carbamoylated or acetylated at the α-nitrogen, or (in the case of dipeptides) linked to the carboxyl terminus of a second amino acid. Both molecular modifications, amidation at C-1 and derivatization of the α-amino group, lead to the formation of a polar non-ionic molecule which is fundamentally different from all other compatible solutes of the zwitterion type. Whereas *N*α-carbamoylglutamine amide is relatively unique (Galinski and Oren, 1991), the neutral dipeptide *N*α-acetylglutaminylglutamine amide has already been described in a number of Proteobacteria.

***N*-acetylated diamino acids.** Diamino acids such as ornithine and lysine are positively charged at physiological pH. Acetylation of one of the amino groups renders the molecule an neutral zwitterionic solute and as such a more suitable compatible osmolyte. The involvement of *N*δ-acetylornithine in osmoadaptation was first documented with one of our own isolates, strain M96/12b (Wohlfarth *et al.*, 1993). This strain has been shown to group with the so-called bacillus-related species and its compatible solute, *N*δ-acetylornithine, was subsequently detected—at least as a minor component—in almost all *Bacillus* species under investigation, including related organisms such as *Sporosarcina halophila* and *Planococcus citreus* (Severin *et al.*, 1992). The homologous *N*ε-acetyllysine, originally isolated and identified from *Sporosarcina halophila*, is also relatively widespread among bacilli and related organisms (Severin *et al.*, 1992; del Moral *et al.*, 1994). Interestingly, the β-isomer *N*ε-acetyl-β-lysine has been described as a compatible solute of halophilic archaebacterial methanogens (Sowers *et al.*, 1991; Lai *et al.*, 1991). Homologues shorter than acetylated ornithine, such as *N*-acetylated diaminobutyric acid, on the other hand, have so far not been connected with osmotic functions, although *N*-acetyldiaminobutyrate in particular resembles the hydrolyzed linear form of ectoine (see below).

Ectoines. 1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid (ec-

toine) was named after its discovery in the phototrophic sulfur bacterium *Ectothiorhodospira halochloris* (Galinski *et al.*, 1985). This solute is without doubt one of the most abundant osmolytes in nature, and whereas betaine can be regarded as the typical product of halophilic phototrophic bacteria, ectoines can be seen as the common solutes of aerobic heterotrophic eubacteria (at least within the Proteobacteria and Firmicutes).

Although not obvious from its chemical formula, ectoine constitutes the cyclic form of *N*-acetylated diaminobutyrate (see above). Because of the delocalization of the π-electrons its amino functions are, however, not reactive and therefore not detectable by standard amino acid analysis, which explains why this compound escaped detection for such a long time. In fact, failure to find any organic osmolytes by conventional techniques has in the past often led to confusion and unnecessary speculation about the composition of a halophile's cytoplasm (Vreeland, 1987; Hart and Vreeland, 1988). The β-hydroxy derivative of ectoine, often found as a minor component in Proteobacteria of the γ-subdivision, seems to be of greater importance in Gram-positive eubacteria such as *Nocardiopsis* species, some brevibacteria, *Marinococcus* species and others (Severin *et al.*, 1992; Frings *et al.*, 1993).

Bacterial products containing ectoine moieties have also been detected in *Rhizobium loti* ("rhizolotine") (Shaw *et al.*, 1986; Scott *et al.*, 1987) and in *Pseudomonas* species (as part of siderophores) (Demange *et al.*, 1990; Gipp *et al.*, 1992; Gwoese and Taraz, 1992), and it remains to be seen if ectoines are common constituents of other secondary metabolites. In addition, both ectoine and hydroxyectoine were found in non-halophilic antibiotic-producing streptomycetes, where they seem to protect the producer strain from its own antimicrobial product (Inbar and Lapidot, 1988). These observations suggest that ectoines are not just dedicated osmolytes but also serve a variety of other functions.

Glycine betaine. Glycine betaine (subsequently named betaine) was one of the first organic solutes known to be involved in osmoregulatory functions in the plant and animal kingdoms (Wyn Jones and Storey, 1981; Blunden *et al.*, 1986; Larher, 1988; Bagnasco *et al.*, 1986; Dragolovich, 1994) as well as in prokaryotes of different ecophysiology. As has been pointed out before, this compound is also one of the preferred external osmolytes, which is readily taken up by non-halophiles in order to extend their range of salt tolerance.

Although the role of betaine as a universal compatible solute is undoubted, the ability to synthesize betaine has been largely overestimated, mainly due to the fact that its presence (or that of suitable precursors) in complex media components was not accounted for and that transport properties for betaine were not known at the time (Imhoff and

Rodriguez-Valera, 1984). In addition, there has been confusion in the literature because the term "betaine synthesis" has often been used indiscriminately to refer to situations where the precursor choline was in fact only converted into betaine by a two-step enzymatic sequence (see later; Fig. 9). Today, we understand that the ability to synthesize betaine *de novo* is rare among chemoheterotrophic eubacteria (one of the few exceptions being *Actinopolyspora halophila*) but typical for both oxigenic and anoxigenic phototrophic eubacteria, especially for those displaying a high salt tolerance (Galinksi and Trüper, 1982; Reed *et al.*, 1984b; Mackay *et al.*, 1984; Imhoff, 1986). Betaine is probably abundant in natural environments where mass development of phototrophic bacteria (e.g. cyanobacterial mats) is very common. It may therefore serve as a convenient source for heterotrophic bacteria that are either unable to synthesize their own compatible solutes or are able to switch to more economical uptake strategies for osmoadaptation (cf. section 4.2.2(d).

Another source for betaine—this time within the anoxic environment—is to be found in a range of halophilic and halotolerant archaebacterial methanogens (Robertson *et al.*, 1990; Lai *et al.*, 1991), which produce significant cytosolic concentrations of glycine betaine. In addition, the demethylated derivative of glycine betaine, *N*,*N*-dimethylglycine, has been detected in three moderately halophilic species of the genus *Methanohalophilus* as the major compatible solute, reaching a cytoplasmic concentration of 2 M (Menaia *et al.*, 1993). It is presently not known, and remains to be seen, if an ineffective final methylation step is the reason for the accumulation of dimethylglycine. In any case, the dimethyl variant of betaine is apparently equally suitable for osmotic adaptation.

(b) *Biosynthetic pathways*. Known and hypothetical pathways of compatible solute synthesis are summarized in Fig. 5. It is shown that apart from glucosylglycerol and trehalose, all nitrogen- and sulfur-containing compatible solutes (as far as we know them) are arranged or believed to be arranged around the cell's amino acid metabolism deriving from oxaloacetate, oxoglutarate and glyoxylate. The aspartate branch provides the building blocks for ectoine (and hydroxyectoine) and probably also for DMSP via the sulfur-containing amino acid methionine. The majority of other compatible solutes, including the glutamine amide derivatives, acetylated diamino acids and proline, are however, derived from glutamate or its precursor oxoglutarate. It has never been shown, but always been assumed, that proline used osmotically is synthesized in the same way as the proteinogenic compound. However, as independent regulation of both functions is vital for osmotic adaptation, the possibility of a separate pathway cannot be excluded. The concomitant presence and interdependence of proline and acetylated diamino acids in many halophilic bacillus-group organisms supports the view that these pathways may be interrelated as shown in Fig. 5, and that

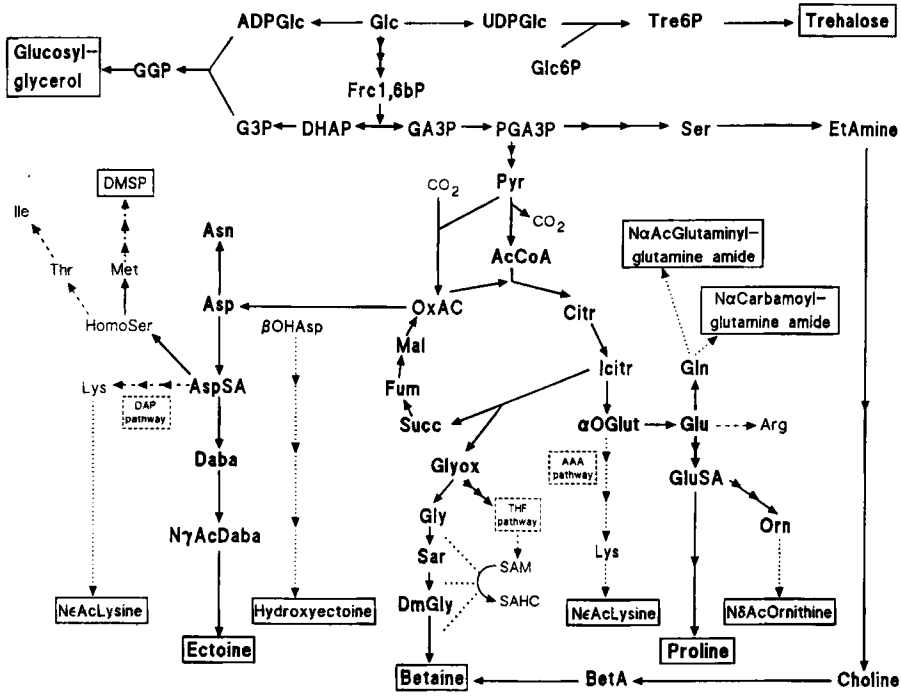


Figure 5 Summary of biosynthetic pathways for compatible solutes in bacteria, including hypothetical reactions (dotted lines). Broken lines illustrate common sequences within the cell's amino acid metabolism. AAA, aminoadipic acid; AspSA, aspartic semialdehyde; BetA, betaine aldehyde; Citr, citrate; Daba, α,γ-diaminobutyrate; DAP, diaminopimelic acid; DHAP, dihydroxyacetone phosphate; DmGly, dimethylglycine; DMSP, dimethylsulfoniopropionate; EtAmine, ethanolamine; Frc1,6bP, fructose-1,6-bisphosphate; Fum, fumarate; GA3P, glyceraldehyde-3-phosphate; GGP, glucosylglycerolphosphate; Glc, glucose; GluSA, glutamic semialdehyde; Glyox, glyoxylate; G3P, glycerol-3-phosphate; Ictr, isocitrate; Mal, malate; NγAcDaba, *N*γ-acetyldiaminobutyrate; αOGlut, α-oxoglutarate; βOHAsp, β-hydroxyaspartate; OxAc, oxaloacetate; Pyr, pyruvate; SAHC, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; Sar, sarcosine; Succ, succinate; THF, tetrahydrofolate; Tre6P, trehalose-6-phosphate.

N-acetylated lysine used for osmoadaptation may be derived from a pathway uncommon in prokaryotes. Similarly, the observation that cytoplasmic levels of *N*α-carbamoylglutamine amide (CGA) rise when glutamine is supplied as a precursor may serve as an indication for glutamine-linked synthesis of *N*α-carbamoylglutamine amide and *N*α-acetylglutaminylglutamine amide (Oren *et al.*, 1991). According to the scheme in Fig. 5, the biosynthesis of betaine follows a straightforward strategy of successive methylation of glycine or, alternatively, a two-step oxidation of its precursor choline.

It should, however, be stressed at this point that only very few bacterial pathways have actually been elucidated in detail. Among those are biosynthetic routes for betaine (Roberts *et al.*, 1992; Tschichholz, 1994), ectoine (Peters *et al.*, 1990; Tao *et al.*, 1992) and glucosylglycerol (Hagemann and Erdmann, 1994). A biosynthetic strategy for DMSP formation has been deduced (but not proven) from recent investigations into its metabolism in plants (Hanson *et al.*, 1994).

Betaine. The biosynthetic pathway of betaine, which is also a convenient compatible solute in salt-tolerant higher plants, had already been investigated in halophytes of the family Chenopodiaceae when this compound was discovered as a solute in bacteria (Galinski and Trüper, 1982). In plants, the synthesis proceeds either directly (spinach) or through phosphoryl intermediates (sugar beet) or as a combination of both via serine and ethanolamine, which is then methylated to yield choline (Coughlan and Wyn Jones, 1982; Hanson and Rhodes, 1983). Choline is subsequently oxidized involving two irreversible enzymatic steps, namely choline oxidase and betaine aldehyde dehydrogenase. The route of betaine synthesis in cyanobacteria still remains to be determined, although Sibley and Yopp (1987) suggested a similar pathway involving *S*-adenosyl methionine as a methyl group donor. An analogous biosynthetic route via the oxidation of choline was, however, ruled out for *Ectothiorhodospira* species (Galinski, 1986; Trüper and Galinski, 1990).

With the help of ^{13}C-labelling techniques, a direct methylation of glycine using *S*-adenosyl methionine as a methyl donor was suggested and subsequently confirmed at enzymatic level (Tschichholz, 1994). Methyl groups are probably supplied by cleavage of glyoxylate and subsequent reduction of formyl-tetrahydrofolate through the THF pathway (Galinksi, 1986; Trüper and Galinski, 1990; Tschichholz, 1994). A diagram of the betaine pathway as we see it now, at least for *Ectothiorhodospira* species, is presented in Fig. 6. It is interesting to note that there has been convincing evidence, again using NMR techniques, for a similar pathway (direct methylation of glycine) in the phylogenetically distant methanogenic Archaea (Roberts *et al.*, 1992; Robertson *et al.*, 1992). However, subsequent elucidation of betaine pathways in halophilic/halotolerant cyanobacteria and chemoheterotrophic eubacteria such as *Actinopolyspora halophila* is needed to complete the picture.

Although regulatory mechanisms have not been investigated as yet, a feedback control as proposed by Sibley and Yopp (1987) may serve as a starting point for future analysis. On the basis of their observations with cyanobacteria, they proposed an intriguing hypothesis of regulation where methylation is inhibited by the reaction product, *S*-adenosyl homocysteine. A reversible hydrolase, which is activated by potassium in the direction of hydrolysis and by betaine in the direction of synthesis of *S*-adenosyl

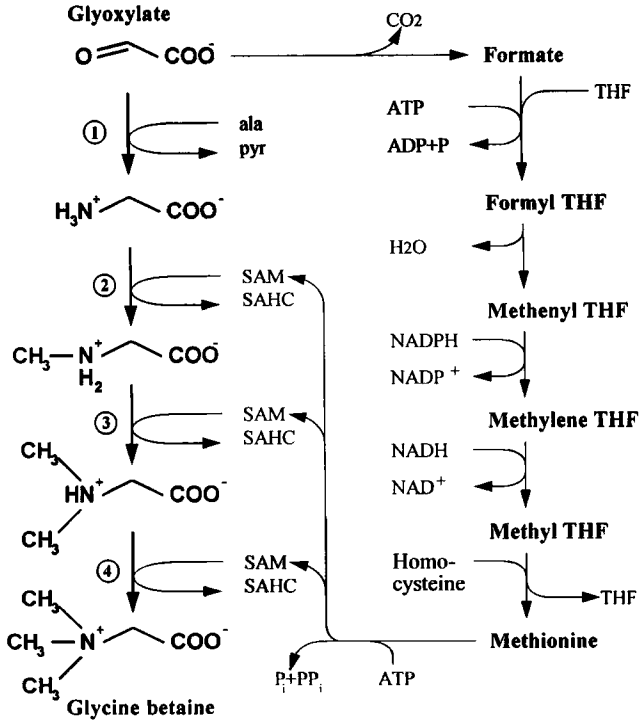


Figure 6 Biosynthetic pathway of glycine betaine in the sulfur bacterium *Ectothiorhospira halochloris* via direct methylation of glycine (provided from glyoxylate). Methyl groups are supplied by the tetrahydofolate pathway with *S*-adenosylmethionine as the terminal donor. Enzymes involved are: (1) glutamate:glyoxylate aminotransferase; (2) glycine transmethylase; (3) sarcosine transmethylase; (4) dimethylglycine transmethylase (data from Tschichholz, 1994). A similar pathway is probably used in methanogenic Archaea (Roberts *et al.*, 1992; Robertson *et al.*, 1992). A regulatory mechanism based on the modulation of *S*-adenosylhomocysteine hydrolase activity as suggested by Sibley and Yopp (1987) is at present hypothetical.

homocysteine, would thus control the level of inhibition and regulate the cytoplasmic concentration of betaine.

Ectoine. According to its chemical structure, ectoine can be classified as a partially hydrated pyrimidine carboxylic acid. Its biosynthetic origin was first investigated in *Ectothiorhodospira* species, again with the help of ^{13}C-labelling techniques, and it was revealed that the C2-unit enclosed by the two ring-nitrogens originated from an acetate unit and that aspartate was the likely precursor for the remainder of the molecule (Galinski, 1986;

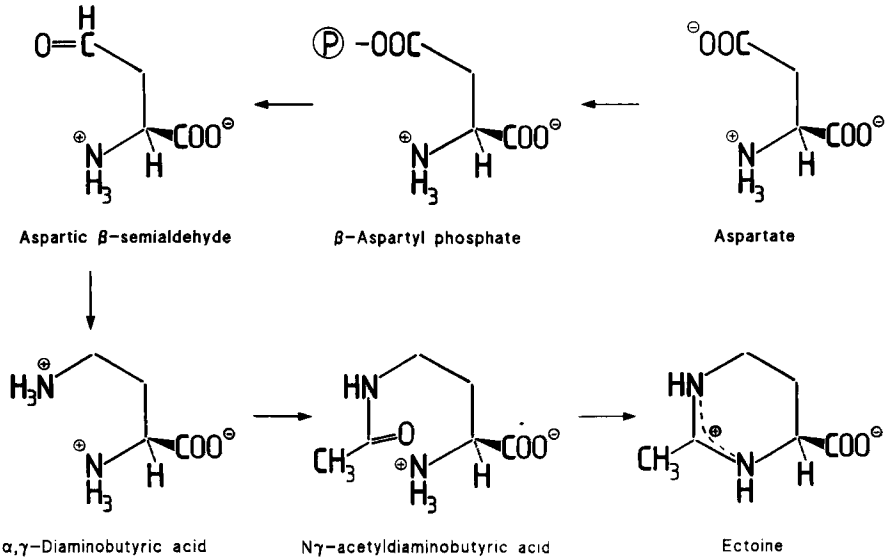


Figure 7 The biosynthetic pathway of ectoine. The first two reactions, yielding aspartic β-semialdehyde, are common sequences of the cell's amino acid metabolism (formation of lysine, methionine, threonine, isoleucine). Subsequent enzymes required for the biosynthesis of ectoine are: 2,4-diaminobutyrate transaminase, 2,4-diaminobutyrate acetylase, *N*γ-acetyldiaminobutyrate dehydratase (ectoine synthase). An analogous pathway for hydroxyectoine is assumed to take a similar route starting from erythro-β-hydroxyaspartate, which has the same steric configuration as hydroxyectoine (*S*,*S*-α-amino-β-hydroxy form). However, subsequent hydroxylation of ectoine or one of its precursors cannot be excluded.

Khunajakr *et al.*, 1989). A biosynthetic route proceeding via aspartic semialdehyde, diaminobutyrate and *N*γ-acetylated diaminobutyrate (Fig. 7) was subsequently confirmed on enzymatic grounds for both phototrophic *Ectothiorhodospira halochloris* and chemoheterotrophic *Halomonas elongata* (Peters *et al.*, 1990). This pathway requires only three additional enzymes, namely diaminobutyrate transaminase, diaminobutyrate acetylase and *N*γ-acetyldiaminobutyrate dehydratase (ectoine synthase) (Peters *et al.*, 1990). The fact that relatively few and common enzymatic reactions form the basis for ectoine biosynthesis probably explains why this solute (and its hydroxy derivative) are so widespread among halophilic bacteria. Pursuing similar objectives, the group of M. Takano and H. Ono has succeeded in purifying and characterizing some of the enzymes involved in ectoine synthesis of *Halomonas elongata*. 2,4-Diaminobutyrate transaminase located at the branching point of the ectoine pathway has an apparent molecular mass of 260 000 Da and is probably composed of six subunits. The presence of potassium ions increased its activity three-fold

in the range of 1–50 mM. In addition, potassium was needed to stabilize the enzyme at the organism's optimal growth temperature (37°C). Regulatory properties within this pathway are still under investigation.

Glucosylglycerol. As glucosylglycerol is structurally and functionally comparable to heterosides, e.g. floridoside (O-α-*D*-galactopyranosyl-(1→2)-glycerol) of red algae, analogous biosynthetic pathways have been assumed. In red algae, condensation of UDP-galactose and glycerol-3-phosphate gives galactosylglycerol-phosphate, which is subsequently dephosphorylated to yield galactosylglycerol (Kremer and Kirst, 1981). The many unsuccessful attempts to prove the involvement of UDP-activated glucose in glucosylglycerol synthesis have recently been explained by Hagemann and Erdmann (1994), who revealed that glucosylglycerol is in fact synthesized from ADP-activated glucose and glycerol-3-phosphate in *Synechocystis* sp. PCC6803. The pathway's dependence on ADP-activated sugars contrasts with both those of heteroside biosynthesis in red algae and trehalose synthesis in *E. coli* (Giæver *et al.*, 1988) and *Ectothiorhodospira halochloris* (Lippert *et al.*, 1993), which are all strictly dependent on UDP-sugars. The glucosyglycerol pathway in *Synechocystis* involves two specific enzymes: ADP-glucose:glycerol-3-phosphate, 2-glycosyltransferase (GGP synthase) and glucosylglycerol-3-phosphate phosphohydrolase (GGP phosphatase). The glucosylglycerol-forming pathway required activation, which was achieved *in vitro* by the addition of NaCl to the assay buffer. It was suggested that the immediate activation of glucosyglycerol synthesis by salt is possibly caused by post-translational covalent modifications, probably via protein phosphorylation/dephosphorylation (Hagemann and Erdmann, 1994).

DMSP. Little is known about the biosynthetic pathway of DMSP in microorganisms, except for the fact that methionine is the source of the sulfur atom and of both methyl groups in *Ulva lactuca* (a green alga) and that the α-carbon of methionine will yield the carboxyl group of DMSP (Greene, 1962; Rhodes and Hanson, 1993). These data have led to the proposal of a pathway where methionine is oxidatively deaminated, decarboxylated and finally methylated (Maw, 1981). However, the actual sequence of reactions has so far not been clarified in microorganisms. A biosynthetic pathway for DMSP in *Wollastonia biflora*, a common Indio-Pacific strand plant, has presented evidence that methylation is the first step in the sequence, not the last (Hanson *et al.*, 1994).

(c) *Downshock adjustment.* In nature, often on a seasonal basis, disturbances of medium osmolality are usually gradual in the direction of increasing salinity (evaporation processes), but dilution of the surrounding medium may well be sudden through heavy rainfall or local underground or open fresh

water supplies. Slow and gradual changes are usually mastered by adjusting synthesis of compatible solutes. A correlation of compatible solute content with external salinity has been demonstrated in many cases and cytoplasmic concentrations have been calculated to be as high as 2.5 mol/kg water (equivalent to approximately 25% of cell dry weight) (Galinski, 1986; Wohlfarth *et al.*, 1990; Severin *et al.*, 1992). In the event of a dilution, the microbial population is often faced with relatively rapid changes. This situation is potentially dangerous for cells full of compatible solutes, as rapid influx of water will dramatically increase turgor pressure (with the risk of cell rupture) unless mechanisms are provided that can deal within minutes with such shock events and increased cytoplasmic turgor. Possible response mechanisms would entail rapid catabolism, including conversion of solutes into osmotically inactive forms and/or extrusion of solutes into the surrounding medium (Galinski and Trüper, 1994).

Catabolism of compatible solutes is very common in the natural environment, both by anaerobic as well as aerobic processes (Oren, 1990). In addition, many halophilic eubacteria (including *Halomonas elongata*) are able to use compatible solutes as both osmoticum and carbon source (Severin, 1993). In *Rhizobium meliloti* and in bacteroids from *Medicago sativa* (induced by *Rhizobium meliloti*), for example, glycine betaine is degraded and used as a carbon source at low salinities only (via successive demethylation to glycine), while at elevated salinity catabolism is blocked and betaine is conserved as an osmoticum (Bernard *et al.*, 1986; Le Rudulier and Bernard, 1986; Smith *et al.*, 1988; Fougère and Le Rudulier, 1990). In a situation of rapid dilution, however, many catabolic processes are seemingly too slow and rapid extrusion mechanisms are used. Marine cyanobacteria such as *Synechocystis* and *Synechococcus* species, for example, upon transfer to a fresh water medium, were shown to release up to 50% of low molecular weight carbohydrates and 73% of cytoplasmic amino acids within 2 min (Reed *et al.*, 1986; Fulda *et al.*, 1989). Furthermore, some anoxigenic phototrophic primary producers are unable to degrade glycine betaine, even under severe nitrogen limitation (Galinski and Herzog, 1990). These organisms depend totally on their rapid extrusion systems, which can master even dramatic events.

Diluting a culture of *Ectothiorhodospira halochloris* from a salinity of 20% to 12% total salts will lead to instantaneous and massive export of betaine in an overshoot-like manner with subsequent re-uptake of solutes until a steady state is achieved (Tschichholz and Trüper, 1990). Among heterotrophic halophilic bacteria, *Halomonas elongata* has served as a model organism for rapid release phenomena. Here, a dilution from 15% to 5% salinity caused rapid extrusion of ectoine without affecting viability of cells (T. Sauer and E. A. Galinski, unpublished). Similar release mechanisms—although on a much smaller scale—have also been observed in *E. coli* (Koo *et al.*, 1991). The nature of such selective extrusion of solutes is at present

far from clear and still under discussion. Evidence has been provided for the action of so-called stretch-activated or mechanosensitive channels, which are supposed to be closed at normal turgor and open above a critical turgor pressure. The work of Sukharev *et al.* (1994) and A. Ghazi (personal communication) has already led to a more detailed characterization of one such family of membrane proteins in *E. coli*, namely MscM, MscS and MscL (selective for cations, anions and unselective, respectively) and even revealed the localization of some of the genes for these channel proteins (downstream of *trkA*). It is therefore only a question of time until rapid release phenomena will be better understood, hopefully also for truly halophilic bacteria displaying a much wider span of osmotolerance.

The excretion of betaine and other solutes into the environment has interesting consequences. The well-known mass developments of phototrophic bacteria (e.g. cyanobacterial mats) in saline environments probably make betaine one of the most abundant solutes in marine ecosystems, where considerable concentrations have been reported in interstitial waters of sediments (Gauthier and LeRudulier, 1990; Ghoul *et al.*, 1990). Besides serving as carbon and nitrogen supply for many chemoheterotrophic aerobes as well as anaerobes (Oren, 1990), osmolytes released into the environment are also used by several halophilic/halotolerant bacteria as an alternative and easily accessible source of compatible solutes, provided they possess the necessary uptake systems. Therefore, compatible solutes play an important part in saline ecosystems, both as alternative osmolytes for solute producers and as vital supplements for others depending on these solutes. Even non-halophilic organisms unable to synthesize compatible solutes themselves may thrive in a saline environment if vital solutes (or suitable precursors) are supplied by *de novo* producers. This intriguing possibility has been extensively demonstrated and it is thanks to research on non-halophilic organisms such as *E. coli*, *S. typhimurium* and recently also *Staphylococcus aureus* and *Bacillus subtilis* that we have a more clear conception of the nature of at least some of the transport systems involved in osmoregulation.

(d) *Uptake systems*. Solute uptake systems are of prime importance for all organisms facing an osmotic challenge. As to be expected, they have been most thoroughly studied in enterobacteria and rhizobia, which, although salt-sensitive by nature, can make use of effective transport mechanisms for betaines, ectoines and other osmolytes and acquire a certain degree of halotolerance (to at least 5% NaCl) provided suitable solutes are supplied with the medium (May *et al.*, 1986; Cairney *et al.*, 1985a,b; Jebbar *et al.*, 1992). Two distinct uptake systems, namely a low-affinity system ProP and a high-affinity system ProU, play an important part in osmotic adaptation of these Proteobacteria. These two systems were originally associated with proline uptake (hence the name) and it was only shown later that they transport a relatively wide spectrum of solutes with much higher affinity than

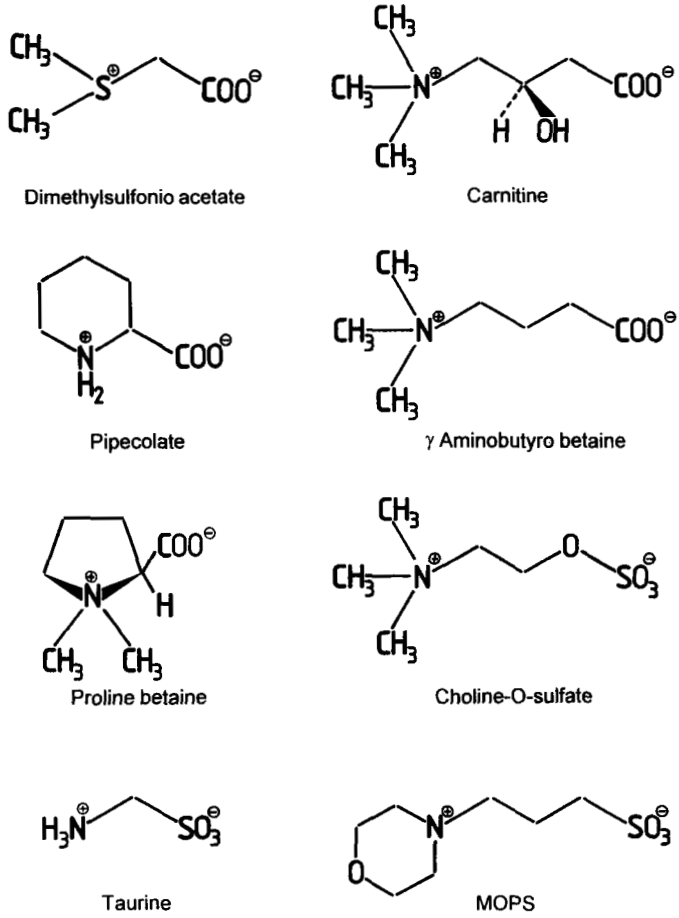


Figure 8 Osmolytes of (apparently) non-bacterial origin accepted by proP/proU transport systems. Natural sources of dimethylsulfonioacetate (the shorter homologue of DMSP), pipecolate choline-O-sulfate and MOPS are at present uncertain (Gouesbet *et al.*, 1992; Frings *et al.*, 1993); proline betaine and γ-butyrobetaine are typical osmolytes of halotolerant plants; carnitine and taurine are derived from animal sources.

proline. While the major focus of attention has so far centred on just a few major bacterial compatible solutes (mainly betaine, proline, ectoine and hydroxyectoine), many more—some clearly not of microbial origin—were found to be accumulated and to provide osmoprotection (Chambers *et al.*, 1987; Mason and Blunden, 1989). It is now known that the osmolyte transport systems described above are relatively unspecific, accepting also other components of plant and animal resources or even man-

made/derivatized compounds such as 3-morpholino-1-propanesulfonic acid (MOPS) and choline-O-sulfate (Fig. 8). Preferred substrates for uptake, as far as they have been compared, are (in order of growth improvement): betaine, ectoine, dimethylsulfoniopropionate, proline betaine, choline-O-sulfate, proline, pipecolate, carnitine and γ-aminobutyrobetaine (Bernard *et al.*, 1986; Fougère and Le Rudulier, 1990; Gloux and Le Rudulier, 1989; Mason and Blunden 1989; Jung *et al.*, 1990; Jebbar *et al.*, 1992; Bernard *et al.*, 1993; Talibart *et al.*, 1994; Gouesbet *et al.*, 1994). The structural similarity of these osmolytes is apparent, and one may speculate that the range of possible substrates and potential osmotic protectants has by no means been fully explored.

Following a recent revival of interest in osmoregulation within Gram-positive representatives, *Listeria monocytogenes*, *Staphylococcus aureus* (both remarkably halo/osmotolerant and a major cause of food poisoning) and *Bacillus subtilis* have become another focus of investigation (Anderson and Witter, 1982; Patchett *et al.*, 1992; Whatmore *et al.*, 1990). Here, too, specific transport systems for proline and betaine have been detected (Pourkomailian and Booth, 1992; Bae and Miller, 1992; Bae *et al.*, 1993; Stimeling *et al.*, 1994; Ko *et al.*, 1994; Patchett *et al.*, 1994). In addition, the crucial role of carnitine as an osmoprotectant (always abundant in meat and milk products) has been shown for *Listeria monocytogenes* (Beumer *et al.*, 1994) and some lactic acid bacteria (Kets *et al.*, 1994). The discovery of this interdependence of salt/osmotolerance and environmental solute supply, especially in complex matrices of low water activity, is bound to have a large impact on our understanding of the pitfalls of food preservation and on the advance of predictive microbiology.

As regards true halophiles, our knowledge of transport mechanisms is at present very limited. In general, halophilic bacteria will also prefer uptake over *de novo* biosynthesis of species-specific solutes for energetic reasons, provided appropriate transport systems are inducible. The betaine synthesizing cyanobacteria *Aphanothece halophytica*, *Dactylococcopsis salina* and *Synechocystis* DUN52, for example, possess a powerful betaine transport system, which is responsible for rapid uptake and subsequent osmotic equilibration (Reed and Stewart, 1988; Moore *et al.*, 1987). Similarly, uptake systems in *Ectothiorhodospira halochloris* have been demonstrated and characterized (Peters *et al.*, 1992). Others, such as the ectoine transport system of Gram-positive chemoheterotrophic eubacteria, have so far not been investigated in depth. The whole range of potential transport systems involved in osmoregulation may well stretch beyond current beliefs. Furthermore, the situation is even more complicated by the fact that many bacteria are able to convert suitable precursors into compatible solutes (see below). Hence an additional range of pre-osmolyte transport systems have to be considered, and may well be osmoregulated.

(e) *Conversion of precursors*. Because of earlier confusion concerning

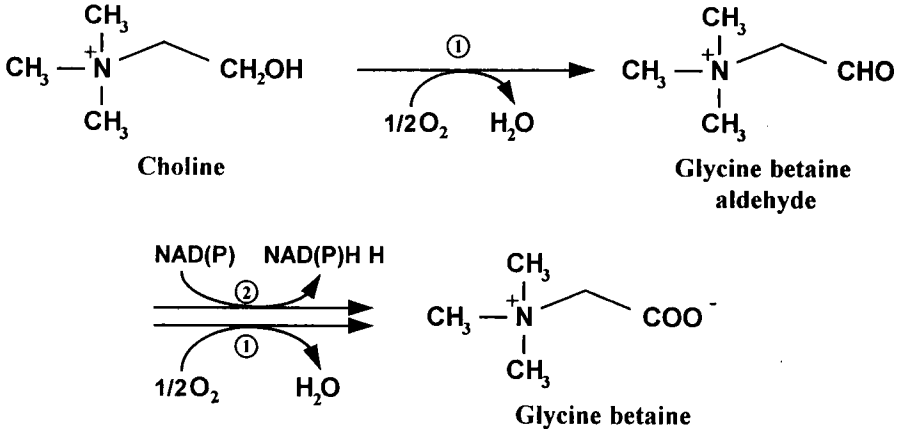


Figure 9 Conversion of precursors into osmolytes: oxidation of choline by an O_2-dependent choline oxidase (1), which also catalyzes the second enzymatic step, and a specific NAD-dependent betaine aldehyde dehydrogenase (2).

microbial abilities to *synthesize* betaine, synthesis *de novo* from simple carbon sources such as CO_2, acetate, pyruvate and glucose should be clearly distinguished from conversion mechanisms involving only a few enzymatic steps and depending on external precursors. One such conversion mechanism, often still misnamed "betaine synthesis", is the enzymatic oxidation of choline (Fig. 9) (Le Rudulier *et al.*, 1984; Strøm *et al.*, 1986). Choline, a common component of complex media derived from the hydrolysation of choline-containing phospholipids, is readily taken up by two independent uptake systems (high affinity and low affinity) and then converted by the action of two enzymes, namely a membrane-bound O_2-dependent (electron transfer-linked) choline oxidase and a specific NAD-dependent betaine aldehyde dehydrogenase (Styrvold *et al.*, 1986; Falkenberg and Strøm, 1990). This pathway, which has been intensively investigated, not only in *E. coli* (Landfald and Strøm, 1986), but also in *Rhizobium meliloti* (Smith *et al.*, 1988), depends on aerobic conditions for the oxidation of choline. It is also found in Gram-positive microorganisms such as bacilli and appears to be quite common among aerobic chemoheterotrophic halophiles (J. Severin and E. A. Galinski, unpublished). Our present state of knowledge concerning genomic organization and regulatory aspects will be discussed in more detail in section 4.3.

Another example of solute acquisition by conversion—this time derived from *osmo*tolerant species—is found in the Gram-negative fermentative ethanol-producing bacterium *Zymomonas mobilis*. It was originally isolated from fermented agave sap (pulque) (Kluyver and Hoppenbrouwers, 1931) and found to tolerate up to 40% (2.2 M) glucose while being sensitive to as

little as 2% NaCl (0.34 M) (Swings and DeLey, 1977). The concomitant formation of sorbitol, which appeared in the medium during growth on sucrose or a mixture of glucose and fructose, led to the proposal of a pathway for sorbitol synthesis and the discovery of a novel periplasmic enzyme (glucose-fructose oxidoreductase, GFOR) which converts fructose into sorbitol (Leight *et al.*, 1984; Zachariou and Scopes, 1986). While the physiological function of this enzyme remained obscure for a long time, it was shown only recently that sorbitol produced in the periplasm is also taken up and used as a compatible solute to balance (at least in part, in combination with cytoplasmic sugars) the osmotic effect of a high sugar-content environment (Loos *et al.*, 1994). Sorbitol was shown to accumulate internally to 700 mM in cells grown in a medium containing 1 M sucrose, and to promote markedly growth under elevated sugar concentrations (Loos *et al.*, 1994), but not at increased salinity (upper limit of 2% NaCl), indicating that adjustment of membrane components may be the limiting factor for growth rather than osmotic adjustment.

The sorbitol formation and uptake system of *Zymomonas* represents the only case of osmoadaptation by polyols in bacteria so far and resembles a combination of a compatible solute uptake strategy (transport system) with biosynthetic properties of osmolyte production from a natural carbon source. Although it cannot be excluded that sorbitol is also produced in small amounts within the cytoplasm by the action of an aldose reductase, the main source of sorbitol is without doubt the enzymatic conversion of fructose by a periplasmic glucose-fructose oxidoreductase (GFOR) and subsequent uptake via energy-dependent transport systems (Fig. 10). The extraordinary low affinity of GFOR for fructose (K_m = 400–1000 mM) (Zachariou and Scopes, 1986) explains why sorbitol production is restricted to hyperosmotic stress only. Although these unusual features of the GFOR system have given rise to speculation on a possible role as an osmosensor (indicating indirectly the presence of high concentrations of sucrose), an involvement of the sorbitol transport system in osmoregulation seems more likely. At present, however, nothing is known about osmosensing and signal transduction in *Z. mobilis*.

4.3. Regulation at the Molecular Level

4.3.1. *Non-halophiles*

The best-studied model systems of osmotic adaptation are clearly *E. coli* and *S. typhimurium* and the reader is referred to excellent reviews by Le Rudulier *et al.* (1984), Epstein (1986), Strøm *et al.* (1986), Booth and Higgins (1990), Csonka and Hanson (1991) and Lucht and Bremer (1994). Thorough investigations into the *E. coli* world of osmoadaptation (as briefly sum-

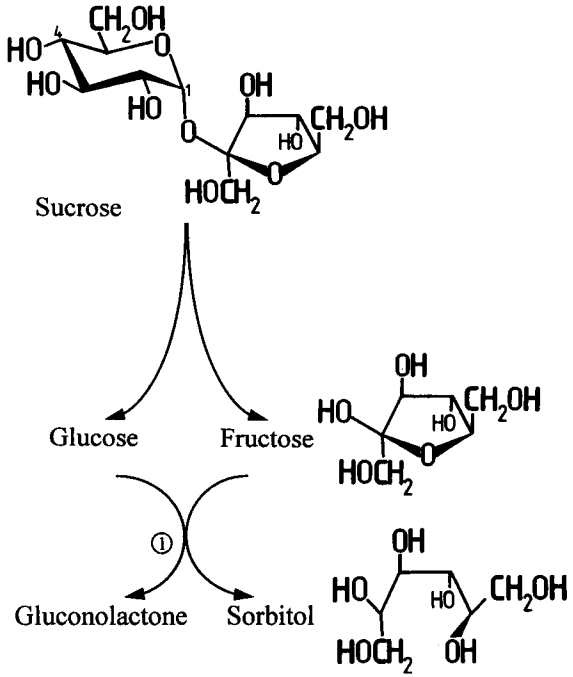


Figure 10 Conversion of sucrose into sorbitol by *Zymomonas mobilis*. Following the cleavage of sucrose a periplasmic glucose-fructose oxidoreductase (GFOR) (1) converts fructose into sorbitol, which is then accumulated as a compatible solute in the cytoplasm.

marized below) are of particular interest because of the central role of turgor maintenance for all living organisms. In addition, one can expect that the principal discoveries of osmoresponse may also apply to much more severe osmotic stress situations such as those experienced by real halo/osmophiles. For *E. coli*, the sequence of events following osmotic upshock is well-documented, and the interrelationship of at least four regulating mechanisms is illustrated in Fig. 11: potassium uptake (and formation of counterion), trehalose synthesis, uptake and conversion of choline, accumulation of external betaine and other compatible solutes.

(a) *Potassium*. The first step is rapid accumulation of potassium (probably triggered by a sensory mechanism), for which three distinct potassium uptake systems have been described (Dosch *et al.*, 1991); namely TrkA, TrkD (both constitutive) and Kdp. Although all three uptake systems display increased activity in response to osmotic upshock, only expression of the high-affinity Kdp system is osmotically regulated and has been characterized in terms of osmoregulation (Altendorf *et al.*, 1992). The Kdp porter apparently con-

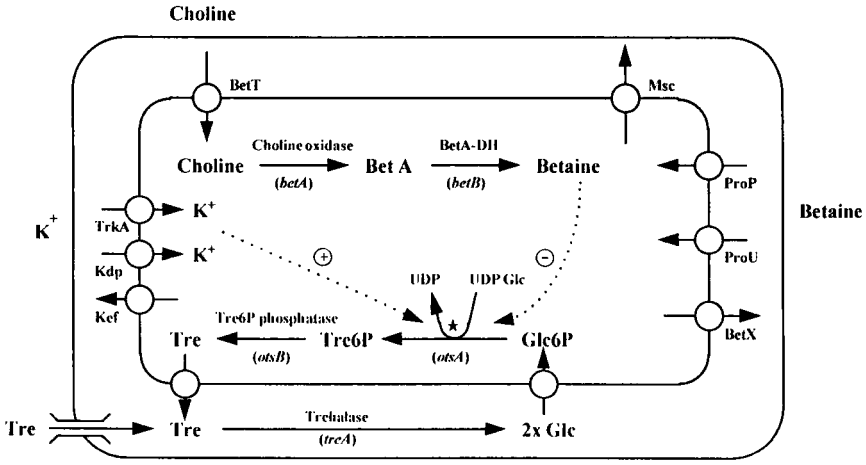


Figure 11 Schematic diagram illustrating the interrelation of several osmoregulated systems in *E. coli*, where cytoplasmic K^+ and betaine exert a regulatory effect on trehalose-6-phosphate synthesis (further explanation in text). TrkA, one of two constitutive K^+ uptake systems (TrkD, not shown); Kdp, inducible osmoregulated K^+ uptake system; Kef, K^+ efflux system; BetT, choline transport system; ProP and ProU, transport systems for compatible solutes such as betaine; BetX, betaine efflux system; Msc, mechanosensitive channel (stretch-activated) for the rapid release of osmolytes; BetA, betaine aldehyde; Glc, glucose; Tre, trehalose; *, trehalose-6-phosphate synthetase; designations in parentheses refer to the genes encoding the enzymes.

stitutes an emergency uptake system, which is expressed only when Trk cannot maintain turgor pressure. The *kdpABC* operon encodes three proteins constituting a membrane transport ATPase (Hesse *et al.*, 1984; Siebers and Altendorf, 1993). The transcription of the *kdpABC* operon is under the control of regulatory genes *kdpD* and *kdpE* located immediately downstream of the *kdpABC* structural genes (Polarek *et al.*, 1992; Walderhaug *et al.*, 1992). Following a still unknown stimulus, the cytoplasmic membrane protein KdpD undergoes autophosphorylation and subsequently transfers the phosphate group to a soluble regulator protein KdpE (phosphorylation cascade), which, thus activated, binds to a *cis*-acting sequence upstream of the *kdpABC* promotor and triggers *kdp* transcription (Sugiura *et al.*, 1994).

Although the importance of the K^+transport system for rapid osmotic adaptation is well established, it has since been shown that K^+ accumulation is not vital for osmotic regulation and that *E. coli* can also adapt, although more slowly, to environmental changes in the presence of uncouplers dissipating the proton motive force (Ohyama *et al.*, 1992). The role and

regulation of at least three additional distinct export systems Kef (Douglas *et al.*, 1991) still need to be elucidated.

The concomitant accumulation of glutamate (and of other organic counter-ions) has unfortunately not been investigated in depth. The chances are that potassium accumulation itself may activate the synthesizing enzymes, but because of the central role of glutamate in the cell's amino acid metabolism, the combined influence of different regulating factors will probably be difficult to resolve. Cytoplasmic K^+-glutamate resulting from this primary response to osmotic stress is subsequently replaced by organic osmolytes, which are known to stabilize the integrity of cell components and the function of proteins in solution. This accumulation of solutes is a universal phenomenon observed across the microbial, plant and animal worlds.

(b) *Trehalose.* In *E. coli* the synthesis of trehalose is achieved by the concerted action of two enzymes: trehalose-6-phosphate synthetase (using UDP-glucose and glucose-6-phosphate as substrates) and trehalose-6-phosphate phosphatase. The activity of the synthetase is activated by K^+-glutamate and other monovalent cations (Giæver *et al.*, 1988; Strøm and Kaasen, 1993). The corresponding genes of the trehalose pathway, *otsA* and *otsB*, are controlled by a regulatory gene (rpo^S), which is induced not only by osmotic stress but also during stationary phase. This regulator, a putative sigma factor (σ^S), was subsequently shown to trigger a whole range of stress response mechanisms including thermotolerance and long-term starvation survival (Lange and Hengge-Aronis, 1991; Hengge-Aronis *et al.*, 1991). These findings clearly support the view that trehalose synthesis indicates a broad and general stress response mechanism, and that its role as an osmolyte may be only secondary (Jenkins *et al.*, 1990). In this context, it may be worthy of note that salt/osmotic stress are factors accompanying water loss and hence very good indicators for the onset of a resting period. Nevertheless, mutants impaired in trehalose synthesis proved more sensitive to osmotic stress than the wild type (Giæver *et al.*, 1988).

A highly active periplasmic trehalase (TreA) apparently works as a scavenger for leaked-out trehalose. It splits trehalose into two glucose units which are then rapidly recovered by a high affinity glucose-specific transport system (Gutierrez *et al.*, 1989; Boos *et al.*, 1990; Styrvold and Strøm, 1991). Expression of the structural gene *treA* is stimulated by high osmolarity and controlled by rpo^S (Hengge-Aronis *et al.*, 1991). Surprisingly, the inducible transport system in *E. coli* for trehalose (PTS) is inhibited by high osmolarity. A probable explanation is that the inducer (trehalose-6-phosphate) is removed by the action of osmoregulated trehalose-6-phosphate phosphatase (*otsB*) (Klein *et al.*, 1991).

(c) *Choline oxidation.* This pathway, which has been intensively investigated both in *E. coli* and *Rhizobium meliloti* (Smith *et al.*, 1988), depends on aerobic conditions for the oxidation of choline and is induced by osmotic stress (Strøm *et al.*, 1986). In *E. coli* the *bet* genes encoding the os-

moregulatory choline-betaine pathway have been cloned (Andresen *et al.*, 1988; Styrvold *et al.*, 1986) and the entire *bet* region has been sequenced (Lamark *et al.*, 1991). The genes *betA* and *betB* encode a membrane-bound choline oxidase (catalyzing both oxidation reactions) and a soluble NAD-dependent betaine aldehyde dehydrogenase, respectively. The *betT* gene encodes a high-affinity transport system energized by proton motive force and activated by high osmolarity; the *betI* gene product resembles a regulator protein for *bet* expression.

Lac-fusion analysis has revealed that the *bet* genes are regulated at the transcriptional level by K^+, betaine, choline, osmolarity and oxygen (Eshoo, 1988), probably via the action of the repressor *betI*. The observed mechanisms of regulation were shown to be slightly different in *R. meliloti* (stimulation), presumably because choline is also used as a carbon source, and only under osmotic stress is the subsequent degradation of betaine blocked (Smith *et al.*, 1988).

The *bet* genes have been successfully transferred into *S. typhimurium*, which has a large chromosomal deletion and lacks this pathway (Andresen *et al.*, 1988). Similarly, a choline-oxidizing (*cox*) gene from *Arthrobacter pascens* has been introduced into an *E. coli* strain defective in betaine synthesis (Rozwadowski *et al.*, 1991). In both cases, gene transfer yielded an osmotolerant phenotype. This opens up the possibility of using the *bet* gene system for the transfer of osmotolerance to osmosensitive micro-organisms or (as a long-term goal) even to salt- or drought-sensitive cultivars.

(d) *Solute transport*. The two transport systems primarily involved in compatible solute accumulation from the medium (ProP and ProU) have been extensively studied and from the wealth of information attainable the whole complexity of osmoregulation becomes apparent. The ProP and ProU solute porters are principally different and probably also serve different functions. The low affinity system ProP comprises a single polypeptide embedded in the cytoplasmic membrane and energized by a proton motive force (Culham *et al.*, 1993). It is responsible for osmotically triggered rapid transport processes, probably on the basis of conformational change and not subject to feedback inhibition (Koo *et al.*, 1991). In addition to proline, the ProP transport system also accepts betaine, ectoine, taurine and other compounds of similar structure and, despite its relatively low affinity, probably accounts for most of the solute uptake from the medium (Stirling *et al.*, 1989). Solute uptake via ProP constitutes one of the most rapid osmoregulatory responses detected so far, and makes this transport system one of the primary respondents to changes in cell turgor (Milner *et al.*, 1988). Sequencing of the *proP* gene has revealed similarities with sequences of genes encoding integral membrane proteins comprising a transporter superfamily (Culham *et al.*, 1993). This type of transport system catalyzes facilitated diffusion or ion-linked transport of organic solutes, giving weight to

the assumption that ProP resembles an osmoregulated solute-ion co-transporter.

ProU on the other hand is a multi-component high-affinity transport system (extensively reviewed by Lucht and Bremer, 1994). This porter also has a broad substrate specificity and, apart from betaine ($K_m = 1.3\ \mu M$), accepts ectoine, proline and taurine, albeit with lower affinity. It has been suggested that the primary function of the ProU system may be to recapture and recycle osmolytes that leak from the cell, serving an osmoprotective role even in the absence of external osmolytes (Stirling *et al.*, 1989).

The *ProU* gene locus of *S. typhimurium* has been characterized and shown to comprise three genes (*proV*, *proW*, *proX*). They are transcribed as a single operon and coordinately expressed under the control of a major osmoregulated promotor displaying homologies to the consensus sequence of σ^{70}-dependent *E. coli* promotors (Gowrishankar, 1989; Stirling *et al.*, 1989). The operon encodes the following gene products: a hydrophilic protein associated with the inner side of the cytoplasmic membrane, which is the energy coupling component able to hydrolyse ATP (ProV), a hydrophobic integrated membrane protein (ProW) and a periplasmic binding protein of very high affinity for a range of osmolytes (K_d around $1\ \mu M$) (ProX). Further periplasmic binding proteins with high affinity for betaine have been identified in *Rhizobium meliloti* (Le Rudulier *et al.*, 1990) and *Azospirillum brasilense* SP7 (Riou *et al.*, 1991).

Deletion analysis within the *proU* operon revealed regulatory sequences in the first gene (*proV*), which are required to shut down expression under low osmolarity growth conditions ("silencer"). Mutants with lesions in another gene *hns* (formerly *osmZ*), which is not linked to the *proU* operon, were shown to increase *proU* transcription to an extent expected for cells sensing a higher osmolarity than actually present (Higgins *et al.*, 1988). A putative role as a specific osmoregulator was, however, later dismissed when it became clear that this H-NS protein is a non-specific "histone-like" DNA binding protein with a preference for curved regions (Hulton *et al.*, 1990; Owen-Hughes *et al.*, 1992). Although a strong affinity towards the putative "silencer" region of the *proU* operon is undoubted, this protein is more likely to have a general pronounced influence on DNA supercoiling, which will in turn affect a large number of genes with diverse functions (Higgins *et al.*, 1990). The specific involvement of H-NS and DNA supercoiling in osmoregulation of the *proU* operon is at present still disputed, being proposed by Higgins *et al.* (1988) and Ni Bhriain *et al.* (1989) and questioned by Ramirez and Villarejo (1991).

Based on the finding that *proU* expression cannot be induced by high osmolarity in media containing limited amounts of potassium (Sutherland *et al.*, 1986) and that expression of the ProU system is triggered osmotically by a great variety of solutes that cannot permeate the membrane, it was suggested that secondary messengers such as K^+ may trigger *proU* induction.

One of the proposals for a cascade of events following osmotic stress assumes that loss of turgor is sensed by a K^+ transport system, and that increased levels of cytoplasmic K^+ (glutamate) are responsible for secondary responses. The following modes of interaction with K^+ (glutamate) seem possible (Lucht and Bremer, 1994) and are open to discussion.

1. K^+-glutamate, which is known to stimulate transcription *in vitro*, facilitates interaction of RNA polymerase and the promotor of the *proU* operon.
2. K^+-glutamate influences the activity of DNA topoisomerase or binding of histone-like proteins (H-NS) to DNA, which results in changes in DNA supercoiling affecting the strength of the promotor ("Higgins model", see above).
3. A downstream repressor protein (possibly H-NS) bound to the "silencer" region is released in the presence of K^+-glutamate and enables transcription of the operon.

Compared with the abundance of information on the regulation of *proU* expression, modulation of the *activity* of the transport system, which is also influenced by osmolarity, is not well understood. As the affinity of the binding protein is not altered in response to osmotic changes (May *et al.*, 1986; Higgins *et al.*, 1987), activation probably occurs through conformational changes of inner membrane components of the transport system. This poses the obvious, but still unanswered, question as to how cells actually sense osmotic changes of the environment. It has often been proposed (but not yet proven) that loss of membrane tension as a result of plasmolysis triggers a variety of response mechanisms, primarily activation of transport systems providing secondary messengers such as K^+ (see above). An even more intriguing model envisages that intrinsic membrane stretch may lead to conformational changes of the embedded proteins, altering their transport activity. Subsequent recovery of turgor would then automatically shut off acquisition of compatible solutes. However, this is at present purely speculative and subject to further research. In fact, there has been recent evidence that the betaine uptake system ProP is not under feedback control (Koo *et al.*, 1991). This has in turn led to a completely different proposal for regulation, placing more emphasis on separate efflux systems for the rapid extrusion of betaine, which have so far been largely ignored.

4.3.2. *Halophilic and halotolerant bacteria*

(a) *Solute uptake and conversion.* Transport systems for the compatible solute betaine have been found and characterized in several halophilic and halotolerant bacteria, such as *Aphanothece halophytica* (Moore *et al.*, 1987), *Listeria monocytogenes* (Ko *et al.*, 1994), *Lactobacillus lactis* (Molenar *et al.*, 1993), *Staphylococcus aureus* (Bae *et al.*, 1993) and *Ectothiorhodospira*

halochloris (Peters, 1994). Comparison of the K_m data revealed that they were either very low, as expected for binding-protein dependent systems (2, 8 and 1.5 μM, respectively for the first three examples), or in a range more typical for a low-affinity system (65 and 66 μM for *S. aureus* and *E. halochloris*, respectively). Still, a periplasmic binding protein similar to that of *E. coli* was clearly demonstrated in *E. halochloris* using immunological techniques. In addition, DNA-hybridization studies revealed that the transport system of *E. halochloris* is different from both ProP and ProU (Peters, 1994).

The presence of genes for choline oxidation (betaine aldehyde dehydrogenase, choline oxidase), similar to the *betA/betB* genes of *E. coli*, has been demonstrated by the use of gene probes in some cyanobacteria (e.g. *Spirulina subsalsa*) and in a whole range of chemoheterotrophic eubacteria, of which *Halomonas elongata* and *Vibrio costicola* showed the strongest hybridization (O. Matan, personal communication). We therefore have reason to believe that the ability to accumulate and oxidize choline is widespread among other bacteria, a predisposition that is reflected by remarkable similarities at gene level, at least within the Proteobacteria.

Among halotolerant microorganisms, the Gram-positive *B. subtilis* and *S. aureus* have only recently become model organisms for investigations into osmotic adaptation at the molecular level (Bae and Miller, 1992; Graham and Wilkinson, 1992; Boch *et al.*, 1994). From the little information available, one can already pinpoint similarities with, as well as differences from, the *E. coli* system. Preliminary results on Gram-positive bacteria suggest very high cytoplasmic potassium levels largely unaffected by changes in osmolarity (Nagata *et al.*, 1991; Graham and Wilkinson, 1992; Whatmore *et al.*, 1990). While the nature of the counter-ion for K^+ is still unclear (possibly Cl^-), a fundamentally different involvement of potassium in turgor regulation seems likely (Whatmore and Reed, 1990). On the other hand, the way in which the betaine precursor choline is taken up via a high-affinity transport system and oxidized to betaine (Boch *et al.*, 1984; Graham and Wilkinson, 1992) is very similar to the situation in *E. coli*. Moreover, at least two effective solute uptake mechanisms seem to be present in *B. subtilis*, OpuA and OpuD (for osmoprotectant uptake), the former a high-affinity binding protein-dependent transport system of the ABC-type and the latter a low-affinity transport system, OpuD, similar in function, but not homologous, to ProP (E. Bremer, personal communication).

The mechanism of accumulation and/or regulation of osmolytes (at least in *Staphylococcus* species) seems to be different from that of the proteobacterial ProP/ProU system. When the organism is subjected to osmotic stress, the low-affinity system is activated (the high-affinity system is not), and when sufficient betaine has been accumulated the activity of the uptake system is reduced (feedback) (Stimeling *et al.*, 1994; Pourkomailian and Booth, 1994). This system must therefore be able to sense internal betaine and/or external

osmolarity (or turgor). Regulation of these solute uptake systems is currently under investigation, and bound to provide us with valuable information about similar and/or alternative modes of regulation in a more distant bacterial branch.

(b) *Solute synthesis*. Surprising though it may seem, there is at present hardly any information on the regulation of synthesis *de novo* of compatible solutes in bacteria, the only exceptions being preliminary studies on the putative regulation of glucosylglycerol synthesis in *Synechococcus* (Hagemann *et al.*, 1993; Hagemann and Erdmann, 1994) and enzymological studies on the synthesis/degradation of trehalose in *Ectothiorhodospira halochloris* (Lippert *et al.*, 1993; Herzog *et al.*, 1990). In *Ectothiorhodospira* species, this sugar is synthesized in a way similar to that in *E. coli* using UDP-glucose as a substrate. With regard to regulatory mechanisms, some properties of the degrading enzyme trehalase are worthy of note. The enzyme is inhibited by salt and activated in the presence of betaine. In addition, it exhibits a very high K_m for the substrate trehalose (0.5 M), which is close to the maximal concentration reported in cells exposed to nitrogen limitation (Galinski and Herzog, 1990). In the presence of glycine betaine, this constant is reduced to 0.16 M, promoting degradation. Hence in *E. halochloris* (as in *E. coli*), glycine betaine and monovalent cations seem to display an opposing regulatory effect on trehalose levels in the cytoplasma. These findings support the physiological observation that trehalose is accumulated only under nitrogen-limited conditions (replacing nitrogen-containing solutes) and immediately metabolized upon relief of nitrogen stress.

Investigations into osmoadaptation at the genetic level have so far focused almost exclusively on the well-studied enterobacteria and some Gram-positive non-halophiles (e.g. *B. subtilis*). Only recently, research on some marine cyanobacteria has diverted some of our attention towards more halophilic solute-producing species (Jeanjean *et al.*, 1990; Apte and Haselkorn, 1990; Hagemann and Zuther, 1992). Although genetic work with cyanobacteria is facilitated by the natural competence of many strains, progress on the solute-production side of the story is still largely at an early stage, focusing on the creation of mutants defective in both structural and regulatory genes.

True halophiles, or extremely halotolerant producers of compatible solute, have been almost totally neglected. This is primarily explained by the lack of basic genetic methods. Except for UV-mutagenesis, the preparation of plasmid DNA and hybridization experiments, mainly with halophilic *Halomonas, Deleya* and *Vibrio* species (Kogut *et al.*, 1992; Fernandez-Castillo *et al.*, 1992; O. Matan, personal communication), typical solute-producing halophilic bacteria have hardly been explored genetically and neither effective DNA transfer mechanisms nor strategies for insertion mutagenesis have been established. One major obstacle is the apparent insensitivity of many halophilic solute-producers to a whole range of

antibiotics when grown at elevated salinity, a characteristic that is especially pronounced within members of the Halomonadaceae (Nieto *et al.*, 1993). As effective mutagenesis systems often employ antibiotic resistance markers for the selection of positive clones, broad resistance would preclude or at least restrict the use of such genetic tools. This limitation, however, was recently overcome when we were able to show that antibiotic resistance of many halophiles largely depends on the salinity of the growth medium (Kunte and Galinski, 1995) and that gene transfer and transposition can be successfully achieved at the lower end of the salinity range (2–3%) for extremely halotolerant species such as *Halomonas elongata*. Thus, the way has been paved for an understanding of osmoregulatory strategies controlling the powerful compatible solute synthesizing machinery of true halophiles.

5. MOLECULAR PRINCIPLES OF COMPATIBLE SOLUTE FUNCTION

The general use of only a small number of compounds as osmotic regulators not just within the bacteria but also in higher forms of life, from amoeba to man (Kinne, 1993), raises the question as to the underlying common principles (reviewed by Low, 1985; Yancey *et al.*, 1982; Timasheff, 1992). Since the original definition of compatible solutes by Brown (1976), compatibility of even high concentrations of osmolytes with enzymes and other cellular functions *in vivo* and *in vitro* has been demonstrated (Pollard and Wyn Jones, 1979; Wyn Jones and Storey, 1981; Coughlan and Heber, 1982; Luard, 1983). Not surprisingly, this has inspired the search for an answer to the question, "What makes these solutes compatible?". From the information available today, three general rules seem to emerge: compatible solutes are very soluble, they have no (net) charge and they do not interact with proteins.

It is this lack of interaction with proteins that is most characteristic for compatible solutes and which explains their compatibility in the form of absence of interference, apparently not only *in vitro* but also in the living cell. This is illustrated at the molecular level by the physicochemical principle of preferential exclusion of osmolytes from the immediate surface of proteins and other cytoplasmic structural elements (Arakawa and Timsheff, 1985). Compatible solutes are therefore more than just inert molecules—they remain well clear of protein surfaces (Fig. 12). A model proposed by Wiggins (1990) explains this behaviour on the basis that water near interfaces is structurally different (more dense), allowing preference of compatible solutes towards the less dense water fraction. Others describe this predominant mechanism of non-specific exclusion in terms of increased surface tension of water, the presence of solutes effecting forces of cohesion between water

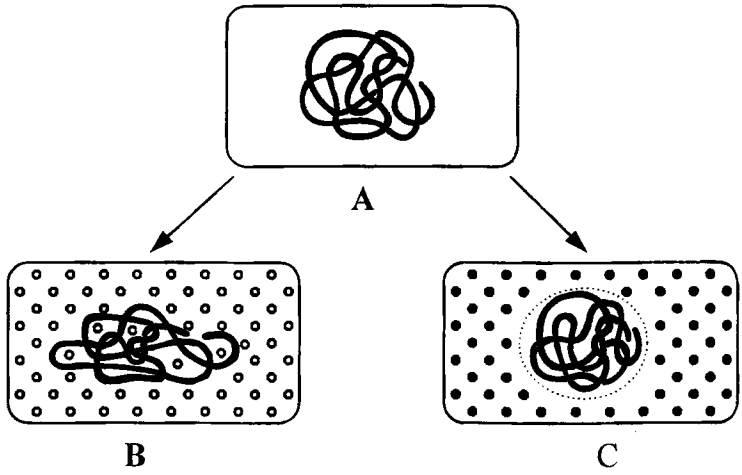


Figure 12 Schematic presentation of the effect of compatible solutes on protein conformation and (subsequently) also stability. (A) Hypothetical protein in its native conformation; (B) denatured form of protein in an environment of disturbing (binding) solutes such as urea; (C) stabilized form of protein in the presence of compatible solutes, which are preferentially excluded from the protein interface.

molecules (Bull and Breese, 1974) and/or in terms of enhancement of water structure by solutes fitting into the water lattice and enforcing the formation of large hydration clusters (Gekko and Timasheff, 1981). As a thermodynamic consequence (minimization of entropy, reinforcement of hydrophobic effect), compatible solutes display a general stabilizing effect opposing the unfolding/denaturation of proteins and other labile macromolecular structures (Galinski, 1993). This is clearly shown by their protective function against a range of stress factors such as heating, freezing and drying (Yancey *et al.*, 1982; Coughlan and Heber, 1982; Jolivet *et al.*, 1982; Carpenter *et al.*, 1987a,b; Lippert and Galinksi, 1992). To continue this train of thought, one would expect, and it has been shown, that the presence of compatible solutes can also compensate for deleterious destabilizing effects of denaturants such as urea (Yancey and Somero, 1979; Yancey *et al.*, 1982; Bagnasco *et al.*, 1986) and salt (Pollard and Wyn Jones, 1979; Luard, 1983; Manetas *et al.*, 1986).

In the living cell, the primary effect of compatible solutes seems at first sight to be osmotic, providing equilibrium across the membrane. Because of the protective effects of compatible solutes mentioned above, however, we would also expect an additional stabilization of cytoplasmic components, especially under drastically changed conditions caused by osmotic upshock (cf. sequence of events as described for *E. coli*). As an increase in the

potassium concentration *in vitro* dramatically reduces the affinity of most proteins for DNA (Record *et al.*, 1978) and as high concentrations of potassium inhibit the activity of many enzymes (Pollard and Wyn Jones, 1979; Warren and Cheathum, 1966; Yancey *et al.*, 1982), it was first assumed that the accumulation of potassium is responsible for the limitation of growth and that the counteracting function of solutes is likely to relieve this stress. However, in the view of Cayley *et al.* (1992), this cannot provide a general explanation for the growth inhibitory effects caused by loss of water, especially as the increased concentration of macromolecules (molecular crowding) should compensate for the effect of potassium, not to mention the fact that many organisms which do not use potassium as their primary osmolyte also display a reduced growth rate under osmotic stress (Yancey *et al.*, 1982).

A thorough investigation into the osmoresponse of *E. coli* performed by Cayley *et al.* (1992), which took into account various interconnected parameters (cytoplasmic volume, solute composition and growth rate), revealed a number of interesting observations: osmotically stressed cells display a markedly reduced cytoplasmic volume (when K^+-glutamate is the only osmolyte). This shrinking of cells was caused mainly by loss of *free* water, while the amount of *bound* water stayed more or less the same at 0.4 μmol/mg dry weight (representing 34% of total volume in the salt-stressed cells). They concluded that retarded biological functions are possibly explained by molecular crowding and concomitantly reduced diffusion rates of proteins and metabolites (Minton, 1981; Muramatsu and Minton, 1988) or by non-specific aggregation of proteins driven by the same effect.

Surprisingly, this initial volume reduction due to water loss was partially relieved by the accumulation of betaine (causing an increase in volume by 50%; from 1.18 μmol/mg to 1.75 μmol/mg dry weight) (Fig. 2). As the total amount of solutes within the cell remained almost unchanged, with no room for a decrease in turgor (Stock *et al.*, 1977), the authors concluded that the betaine replacing potassium, glutamate and trehalose must have a significantly higher osmotic coefficient ϕ, probably explained by differences in the degree of exclusion from the partial volume of bound water. In other words, replacement of relatively evenly distributed solutes such as K^+-glutamate by compatible solutes that are excluded to a greater extent from the hydration shells of cytoplasmic macromolecules and membrane constituents leads to the observed increase in cell volume.

This explanation is fully consistent with the findings of Arakawa and Timasheff (1985), reviewed by Timasheff (1992), who have demonstrated preferential exclusion of betaine and other osmolytes from protein hydration shells (as described above). As a consequence, one can conclude that it is primarily *free* water (as opposed to *bound* water) which responds to osmotic changes of the environment and that the compatibility of compatible solutes is based on the fact that they specifically adjust osmotic equilibrium of the

free-water fraction. Because of their exclusion from interfaces, they do not interfere with cellular function but at the same time restore cell volume.

In contrast to other authors claiming beneficial/protective effects of osmolytes on cellular macromolecules, Cayley *et al.* (1992) suggest that it is the replacement of other less compatible solutes and the subsequent increase in cytoplasmic volume that is responsible for its beneficial effect. Based on a remarkable correlation between growth rate and cytoplasmic volume (irrespective of its composition), the authors postulate that the free cytoplasmic volume (unbound water) is the fundamental determinant of growth under hyperosmotic stress and that the secondary effect of volume increase by compatible solute accumulation is the key for their osmoprotective function (at least in *E. coli*).

As is often the case, the truth may be somewhere in the middle, and I would not be surprised if supporters of both the solute-protection and the cytoplasmic-volume concepts would at some time in the future be found to converge on a joint solution. Following the present belief that compatible solutes by nature of exclusion are also always stabilizing (i.e. compensating the effect of denaturants), it is not hard to imagine that halophilic compatible solute producers and/or accumulators do make use of that stabilizing action in the way that they accept and tolerate higher cytoplasmic levels of salts, and in doing so economize on valuable solutes.

6. CONCLUDING STATEMENTS

Given the complexity of the subject matter, one is always wary of portraying it in too simplified terms. However, a few general statements may be allowed to remind the reader of the most important points.

1. Bacterial ability to osmoadapt seems to be the norm rather than the exception. Under the right environmental circumstances (external osmolytes or precursors thereof), many bacteria, so far believed to be salt-sensitive, will show considerable osmotolerance.
2. Osmoadaptation is a complex business but fundamental for all living cells. It has far-reaching consequences for everyday life, such as in medicine, the food industry and agriculture.
3. Compatible solutes used and produced by halo/osmophilic bacteria are useful tools to study and understand water solvent interactions, and to resolve principles of enzyme stabilization. They are bound to offer a range of technological applications as universal stress protectants.
4. The organisms displaying the most impressive ability to adapt and regulate over a large gradient of osmotic stresses (halotolerant eubacteria) have largely been neglected. However, the way has been paved for more thorough investigations.

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