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ROBERT K. POOLE

West Riding Professor of Microbiology
Department of Molecular Biology and Biotechnology
The University of Sheffield
Firth Court, Western Bank
Sheffield, UK

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Contributors to Volume 55

MICHAEL BERNEY, Department of Microbiology and Immunology, Otago School of Medical Sciences, University of Otago, Dunedin, New Zealand

GREGORY M. COOK, Department of Microbiology and Immunology, Otago School of Medical Sciences, University of Otago, Dunedin, New Zealand

ROBERT A. COX, Division of Mycobacterial Research, National Institute for Medical Research, London, UK

OLGA DANILCHANKA, Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL, USA

MARK DOPSON, Department of Molecular Biology, Umeå University, Umeå, Sweden

MAKOTO FUJISAWA, Department of Pharmacology and Systems Therapeutics, New York, NY, USA

SUSANNE GEBHARD, Department of Microbiology and Immunology, Otago School of Medical Sciences, University of Otago, Dunedin, New Zealand

MATTHIAS HEINEMANN, Institute of Molecular Systems Biology, ETH Zürich, Switzerland

TERRY A. KRULWICH, Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, New York, NY, USA

MICHAEL NIEDERWEIS, Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL, USA

MICHAEL W. PECK, Institute of Food Research, Norwich Research Park, Colney, Norwich, UK

JOAN L. SLONCZEWSKI, Department of Biology, Kenyon College, Gambier, OH, USA

Cytoplasmic pH Measurement and Homeostasis in Bacteria and Archaea

Joan L. Slonczewski[1], Makoto Fujisawa[2], Mark Dopson[3] and Terry A. Krulwich[4]

[1]*Department of Biology, Kenyon College, Gambier, OH, USA*
[2]*Department of Pharmacology and Systems Therapeutics, New York, NY, USA*
[3]*Department of Molecular Biology, Umeå University, Umeå, Sweden*
[4]*Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, New York, NY, USA*

ABSTRACT

Of all the molecular determinants for growth, the hydronium and hydroxide ions are found naturally in the widest concentration range, from acid mine drainage below pH 0 to soda lakes above pH 13. Most bacteria and archaea have mechanisms that maintain their internal, cytoplasmic pH within a narrower range than the pH outside the cell, termed ''pH homeostasis.'' Some mechanisms of pH homeostasis are specific to particular species or groups of microorganisms while some common principles apply across the pH spectrum. The measurement of internal pH of microbes presents challenges, which are addressed by a range of techniques under varying growth conditions. This review compares and contrasts cytoplasmic pH homeostasis in acidophilic, neutralophilic, and alkaliphilic bacteria and archaea under conditions of growth, non-growth survival, and biofilms. We present diverse mechanisms of pH homeostasis including cell buffering, adaptations of membrane structure, active ion transport, and metabolic consumption of acids and bases.

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ABBREVIATIONS

CFAs	cyclopropane fatty acids
cFSE	carboxyfluorescein diacetate succinimidyl ester
GFP	green fluorescent protein
OMPs	outer membrane porins
PMF	protonmotive force
RSO	right-side-out
SCWPs	secondary cell wall polymers

1. INTRODUCTION

All microbes have evolved to grow within a particular range of external pH. Historically, pH has played many key roles in the development of

microbiology. Since ancient times, fermentation has produced storable food products containing inhibitory acids, such as dairy products and vinegar (Buckenhüskes, 2001; Johnson and Steele, 2001) or foods containing alkali, such as *natto* from soybeans and *dadawa* from locust beans (Wang and Fung, 1996). Acidophiles have been exploited for thousands of years for the recovery of valuable minerals (Olson *et al.*, 2003; Rohwerder *et al.*, 2003) but they also cause pollution and corrosion (Johnson and Hallberg, 2003). Alkaliphiles have been exploited for natural products, especially enzymes with high pH optima (Horikoshi and Akiba, 1982; Horikoshi, 1999).

The external pH partly determines the cytoplasmic or intracellular pH, which affects enzyme activity and reaction rates, protein stability, structure of nucleic acids, and many other biological molecules. However, most microbes maintain some degree of pH homeostasis such that the cytoplasmic pH is maintained within a narrower range than external pH, usually closer to neutrality (Fig. 1). For example, *Escherichia coli* during optimal growth conditions maintains its cytoplasmic pH within a range of pH 7.4–7.8 over an external pH range of 5.0–9.0 (Slonczewski *et al.*, 1981; Zilberstein *et al.*, 1984; Wilks and Slonczewski, 2007), and a similar range is observed for *Bacillus subtilis* (Shioi *et al.*, 1980). On the other hand, the alkaliphile *Bacillus pseudofirmus* OF4 shows a range of cytoplasmic pH 7.5–8.3 over the range of external pH 7.5–10.6 (Sturr *et al.*, 1994; Krulwich, 1995) and resting cells of *Acidithiobacillus ferrooxidans* maintain an internal pH between 6.0 and 7.0 over an external pH range of 1.0–8.0 (Cox *et al.*, 1979).

Microbes grow in different ranges of environmental pH, from pH 0 (Nordstrom and Alpers, 1999; Nordstrom *et al.*, 2000) to above pH 13 (Roadcap *et al.*, 2006). It is intriguing that over the range of microbes studied, the crossover point where cytoplasmic pH equals external pH lies between pH 7 and 8, although not all species can actually grow at this point. Actual pH ranges vary in breadth and transmembrane pH difference (ΔpH) but, in general, acidophiles are defined as organisms growing optimally within the pH range 0.5–5, neutralophiles within pH 5–9, and alkaliphiles within pH 9–12. Bacteria and archaea adapted to different habitats in different ranges of environmental pH have evolved diverse mechanisms of pH homeostasis, while some common principles of homeostasis apply across the pH spectrum. In this review, we compare and contrast the mechanisms of cytoplasmic pH homeostasis in acidophilic, neutralophilic, and alkaliphilic bacteria and archaea. We aim to synthesize patterns of cytoplasmic pH behavior across the range of pH environments, seeking connections that may be overlooked when only one range is considered. For example, the relationship between the ΔpH and the transmembrane electrical potential ($\Delta\Psi$) in maintaining pH homeostasis shows a striking pattern of pH dependence across many species, as discussed below.

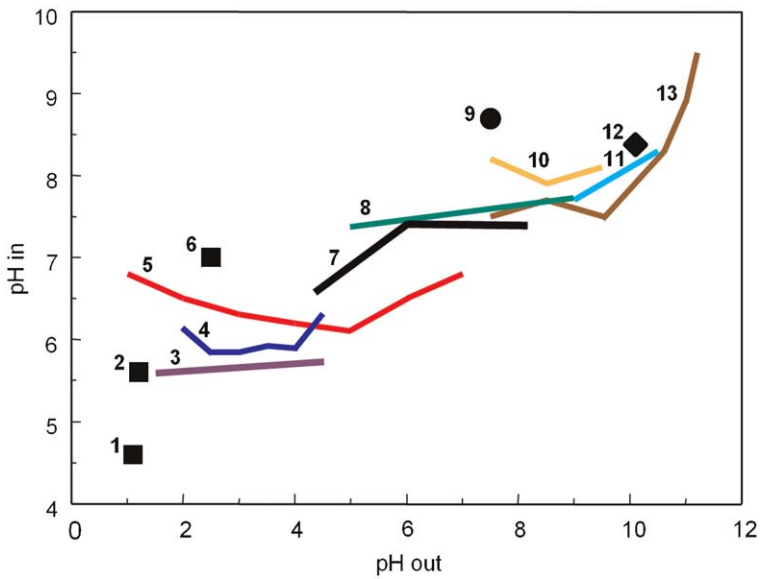


Figure 1 Cytoplasmic pH as a function of the external pH among acidophiles (■), neutralophiles (●), and alkaliphiles (♦). Acidophiles: (1) *Picrophilus torridus* (Fütterer *et al.*, 2004); (2) *Ferroplasma acidarmanus* (Baker-Austin and Dopson, 2007); (3) *Acidiphilium acidophilum* (dark purple; external pH 1–4.5; Matin *et al.*, 1982); (4) *Bacillus acidocaldarius* (dark blue; pH 2–4.5; Krulwich *et al.*, 1978); (5) *Acidithiobacillus ferrooxidans* (red; pH 1–7; Cox *et al.*, 1979); and (6) *Acidithiobacillus thiooxidans* (Baker-Austin and Dopson, 2007). Neutralophiles: (7) *Bacillus subtilis* (black; pH 4.5–8; Shioi *et al.*, 1980); (8) *Escherichia coli* (green, pH 5–9; Slonczewski *et al.*, 1981); and (9) *Bacillus licheniformis* (Hornbæk *et al.*, 2004). Alkaliphiles: (10) *Bacillus cohnii* (orange; pH 7.5–9.5; Sugiyama *et al.*, 1986); (11) *Bacillus pseudofirmus* RAB (turquoise; pH 9–10.5; Kitada *et al.*, 1982); (12) *Bacillus alcalophilus* (Hoffmann and Dimroth, 1991); and (13) *B. pseudofirmus* OF4 (brown; pH 7.5–11.2; Sturr *et al.*, 1994). (See plate 1 in the color plate section.)

1.1. Perturbation of Cytoplasmic pH

The cytoplasmic pH is buffered by small organic molecules such as amino acids, as well as by ionizable groups on proteins and inorganic polymers such as polyphosphate; typical buffering capacities for different species range from 50 to 200 mM protons per pH unit shift (Slonczewski *et al.*, 1982; Zychlinsky and Matin, 1983b; Krulwich *et al.*, 1985a; Rius *et al.*, 1995; Rius and Lorén, 1998; Leone *et al.*, 2007). As discussed in detail below, the

approximate degree of cytoplasmic buffering appears remarkably similar for most bacteria across the range of environmental pH.

Despite the various sources of buffering, in the absence of active mechanisms of pH homeostasis, ionophores or permeant acids or bases rapidly shift the cytoplasmic pH via influx or efflux of protons. Membrane-permeant acids such as fermentation acids become concentrated within the cytoplasm. This concentrative effect is observed when there is a pH difference across the membrane with the cytoplasmic pH higher than the external pH. Such a pH difference is generally small in neutralophiles but is very large in acidophiles, thus making membrane-permeant acids correspondingly more toxic in acidophiles. The accumulation of acids in the cytoplasm results in partial or complete failure of pH homeostasis, and may increase the concentration of an organic acid that can retard growth (Kihara and Macnab, 1981; Salmond *et al.*, 1984; White *et al.*, 1992). Similarly, the accumulation of membrane-permeant bases such as polyamines is favored under conditions in which the cytoplasmic pH is lower than the external pH. Under these conditions, the uncharged base that enters the cell is protonated in the cytoplasm and this consumption of cytoplasmic protons can impair pH homeostasis (Repaske and Adler, 1981; Yohannes *et al.*, 2005).

In growth media with high organic amine content, alkaliphilic bacteria are at special risk for cytoplasmic accumulation of ammonium at the expense of cytoplasmic protons, thus compromising pH homeostatic mechanisms. In alkaliphilic *B. pseudofirmus* OF4, an ammonium efflux system has a role in facilitating growth under these conditions (Wei *et al.*, 2003). Müller *et al.* (2006) studied the effect of high ammonium concentrations on several neutralophilic bacteria and concluded that high ammonium concentrations were not generally detrimental. However, *B. subtilis* mutants lacking one of the cation/proton antiporters important for alkaline pH homeostasis show a large decrease in expression of ammonium transport system, for example, 17-fold decrease in *nrgA* (Wei *et al.*, 2006). The down-regulation of the ammonia transport in the antiporter mutant implies that ammonia is detrimental to the cell under alkali stress. It would be of interest to re-visit the issue of general ammonium toxicity for neutralophiles under alkaline conditions.

Depending upon whether the cytoplasmic pH is higher or lower than the external pH, the ΔpH contributes or detracts from the total protonmotive force (PMF) that drives bioenergetic work. The PMF is an electrochemical or chemiosmotic gradient of protons that is established by active proton pumping and modulated by secondary ion movements (Mitchell, 1961; West and Mitchell, 1974; Booth, 1985; Macnab and Castle, 1987). The PMF

comprises the chemical gradient of protons (ΔpH) and the transmembrane electrical component (ΔΨ). The PMF in millivolts (negative) is equal to ΔΨ−60ΔpH at 25 °C; thus, a ΔpH (alkaline inside) contributes to the PMF. The PMF energizes most active processes that contribute to bacterial pH homeostasis; drives ATP synthesis and ion-coupled solute uptake or efflux; and motility. The PMF must be sufficiently persistent to drive energy requiring processes and therefore, the membrane is proton impermeable such that the PMF is not rapidly dissipated by proton and/or charge movements across the membrane. While there are mechanisms for maintaining homeostasis of membrane ion permeability, especially proton permeability (Albers *et al.*, 2001), certain membrane lipids (such as the tetraethers in acidophilic archaea) confer greater proton impermeability on some species than others.

Other conditions can deplete the PMF such as the presence of an ionophore. For example, dinitrophenol or combination of permeant ions can cause a failure of cytoplasmic pH homeostasis (Khan and Macnab, 1980; MacLeod *et al.*, 1988). Another factor to consider in connection with PMF generation by proton-pumping complexes is that bacteria and archaea exhibit an amazing range of electron donors used for energy generation. Interestingly, use of some substrates mandates an extreme lifestyle. An example noted by Ferguson and Ingledew (2008) is that oxidation of ferrous iron by microorganisms such as *A. ferrooxidans* occurs at low pH because low pH minimizes auto-oxidation and maximizes solubility but also because the mid-point potential of the oxygen/water couple is higher at pH 2 than at pH 7. Since the mid-point potential for the ferrous/ferric couple does not change concomitantly, more energy is available from ferrous oxidation, which improves the energetics at low pH.

The balance between ΔpH and ΔΨ across many species shows distinctive patterns that reflect dependence upon pH. Acidophiles, as well as neutralophiles that survive under extremely acidic conditions (below pH 3) maintain a cytoplasmic pH that is much less acidic than the external pH, and they exhibit an inverted ΔΨ (inside positive) which subtracts from the PMF while helping to maintain a cytoplasmic pH that is only mildly acidic (Foster, 2004; Baker-Austin and Dopson, 2007). Thus, in extreme acid, the ΔpH solely contributes to the PMF in the chemiosmotically productive direction. Conversely, at high external pH, alkaliphiles as well as neutralophiles have an inverted ΔpH (acid inside) that subtracts from the PMF but results in a cytoplasmic pH that is only mildly alkaline even when the external pH is above pH 9 (Yumoto, 2002; Saito and Kobayashi, 2003; Padan *et al.*, 2005). Under these conditions, the ΔΨ is the only chemiosmotically productive component of the bulk PMF. However, there

is evidence that proton sequestration occurs near the outside of the bacterial membrane and/or proton translocation near the outer membrane surface occurs faster than the equilibration of pumped protons with the bulk liquid phase outside the cell (Heberle *et al.*, 1994; Mulkidjanian *et al.*, 2006; Brändén *et al.*, 2006). During growth under alkaline conditions, the concentration of such surface-associated protons relative to the cytoplasmic proton concentration could constitute a ΔpH that is more chemiosmotically favorable than the bulk ΔpH (Krulwich, 1995; Mulkidjanian *et al.*, 2006).

Outside the range of pH permitting growth, pH homeostasis gradually or abruptly fails. Nevertheless, many species can survive (remain viable) for extended periods at pH values outside their growth range, ready to grow again when the pH returns to the optimum. For instance, clinical isolates of *E. coli* can survive several hours of exposure below pH 2 (Gorden and Small, 1993; Buchanan and Edelson, 1999; Price *et al.*, 2000). *E. coli* as well as *Vibrio cholerae* also survive but do not grow in some of the alkaline–saline waters into which they are released during their passage from animal hosts (Colwell and Huq, 1994; Rozen and Belkin, 2001). Acid- or base-resistant strains generally express inducible mechanisms of ''acid resistance'' or ''base resistance,'' enabling them to survive without growth at extreme pH (Small *et al.*, 1994; Foster, 2004). These mechanisms have some factors in common with the pH homeostasis mechanisms of extreme acidophiles and alkaliphiles. It is also evident that different bacteria vary not only in their capacity for pH homeostasis, but also in their ability to survive or even grow when their cytoplasmic pH is significantly below or above the pH range tolerated by most bacteria (see Fig. 1).

1.2. Mechanisms of pH Homeostasis

Diverse mechanisms that maintain active pH homeostasis greatly supplement the contribution of passive cytoplasmic buffering. Major categories of active pH homeostasis mechanisms include: coupling transmembrane proton movements to an energetically favorable exchange with cations (K^+, Na^+) or anions (Cl^-), a strategy that is the central active component of alkaline pH homeostasis (Macnab and Castle, 1987; Padan *et al.*, 2005; Krulwich *et al.*, 2007); metabolic switching to generate acidic or neutral end-products (Stancik *et al.*, 2002; Wei *et al.*, 2006); acid-induced amino acid decarboxylases, and base-induced amino acid deaminases (Blankenhorn *et al.*, 1999; Foster, 2004; Richard and Foster, 2004); use of urease activity, sometimes working together with carbonic anhydrase activity, to regulate cytoplasmic and periplasmic pH (Stingl *et al.*, 2001, 2002; Sachs *et al.*, 2005,

2006); synthesis of acid-resistant membrane structures such as cyclopropane fatty acids (CFAs; Cronan, 2002) and tetraether lipids (Baker-Austin and Dopson, 2007) or increased synthesis of anionic phospholipids or specific neutral lipids at high pH (Clejan *et al.*, 1986); and chaperone protection from temporary damage due to pH shift (Stancik *et al.*, 2002). Some mechanisms are regulated as components of larger regulons, such as the RpoS-dependent acid resistance Gad regulon (Ma *et al.*, 2004). Others respond to pH with a combination of transcriptional responses together with a substantial component of activity control by pH. For example, the major Na^+/H^+ antiporter of *E. coli*, NhaA, is transcriptionally regulated by both sodium and by an RpoS-mediated response to the growth phase (Karpel *et al.*, 1991; Dover and Padan, 2001) but antiport activity is also dramatically and directly enhanced by alkaline pH activation (Padan *et al.*, 2004; Padan, 2008). High-resolution structural information recently obtained for NhaA (Hunte *et al.*, 2005) has made it possible to integrate extensive biochemical and genetic data into a detailed model for the mechanism of activity control of the antiporter by pH (Padan, 2008). Structural biological studies will similarly be central to understanding the specific adaptations in the proton-translocating complexes of the proton cycles that support pH homeostasis in acidophiles and alkaliphiles.

Some extremophile adaptations were found in functional assays, but additional examples are emerging as more extremophile genome data becomes available. Examples of adaptations in proton pumps include: the sequence-based proposal that the cytochrome oxidase of two iron-oxidizing acidophiles lacks one of the proton channels found in homologues from neutralophiles (Ferguson and Ingledew, 2008); adaptions of extremely alkaliphilic *Bacillus* species in cytochromes, including cytochrome *c*, that greatly reduce the mid-point potentials of the alkaliphile proteins relative to neutralophile homologues (Lewis *et al.*, 1981; Yumoto *et al.*, 1991; Hicks and Krulwich, 1995; Goto *et al.*, 2005); alkaliphile-specific sequence motifs in both the caa_3-type cytochrome oxidases (Quirk *et al.*, 1993); and alkaliphile-specific motifs in the proton-translocating *a*- and *c*-subunits of the ATP synthase that are important in proton capture and retention during ATP synthesis at high pH (Ivey and Krulwich, 1992; Arechega and Jones, 2001; Wang *et al.*, 2004; Liu *et al.*, 2009).

The multiplicity of mechanisms and adaptations observed for bacterial pH homeostasis is perhaps to be expected, given that in principle, every macromolecule with pH-titratable residues is a potential "pH sensor." Thus, evolution has generated numerous pH-detecting devices that operate independently. Some categories of pH protection mechanisms are ubiquitous, such as cytoplasmic buffering or almost ubiquitous, such as

transmembrane proton transport of some type that is observed in all but a few bacteria that confine themselves to a narrow range of pH for example, *Clostridium fervidus* (Speelmans *et al.*, 1993). Other mechanisms are associated with groups of microorganisms that are adapted to particular ranges of environmental pH (for reviews see Slonczewski and Foster, 1996; Padan *et al.*, 2005; Baker-Austin and Dopson, 2007). Acidophiles include iron and sulfur bacteria such as *A. ferrooxidans*, as well as archaea such as *Ferroplasma acidiphilum* that grow under the most extreme acid conditions (close to pH 0). Archaeal acidophiles possess tetraether membranes that are highly impermeable to protons. Neutralophiles include the majority of organisms that grow in association with human bodies as well as a majority of those that inhabit soil and most freshwater habitats. Neutralophiles show a wide range of pH-regulating mechanisms that involve heterotrophic metabolism and inorganic ion exchange. Alkaliphilic bacteria that grow well at pH values up to 10.5, for example, the extensively studied *B. pseudofirmus* OF4 or *Bacillus halodurans* C-125, were isolated from soil or marine environments that are not consistently extremely alkaline (Krulwich and Guffanti, 1989; Takami *et al.*, 1999). Even more extreme alkaliphiles have been isolated from natural enrichments such as soda lakes that typically have a pH above 10 or industrial enrichments, for example, indigo dye plants (Jones *et al.*, 1998; Wiegel, 1998; Roadcap *et al.*, 2006). In the alkaliphiles studied to date, a Na^+ transport cycle that is coupled to cytoplasmic proton accumulation plays a major and indispensable role in pH homeostasis (Padan *et al.*, 2005; Krulwich *et al.*, 2007). However, extreme alkaliphiles have been isolated from non-saline groundwater with a pH of 11.4. Some of these bacteria, for example, *Bacillus foraminis* CV53T, grow better in the absence than in the presence of sodium (Tiago *et al.*, 2004, 2006). Therefore, net proton uptake cycles that are based on coupling ions other than sodium are likely to be employed in some environments.

2. CYTOPLASMIC pH MEASUREMENT

The study of cytoplasmic pH requires careful means of controlling and measuring pH, both inside and outside the cell. The pH of the medium must be maintained either in batch culture, through use of buffers that are pH-appropriate and non-metabolized, or through continuous culture (chemostat). Cytoplasmic pH is measured either by membrane-permeant radiolabeled probes of ΔpH, or by indicators of cytoplasmic pH that are

independent of external pH, such as fluorimetry and phosphorus NMR. The relative merits and limitations of these approaches are discussed below.

2.1. Buffered Batch Culture Versus Chemostat Culture

The first requirement for any study of cytoplasmic pH is to establish effective means of maintaining a constant pH of the extracellular medium or to incorporate changes in external pH into the study. In either case, accurate measurements of external and cytoplasmic pH are essential. Most commonly, pH homeostasis is assessed under conditions in which the pH is held constant. This establishes the homeostatic capacity under a specific condition in studies of different mutants or inhibitory conditions. However, there is sometimes merit in studying pH homeostasis during either acidification or alkalinization of the medium caused by bacterial metabolism. Such conditions mimic the niches of bacteria that have relatively small volumes and/or buffering capacities, or that grow in biofilms and in high-density habitats such as the colon.

When the external pH is fixed, the external medium must have sufficient buffering to overcome the effects of metabolism on external pH. The most common approaches to this problem involve batch culture with non-metabolizable buffers, or the use of chemostat cultures. Usually, changes in absorbance and/or cell protein measurements are used to monitor growth. However, there is enormous value in tracking the viable count since this provides an indication of the proportion of cells in the population that retains the capacity for colony formation (Padan *et al.*, 2005). Limitations of batch culture are that (1) the buffer(s) must have appropriate pK_a for the pH range of interest but not be inhibitory to the bacteria in question; (2) concentrations as high as 50–100 mM are required for extended culture of rapidly growing cells; and (3) metabolic breakdown of buffers may occur when working with organisms whose full spectrum of degradative capacities are unknown, generating unknown byproducts (for discussion see Slonczewski and Foster, 1996). Limitations of chemostat culture include (1) the difficulty of establishing biologically independent replicate cultures; (2) the need to sample the chemostat in order to assess the cytoplasmic pH of the culture at intervals until it becomes feasible to use continuous measurements routinely, for example, by fluorimetry. In a review that highlights recent applications of chemostat cultures to studies of bacterial populations, mutations, and evolution, Ferenci (2008) points out that these cultures do not achieve a true steady state for bacterial populations with fixed characteristics that can be readily compared.

2.2. Radiolabeled Membrane-Permeant Probes

Radiolabeled membrane-permeant weak acids and weak bases are used to measure the bulk transmembrane ΔpH, which is added to the extracellular pH to yield cytoplasmic pH. Cells or membrane vesicles of either the right-side-out (RSO) orientation or inside-out orientation can be used for the assays (Padan and Schuldiner, 1986). The cells are collected by filtration or by centrifugation; the cytoplasmic volume must be determined simultaneously by a different radiolabeled probe (Small *et al.*, 1994; van den Vossenberg *et al.*, 1998b; Richard and Foster, 2004).

The weak acid or weak base probe must not be actively transported by the bacteria under study, as this would confound the measurement. Rather, the principle behind use of weak acid and weak base probes depends upon the relative impermeability of the membrane to the charged species of the acid or base in the absence of a transporter. In the absence of a transporter, the charged species do not significantly enter the cell or RSO vesicle and it is the uncharged species that is presumed to diffuse. If the cytoplasmic or intravesicular pH is higher than the external pH, the uncharged protonated weak acid that enters will dissociate more to the charged form than the probe that remains outside. The charged form that is generated inside is then trapped there and so its concentration relative to the probe concentration outside can be assayed. Weak base probes also enter in the uncharged form if the internal pH is lower than the outside pH, and more of the internalized probe than the probe remaining outside will become protonated. The charged protonated form of the basic probe traps the probe inside the cell and facilitates measurement of the inside versus outside probe concentration. If the pH gradient is small, it is advisable to conduct separate assays with a weak acid and weak base on the same preparations. The results should be the inverse of one another and such results help to validate findings of small gradients.

In all instances, a major challenge is correcting the total non-internalized cell- or vesicle-associated probe. The challenge is especially great because the amount of binding may be different among preparations from different mutants or may be affected by different treatments. Usually binding controls are conducted by permeabilization with either solvent, for example, toluenized or butanol-treated cells or membranes or with treatment of the preparation with an ionophore or combination of ionophores that abolishes the pH gradient (Rottenberg, 1979). To assess the optimal binding control with different cells and preparations, it is best to conduct preliminary assays with pH-equilibrated cells or vesicles in which pH gradients of known dimension are established by a sudden shift in external

pH. The other parameter that can be established in such preliminary experiments is the lowest concentration of probe that will be sufficient to saturate the non-specific binding sites while providing a measurement of the imposed pH gradient. It is important to ascertain this value because if the probe concentration is too low, it does not measure the actual gradient but is largely bound non-specifically. If the concentration is too high, the weak acid or base probe will change the pH gradient too much.

Calculation of the intracellular or cytoplasmic pH value (pH_{int}) requires the following equation:

$$pH_{int} = \log\left\{\left(\frac{[A_{in}]}{[A_{out}]}\right)\left(10^{pK_a} + 10^{pH_{out}}\right) - 10^{pK_a}\right\}$$

where $[A]$ represents the concentration of the radiolabeled permeant-acid probe, such as benzoate ($pK_a = 4.2$) or salicylate ($pK_a = 3.0$). At extracellular pH values higher than the pH_{int}, an analogous equation applies to a permeant base such as methylamine ($pK_a = 10.6$). For acid conditions, the pK_a of the probe should be sufficiently low that the protonated concentration is small (preferably well below the cytoplasmic pH) but not so low that the membrane permeation rate of the anion becomes comparable to that of the protonated species. Thus, for example, benzoate is an appropriate probe for use at external pH 5–7, whereas salicylate is optimally used at pH 3–6 (Small *et al.*, 1994; van den Vossenberg *et al.*, 1998b; Richard and Foster, 2004). At pH values below pH 3, the sensitivity of salicylate decreases because most of the probe is protonated.

As permeant acids and bases provide only indirect measures of the pH gradient, the results should be validated to the extent possible with other data about the system. The advantage of permeant-acid and -base probes is that a large number of samples can be measured conveniently. These measurements are reasonably sensitive, that is, over a large pH range, ΔpH values can be reproducibly assessed within 0.2 unit and such measurements correlate with fluorescence-based assays (Ito and Aono, 2002). A limitation is that these are steady-state type measurements and thus do not support kinetic analysis so that studies of pH homeostasis over time are limited to relatively long time intervals.

2.3. Fluorimetry and Fluorescence Microscopy

Fluorescence of a pH-titratable fluorophore yields a direct measure of cytoplasmic pH that is independent of extracellular pH (Olsen *et al.*, 2002; Wilks and Slonczewski, 2007). Fluorescent proteins provide highly sensitive

detection, do not require indicator loading, and lack phototoxicity. The most effective methods use pH-sensitive derivatives of green fluorescent protein (GFP) such as GFPmut3* and YFP (Andersen *et al.*, 1998; Kneen *et al.*, 1998; Llopis *et al.*, 1998; Robey *et al.*, 1998; McAnaney *et al.*, 2005). The pH dependence of fluorescence intensity is based on protonation of the phenolate moiety of the fluorophore derived from tryptophan, an exchange reaction occurring in less than a millisecond (Dickson *et al.*, 1997; McAnaney *et al.*, 2005). Thus, GFP observation allows assessment of the cellular rate of pH change in response to a rapid shift in extracellular pH, or to addition of a permeant acid. Fluorescence spectroscopy or fluorimetry of *E. coli* and *B. subtilis* cell suspensions yields kinetic data on a timescale as short as 4 seconds (Fig. 2A) (Wilks and Slonczewski, 2007; Kitko and Slonczewski, unpublished data). For optimal observation of cytoplasmic pH, the GFPmut3* is best expressed from a high-copy plasmid using a constitutive promoter (Kitko and Slonczewski, unpublished data). Use of current GFP probes is limited to cytoplasmic pH values above pH 5, as the protein denatures in acid. The optimal range of pH sensitivity of current proteins is about pH 5.5–8.5.

GFP fluorimetry can also be used to measure the periplasmic pH independently from the cytoplasmic and external pH values (Wilks and Slonczewski, 2007). The GFPmut3* fusion strain is expressed with a TorA signal peptide that can either retain the fusion protein in the cytoplasm or direct its transport to the periplasm via *tat* transport, a system in which the pre-folded GFP is transferred across the membrane (Thomas *et al.*, 2001; Barrett *et al.*, 2003; Mullineaux *et al.*, 2006). Arabinose incubation induces GFP transport, and eliminates GFP from the cytoplasm; thus, the periplasmic signal can be isolated. This method was used to show that periplasmic pH (unlike cytoplasmic pH) shifts according to external pH, with no apparent homeostasis (Fig. 2B).

pH-dependent fluorescence of cells is also observed by microscopy. For example, Olsen *et al.* (2002) used microscopy to observe GFP fluorescence as a function of pH in isolated cells of *E. coli* and *Lactococcus lactis*. The advantage of microscopy over fluorimetry is that it enables observation of individual cells within a population. Low noise level relative to signal permits ratiometric analysis, in which the ratio is obtained between fluorescence intensity at two excitation wavelengths, one of which increases with pH, and one of which decreases. The ratiometric method improves accuracy by eliminating intensity differences not associated with pH. On the other hand, microscopy necessitates cumbersome quantitation procedures that introduce error and limit the timescale of observable signal such that kinetic observations are limited compared to fluorimetry.

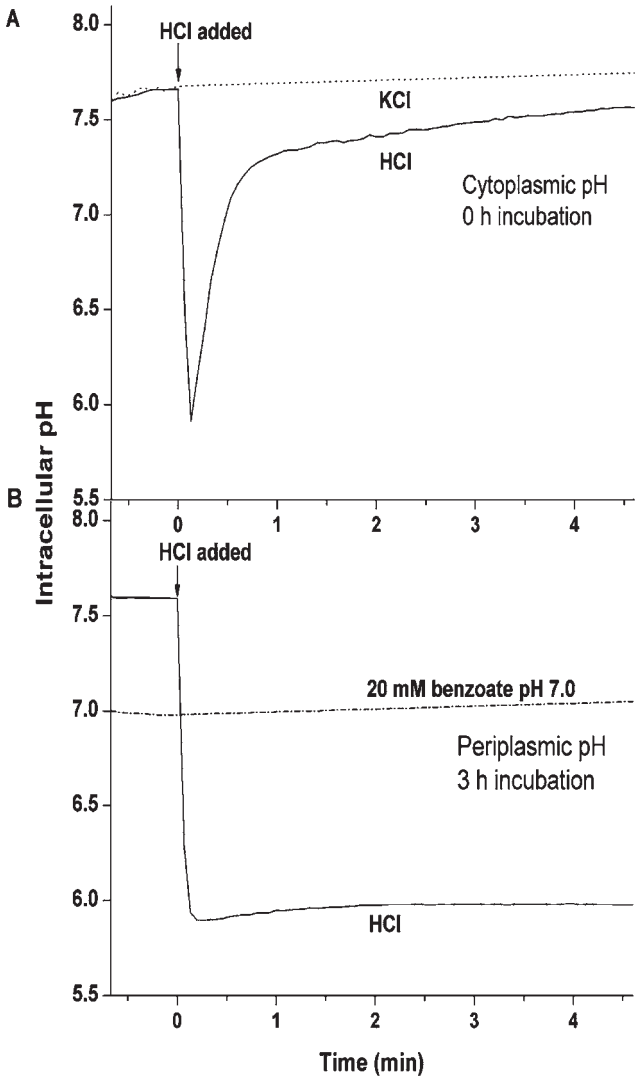


Figure 2 Cytoplasmic pH and periplasmic pH of *E. coli* K-12 observed by fluorimetry. Strain MC4100AR TorA-GFPmut3*. Cultures suspended in pH 7.5 M63 medium (5 mM HOMOPIPES) were tested (A) without further incubation and (B) after 3 h of incubation in the absence of arabinose. At time zero, the external pH was shifted from pH 7.5 to 5.5 by adding 8.5 mM HCl. For the control, at time zero, (A) 8.5 mM KCl (pH 7.5) was added or (B) 20 mM sodium benzoate (pH 7.0) was added to cultures suspended in pH 7.0 M63 medium (50 mM HOMOPIPES). The fluorescence signals from three independent cultures were averaged for each condition, and pH values determined from a standard curve based on cells with ΔpH collapsed by 20 mM sodium benzoate. [Adapted from Fig. 8 of Wilks and Slonczewski (2007).]

In Gram-positive bacteria, pH has been measured by microscopy using exogenous fluorophores such as derivatives of carboxyfluorescein. For an exogenous fluorophore, the challenge is to introduce a non-toxic probe into the cells that has an appropriate pK_a near that of cytoplasmic pH, and that remains in the cell without breakdown or excretion. Breeuwer *et al.* (1996) devised a method based on the use of carboxyfluorescein diacetate succinimidyl ester (cFSE) in *L. lactis* and *B. subtilis*. Within the cell, cFSE becomes hydrolyzed to carboxyfluorescein succinimidyl ester and is subsequently conjugated to aliphatic amines. The conjugated form appears to remain stable within the cell. Fluorescence is observed ratiometrically based on excitation wavelengths of 490 and 440 nm, with an approximate pK_a of pH 7.0. This method has been used to measure the internal pH of *Bacillus cereus* (Ultee *et al.*, 1999; Thomassin *et al.*, 2006) and *Lactobacillus* species (Siegumfeldt *et al.*, 1999), including dynamic changes on a timescale of 30–60 seconds (Siegumfeldt *et al.*, 2000).

Fluorescence microscopy can reveal the pH of a bacterial cellular compartment, such as the forespore compartment of *Bacillus megaterium* (Fig. 3) (Magill *et al.*, 1994, 1996). The fluorophore used is 2′,7′-bis-(2-carboxyethyl)-5-(and 6-)carboxyfluorescein (BCECF). Cells are loaded with the acetomethoxyester of BCECF (BCECF-AF), which is membrane-permeant and non-fluorescent. The probe becomes hydrolyzed by cellular esterases, releasing the pH-dependent fluorophore. Fluorescence excitation

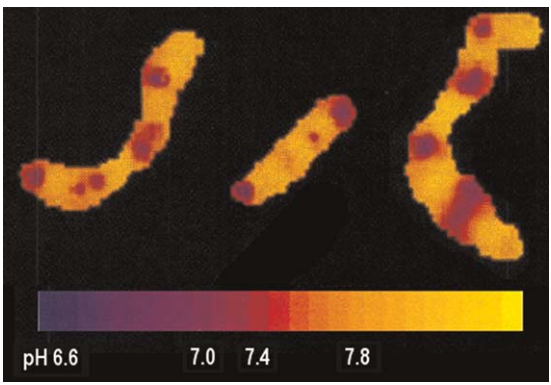


Figure 3 Fluorescent ratio image analysis of intracellular pH in sporulating *Bacillus megaterium.* Sporulating cells were loaded with BCECF-AM. Fluorescence ratios were measured and converted to pseudocolor images calibrated to pH as shown. Bacteria appear as chains of four to eight cells, in which the forespore components show lowered pH. The figure was adapted from Fig. 4 of Magill *et al.* (1994) with permission from American Society for Microbiology. (See plate 2 in the color plate section.)

ratios between 500 and 440 nm are calibrated to pH and represented by false color in the micrograph. During forespore development in the mother cell, the pH declines from pH 8.1 in the mother cell to pH 7.0 in the forespore.

2.4. Phosphorus NMR

Cytoplasmic pH can be measured simultaneously with extracellular pH using ^{31}P NMR observation of titratable phosphate resonances (Fig. 4) (Slonczewski *et al.*, 1981, 1982). NMR shifts are observed for inorganic phosphate (pK_a 7.1) and methylphosphonate (pK_a 7.5), which is taken up from the medium by *E. coli* cells. The observation of cytoplasmic pH requires highly concentrated cell suspensions, typically 20–200 optical density units at 600 nm, as the relative signal intensities of intracellular and extracellular phosphates depend on the cell volume. Cell suspensions are

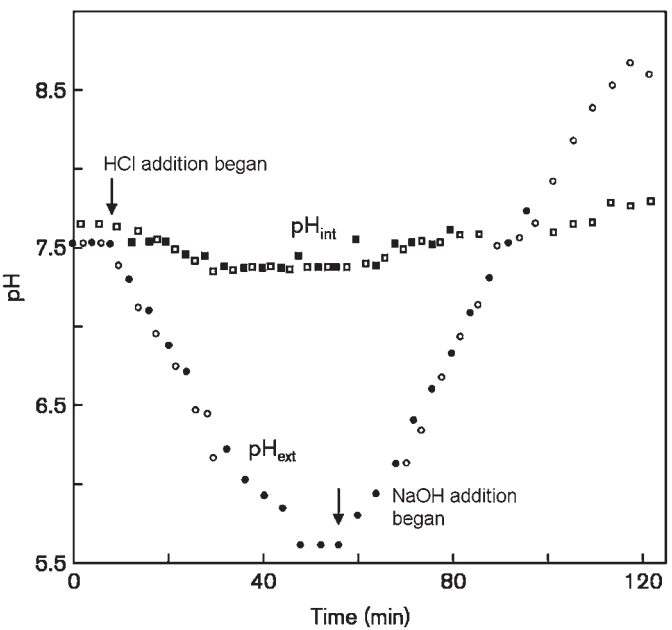


Figure 4 Cytoplasmic pH (pH$_{int}$) of *E. coli* K-12, and extracellular pH (pH$_{ext}$), measured by ^{31}P NMR. The chemical shifts were observed for inorganic phosphate (closed symbols) and methylphosphonate (open symbols). The pH was altered continuously by addition of acid (1 M HCl), then base (1 M NaCl), starting at the times indicated. [Adapted from Fig. 2 of Slonczewski *et al.* (1981).]

observed with aeration in a wide-bore tube of an NMR spectrometer, with field strength of at least 145 MHz. The use of two phosphorus probes with different pK_a values provides an intracellular confirmation of the pH calibration scale. Another advantage of NMR is that phosphorus resonances can be measured simultaneously for nucleotide phosphoryl groups and glycolytic intermediates, enabling assessment of the energetic state of the cell (Slonczewski *et al.*, 1981; Lohmeier-Vogel *et al.*, 2004).

^{31}P NMR studies can yield kinetic data on rapid perturbation of cytoplasmic pH, with a 12-second time resolution (Slonczewski *et al.*, 1982). However, in these experiments the cytoplasmic pH does not begin to recover from an external pH shift until approximately 2 minutes after HCl addition. By contrast, the fluorimetry data show a dip and initiation of recovery within 10–20 seconds (Fig. 2). A likely reason for this difference may be the relatively stressed condition of the cells in the NMR experiment, which are harvested in late log phase and/or are re-suspended at high density so that aeration comparable to optimal growth conditions is not feasible.

3. pH HOMEOSTASIS DURING GROWTH

pH homeostasis is required during growth of acidophiles (Fig. 5), neutralophiles (Figs. 6 and 7A), and alkaliphiles (Fig. 7B), in part due to the production and consumption of protons during metabolism. In most bacteria and archaea, primary pumps produce a PMF, although some marine bacteria and specialized fermentative bacteria also generate and drive bioenergetic work with a sodium motive force (Dimroth and Schink, 1998; Häse and Barquera, 2001; Müller *et al.*, 2001; Hayashi *et al.*, 2001; Dimroth, 2004). The PMF is generated by respiration, by other primary proton pumps, or by PMF-generating solute efflux cycles (Mitchell, 1961; Konings *et al.*, 1997; Schäfer *et al.*, 1999; Friedrich and Scheide, 2000). The modulation of the PMF and specific mechanisms to achieve pH homeostasis differ among acidophiles, neutralophiles, and alkaliphiles. The centrality of pH homeostasis mechanisms for most bacteria and archaea also has secondary effects on the choice of protons versus sodium used to power ion-coupled solute uptake and flagellar-based motility, as described below.

3.1. Acidophiles

Acidophiles maintain a more alkaline cytoplasmic pH that is typically 4–5 pH units above the external pH (Fig. 1). Additional acidophile cytoplasmic

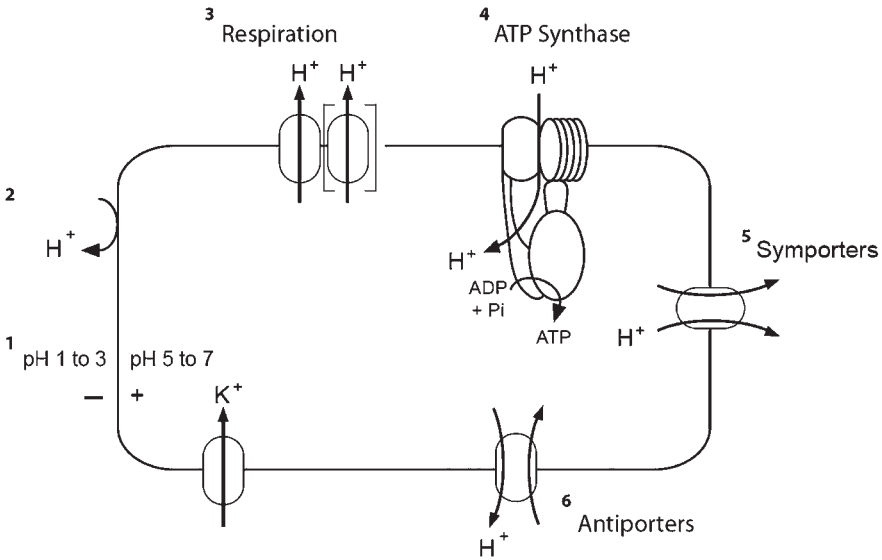


Figure 5 Schematic of general acidophile pH homeostatic mechanisms creating a near-neutral cytoplasmic pH despite an acidic external environment. [1]The internal positive membrane potential (potentially generated by potassium uptake) creates a chemiosmotic gradient that protons have to be transported against to enter the cytoplasm. [2]The cytoplasmic membrane is extremely resistant to the influx of protons. [3]Respiration-dependent primary proton pumps remove protons from the cytoplasm that [4]re-enter to generate ATP via the F_0F_1-ATPase. [5,6]Secondary symporters and antiporters can be used to remove excess protons from the cytoplasm. [Adapted from Fig. 1 of Baker-Austin and Dopson (2007).]

pH values to those given in Fig. 1 include *Thermoplasma acidophilum* that maintains an internal pH around pH 5.5–6.5 (Hsung and Haug, 1977), *Picrophilus oshimae* has an internal pH 4.6 at external pH values between 0.8 and 4.0 (van den Vossenberg *et al.*, 1998b), and *Sulfolobus solfataricus* grows at pH 2–4 with a cytoplasmic pH $\approx$6.5 (Moll and Schafer, 1988). Acidophiles maintain an inverted transmembrane potential ($\Delta\Psi$, discussed below); thus, the pH gradient across the cytoplasmic membrane is the only chemiosmotic productive parameter of the PMF. pH homeostasis requires that any influx of protons for support of bioenergetic work such as ATP synthesis and proton-coupled solute uptake (as well as proton-coupled motility for some eubacterial acidophiles) must be balanced by proton extrusion by electron transport or by an alternative primary pump. Consistent with this expectation, inhibition at any point of electron transport halts metabolism in *Acidithiobacillus caldus* (Dopson *et al.*, 2002),

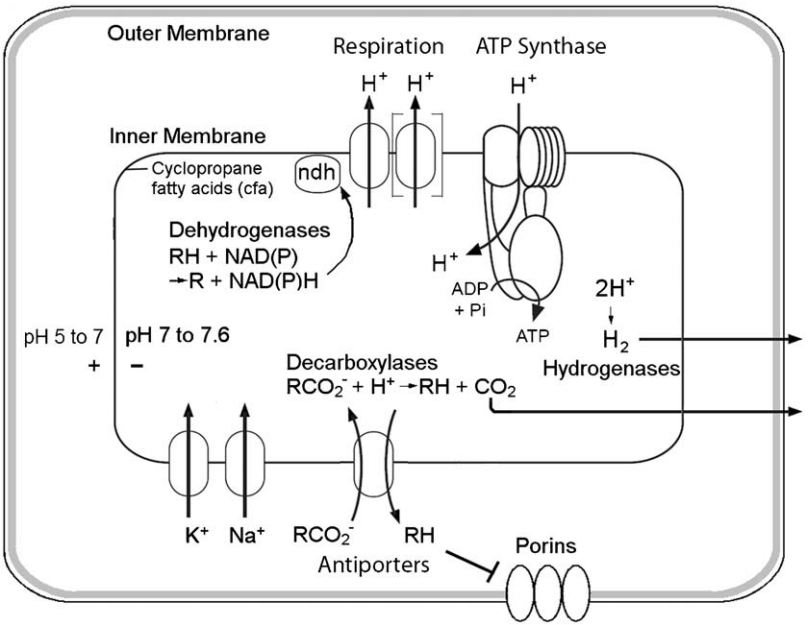
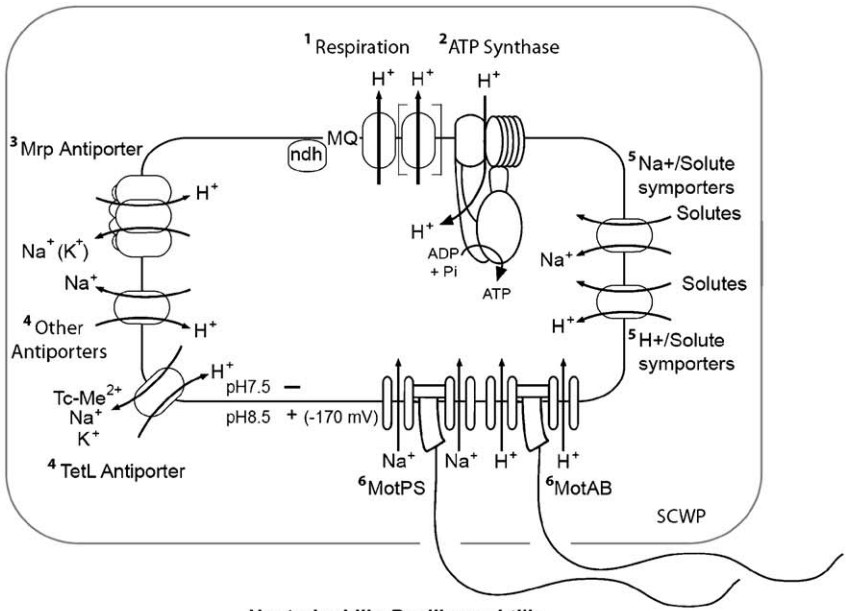


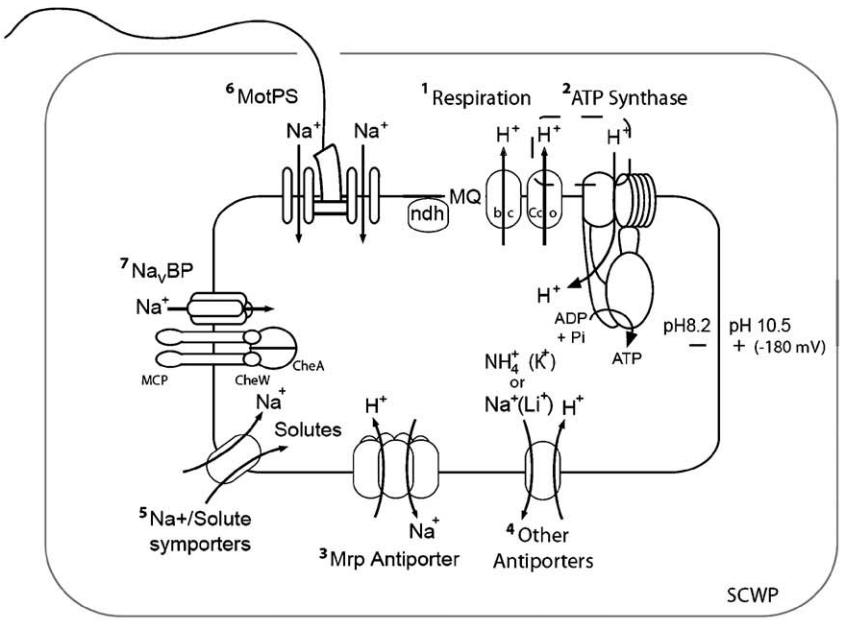
Figure 6 Acid stress during growth: mechanisms contributing to pH homeostasis in respiratory neutralophiles *E. coli* and *B. subtilis.* Acid up-regulates NAD(P)H-dependent substrate dehydrogenases, which enhance proton export. The F_1F_0-ATP synthase is partly down-regulated to limit proton re-entry. K^+ and Na^+ flux balances the charge flow. Hydrogenases convert protons to H_2, which diffuses from the cell. Amino acid and other substrate decarboxylases consume protons and generate polyamines, which are exported from the cell. In *E. coli*, polyamines block outer membrane porins from uptake of antimicrobial agents, including permeant organic acids. Acid up-regulates *cfa*, which synthesizes cyclopropane fatty acids (CFAs) in the inner membrane. (Based on Neely and Olson, 1996; Chang and Cronan, 1999; Tanner and Bornemann, 2000; Foster, 2004; Hayes *et al.*, 2006; Pagès *et al.*, 2008; Wilks *et al.*, 2009; others cited in text.)

Acidiphilium acidophilum (Matin *et al.*, 1982), and *Sulfolobus acidocaldarius* (Lübben and Schäfer, 1989). It was also demonstrated that metabolically active *A. acidophilum* cells maintained a stable external pH, consistent with the anticipated balance between proton consumption and proton pumping (Matin *et al.*, 1982).

Although the cytoplasmic membrane must allow flow of ions and molecules to support ATP synthesis, transport, and metabolism, the acidophile membrane must hinder the entry of protons (Fig. 5). Indeed, acidophile cell membranes (and in particular archaeal membranes) are



(A) **Neutralophilic *Bacillus subtilis***



(B) **Alkaliphlic *Bacillus pseudofirmus* OF4**

highly impermeable to passive influx of protons down the concentration gradient from the outside to the inside of the cell (Konings *et al.*, 2002). This impermeability is vital as protons are more able to cross the membrane than other monovalent cations (van den Vossenberg *et al.*, 1995). It has been demonstrated that the proton permeability of archaeal membranes increases by only a factor of 10 from pH 1 to 11 (Nichols and Deamer, 1980; Nagle *et al.*, 2008), whereas there is a linear increase in the permeability of other ions. Proton translocation is suggested to be mediated by chains of hydrogen-bonded water (Deamer and Nichols, 1989) and therefore, archaeal membranes also have a low influx of water (Dannenmuller *et al.*, 2000). The cytoplasmic membrane of most bacteria consists of a lipid bilayer containing fatty acids ester-linked to a glycerol moiety whereas, archaea have ether linkages (Gliozzi *et al.*, 2002). Bipolar

Figure 7 Interacting proton and sodium cycles in neutralophilic *B. subtilis* and alkaliphilic *B. pseudofirmus* OF4. The pH_{in}, pH_{out}, and $\Delta\Psi$ (inside negative) are shown for an alkaline pH that supports growth. [1]Both species have exclusively proton-pumping respiratory chains; the caa_3-type cytochrome *c* oxidase (Cco) of the alkaliphile plays a major role in oxidative phosphorylation in the alkaliphile but not in the neutralophile. [2]Both organisms have proton-coupled ATP synthases. The alkaliphile synthase has specific adaptations of the proton-translocating *a*- and *c*-subunits that are required for ATP synthesis and/or cytoplasmic pH homeostasis at pH 10.5 (Ivey and Krulwich, 1992; Wang *et al.*, 2004; Liu *et al.*, 2009). The dashed lines connecting the cytochrome oxidase of the alkaliphile respiratory chain and the ATP synthase indicate the existence of incompletely elucidated mechanisms for sequestered proton transfer between the respiratory chain and the synthase in the alkaliphile (Krulwich *et al.*, 2007). [3]The multi-subunit Mrp Na^+/H^+ antiporter has a major role in Na^+ resistance in *B. subtilis* (Kosono *et al.*, 1999; Ito *et al.*, 1999) and a major role in pH homeostasis and in Na^+ resistance in alkaliphilic *B. halodurans* C-125 (Hamamoto *et al.*, 1994). [4]The TetL antiporter plays a major role in pH homeostasis of *B. subtilis* strains that have this tetracycline and monovalent cation/proton antiport system and additional antiporters with supportive roles in pH homeostasis are found in both organisms (Padan *et al.*, 2005). [5,6]Ion-coupled solute uptake is exclusively coupled to sodium in the alkaliphile whereas *B. subtilis* can couple these processes to either protons or sodium. Sodium entry by these routes supports ongoing Na^+/H^+ antiport at high pH. [7]A polarly localized voltage-gated sodium channel, Na_VBP is found in the alkaliphile that contributes to sodium entry to support pH homeostasis at high pH. Its function is required for normal chemotaxis and it co-localizes with chemotaxis receptors (shown as an MCP-CheW,CheA complex) (Ito *et al.*, 2004b). The SCWPs indicated by the line surrounding both cells indicates the presence of secondary cell wall polymers. In alkaliphilic *B. pseudofirmus* OF4, the S-layer protein SlpA supports pH homeostasis (Gilmour *et al.*, 2000) and in alkaliphlic *B. halodurans* C-125, teichuronic acid components of the SCWPs have a major role in pH homeostasis and alkaliphily (Aono and Ohtani, 1990; Aono *et al.*, 1999).

tetraether archaeal lipids have a monolayer organization with cyclopentane rings and a network of hydrogen bonds between the sugar residues on the outer face of the membrane (Elferink *et al.*, 1994) and tight lipid packing (Komatsu and Chong, 1998). Most acidophilic archaea contain tetraether-linked lipids (Macalady *et al.*, 2004) that have been identified in *F. acidiphilum* Y^T and Y2 (Batrakov *et al.*, 2002; Golyshina *et al.*, 2000; Pivovarova *et al.*, 2002), "*Ferroplasma acidarmanus*" (Macalady *et al.*, 2004), *P. oshimae* (van den Vossenberg *et al.*, 1998b), *S. solfataricus* (van den Vossenberg *et al.*, 1998a), and *T. acidophilum* (Shimada *et al.*, 2002). These results are supported by data from *S. acidocaldarius* liposomes (Elferink *et al.*, 1994; Komatsu and Chong, 1998) that were less proton permeable than liposomes from *E. coli* and *Bacillus stearothermophilus*.

Related to pH homeostasis and maintaining a pH gradient across the membrane is the size and permeability of membrane channels in either the outer or inner membrane that allow the influx of protons. The response of *A. ferrooxidans* to acid stress–identified up-regulated genes coding for putative lipoproteins and outer membrane proteins, suggesting that this bacterium may alter its outer membrane structure to reduce proton permeability (Chao *et al.*, 2008). In another study, the *A. ferrooxidans* outer membrane protein Omp40 was up-regulated as a result of a pH drop from pH 3.5 to 1.5 in a similar response to heat shock (Amaro *et al.*, 1991). Outer membrane porins (OMPs) from Gram-negative neutralophiles are trimeric structures that form water filled channels through which nutrients may pass (Nikaido, 2003). The *A. ferrooxidans* Omp40 has a large external L3 loop that may control the size and ion specificity at the entrance of the porin (Guiliani and Jerez, 2000). It also has an estimated isoelectric point of 7.21 and the L3 loop has a charge of +2 at pH 2.5 (compared to −4 for the *E. coli* homologue at pH 7) that may be adaptations to limit the passage of protons into the cytoplasm (Guiliani and Jerez, 2000). Omp40 has been shown to be potentially involved in Fe^{2+} oxidation by interaction with the substrate (Castelle *et al.*, 2008) although its role in response to a sudden drop in pH has not been elucidated. *A. ferrooxidans* is thus far the only acidophile in which the membrane is known to change in response to acid stress. There is a need for further studies of the porins of acidophile membranes in relation to acid stress, especially in an acidophilic archaea.

A further feature of pH homeostasis in acidophiles is the generation of an inverted transmembrane potential ($\Delta\Psi$), positive inside relative to outside (Fig. 5). This is opposite to the orientation of the $\Delta\Psi$, inside negative, of neutralophiles and alkaliphiles. The $\Delta\Psi$ has been measured in a number of acidophiles including +10 mV in *A. ferrooxidans* (Cox *et al.*, 1979), +73 mV in *A. acidophilum* (Matin *et al.*, 1982), and approximately

+20 mV in *T. acidophilum* (Michels and Bakker, 1985). The inside-positive $\Delta\Psi$ in acidophiles helps to maintain the large ΔpH and hence pH homeostasis by impeding entry of protons down their chemical gradient. A small internal positive potential is also observed in de-energized cells that counterbalance the pH gradient (ΔpH) across the membrane such that the PMF is zero in *T. acidophilum* (Hsung and Haug, 1977), *Bacillus acidocaldarius* (Oshima *et al.*, 1997), *A. caldus* (Dopson, 2001), *A. ferrooxidans* (Cox *et al.*, 1979), and *A. acidophilum* (Goulbourne *et al.*, 1986). Problems of accurately measuring such small potentials and correcting for probe binding were noted (Michels and Bakker, 1985). However, as recently described by Ferguson and Ingledew (2008), the inside-positive $\Delta\Psi$ in acidophiles is necessary to maintain a cytoplasmic pH much closer to neutrality than the outside pH, and this is clearly critical for cell metabolism and growth. Further, they note that under survival – only conditions such as de-energization or after treatments that increase proton permeability, the pattern of a counterbalanced inside-positive $\Delta\Psi$ and a ΔpH, acid outside, would be expected to persist, as has been reported, unless a permeant anion is present. A permeant anion would collapse the inside-positive $\Delta\Psi$ and that in turn would lead to collapse of the ΔpH.

There have been suggestions that potassium ions play a role in generation of the acidophile PMF and its inside-positive $\Delta\Psi$ (Fig. 5). For example, proton translocation during respiration and PMF generation in *Sulfolobus* spp. is dependent on the presence of K^+ ions (Schäfer, 1996) and generation of the $\Delta\Psi$ in *Acidithiobacillus thiooxidans* depends upon the presence of cations with K^+ being most effective (Suzuki *et al.*, 1999). Some of the effects of K^+ could result from the stimulation of respiration-dependent PMF generation secondary to PMF depletion during K^+ uptake as described by Bakker and Mangerich (1981). Richard and Foster (2004) describe a series of reactions, including amino acid carboxylases and electrogenic substrate/product exchange during acid adaptation of *E. coli* that could contribute to formation of the inside-positive $\Delta\Psi$ generated in this neutralophile (see Section 4.1). The nature and origin of the inside-positive $\Delta\Psi$ merits further investigation across a spectrum of acidophiles and acid-tolerant neutralophilic bacteria, as this potential plays a key role in maintaining pH homeostasis.

3.2. Neutralophiles: pH Homeostasis in Acid

Bacteria growing at near-neutral pH show fewer membrane adaptations than do acidophiles. At near-neutral values of external pH, both

respiratory neutralophiles and neutralophiles that lack respiratory chains are able to grow without pH homeostasis when the PMF is diminished or eliminated by an uncoupler. *E. hirae* grows at pH 7.1–7.8 with ΔpH and the proton potential eliminated by 1 μg/ml gramicidin D (Harold and Van Brunt, 1977). Respiratory bacteria such as *E. coli* grow at pH 7.4–7.6 with the proton potential collapsed by 50 μM of the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (Kinoshita *et al.*, 1984) or at pH 7.0–8.0 in the presence of benzoate, which diminishes or eliminates ΔpH (White *et al.*, 1992). In these cases, growth requires rich medium, with energy obtained through substrate-level phosphorylation on catabolites such as glucose or amino acids. Some organisms may in fact sacrifice pH homeostasis in order to avoid low pH-driven uptake of fermentation acids (discussed below, Section 3.3). For instance, the pathogen *E. coli* O157:H7 allows its internal pH to decrease below pH 7 in order to avoid acetate accumulation (Diez-Gonzalez and Russell, 1997). However, in most cases the loss of pH homeostasis allows growth only when the external pH is at or near the optimal value of cytoplasmic pH.

Exposure of neutralophiles to low pH requires adjustments, as discussed below, and induces structural changes in membranes, most notably an increase in production of CFAs (Grogan and Cronan, 1997). The means by which CFAs strengthen the membrane are unclear, but the lowering of membrane proton conductance may play a role. *E. coli* cells defective for the enzyme CFA synthase (*cfa*) lose the ability to survive an acid shift from neutral pH to pH 3 (Chang and Cronan, 1999). In *E. coli*, *cfa* is up-regulated both by low pH (Hayes *et al.*, 2006) and by acetate, which acidifies the cytoplasm at neutral pH (Rosenthal *et al.*, 2006). Up-regulation of *cfa* is RpoS-dependent, as are major mechanisms of acid resistance such as the Gad regulon (discussed below). The *cfa* enzyme is similarly up-regulated by acid in *Salmonella enterica* (Kim *et al.*, 2005), *L. lactis* (Budin-Verneuil *et al.*, 2005), and *Oenococcus oeni* (Grandvalet *et al.*, 2008). External acid and base also differentially regulate a number of outer membrane proteins, whose role in acid resistance remains unclear (Stancik *et al.*, 2002; Maurer *et al.*, 2005; Hayes *et al.*, 2006). As in the acidophile *A. ferrooxidans*, the OmpR/EnvZ regulation of porins enhances *E. coli* growth at low pH (Sato *et al.*, 2000). Some amino acid decarboxylases maintain pH homeostasis in enteric bacteria surviving at pH values below their growth range, in part through generation of an inverted electrical potential (Foster, 2004; see Section 5.1).

Two general mechanisms of acid pH homeostasis occur among neutralophiles: (1) ATPase-dependent proton extrusion in non-respiratory bacteria and (2) ATPase-independent ion transport and catabolic acid

consumption in respiratory bacteria. The first class includes bacteria lacking a respiratory chain, such as *Enterococcus hirae* (formerly *Streptococcus faecalis*), which inhabits animal intestines (Kakinuma, 1998). *E. hirae* primarily uses the F_1F_0-ATPase to expend ATP to expel excess protons (Kobayashi and Unemoto, 1980; Kobayashi *et al.*, 1982; Shibata *et al.*, 1992). Both the expression and activity of the ATPase are increased at low external pH; and mutants in the ATPase fail to grow below pH 7 (Suzuki *et al.*, 1988). The ATPase similarly serves as the primary means of acid reversal for lactococci (Hutkins and Nannen, 1993) and for oral streptococci (Kuhnert and Quivey, 2003), although supplemented by catabolic mechanisms such as amino acid decarboxylases (Curran *et al.*, 1995).

In most respiratory neutralophiles, no one mechanism is known to be essential for growth below pH 7 (Fig. 6). The F_1F_0-ATPase acts primarily as the ATP synthase coupled to electron transport. In fact, the *atp* operon is up-regulated at high external pH, suggesting a need to compensate for the lower PMF under conditions of inverted ΔpH (in *E. coli* K-12, Maurer *et al.*, 2005; in *B. subtilis*, Wilks *et al.*, 2009; in *Desulfovibrio vulgaris*, Stolyar *et al.*, 2007). In *E. coli*, potassium transport has been associated with pH homeostasis (Bakker and Mangerich, 1981; Roe *et al.*, 2000; Buurman *et al.*, 2004). Nevertheless, no one potassium transport system is essential for growth at low pH. A triple mutant for potassium transport systems, deleted for *kdpABC trkA trkD*, requires high extracellular K^+ for pH homeostasis and growth below pH 7.5 (White *et al.*, 1992). In standard laboratory strains, K^+ is not required for reversal of transient effects of acid exposure on cytoplasmic pH, even when the exposure is an abrupt acid shift (Wilks and Slonczewski, unpublished data).

A diverse collection of catabolic enzymes and substrate transporters are transcriptionally regulated so as to favor acid consumption and base production at low pH (and the reverse at high pH). The best characterized example is that of lysine decarboxylase, *cadAB*, and the regulator *cadC* (Slonczewski *et al.*, 1987; Meng and Bennett, 1992a, b; Dell *et al.*, 1994; Neely *et al.*, 1994; Neely and Olson, 1996). Amino acid decarboxylases consume an acidic group, usually with release of a polyamine (for details, see Section 4.4). Low pH also up-regulates hydrogenase complexes, which may consume protons to release as hydrogen gas (King and Przybyla, 1999; Hayes *et al.*, 2006). Both decarboxylases and hydrogenases show acid-enhanced expression primarily under anaerobiosis. Aerobic conditions lead to acid-up-regulation of proton pumps such as the NADH dehydrogenases and cytochrome *d* oxidase (Maurer *et al.*, 2005). In *B. subtilis*, acid also up-regulates numerous NAD(P)H-dependent substrate dehydrogenases, which could channel electrons into respiration and accelerate proton export

(Wilks *et al.*, 2009). The mechanism of the acid-stress decarboxylase induction response of *B. subtilis* is beginning to be unraveled with the finding that the σ-like protein YvrI works with a pair of co-regulators to control levels of the oxalate carboxylase OxdC that is implicated in the acid-stress response (Tanner and Bornemann, 2000; MacLellan *et al.*, 2009).

A special case is that of *Helicobacter pylori*, a neutralophilic epsilon proteobacterium that has the ability to persistently colonize the epithelium of the extremely acidic human stomach, where it is associated with peptic ulcers and gastric cancer (Fig. 8). Under laboratory culture conditions in the absence of urease, *H. pylori* behaves like a neutralophile in that it grows primarily between external pH 5 and 7.5 (Bauerfeind, *et al.*, 1997; Slonczewski *et al.*, 2000). By contrast, other pathogenic neutralophiles survive while passing through the stomach but do not grow in highly acidic environments or colonize them (Blaser and Atherton, 2004; Sachs *et al.*, 2005). The median pH of the gastric lumen in the absence of food is about pH 2; although there is controversy about the pH at the gastric surface, consistency between genes induced upon an *in vitro* acid shift and exposure to the *in vitro* environment support the conclusion that the acid pH challenge to *H. pylori* is formidable (Wen *et al.*, 2003; Scott *et al.*, 2007). *H. pylori* differs from both *bona fide* acidophiles and other neutralophiles that can survive an acid challenge in not exhibiting the inside-positive $\Delta\Psi$ found in these other organisms. Instead, *H. pylori* maintains a small $\Delta\Psi$ (~ -25 mV) in the chemiosmotically productive orientation (inside negative) while also maintaining a very large ΔpH (alkali inside), and a cytoplasmic pH in the near-neutral range during colonization of the acidic gut (Sachs *et al.*, 2005, 2006).

Not surprisingly, *H. pylori* use a unique pH homeostasis strategy. An important initial clue was the finding of particularly high constitutive levels of urease (Mobley *et al.*, 1995; van Vliet *et al.*, 2001), which is increased to even higher levels during growth in acid (Slonczewski *et al.*, 2000). In acidic media containing urea, urea passes through an OMP and is taken up by the UreI inner membrane urea channel that is proton gated and is associated on the cytoplasmic side with the cytoplasmic urease (Sachs *et al.*, 2005, 2006). The combined actions of the cytoplasmic urease and a cytoplasmic carbonic anhydrase release ammonia and carbon dioxide. Some ammonia may scavenge protons in the cytoplasm, but it is largely expected to diffuse into the more acidic periplasm along with the carbon dioxide. Some of the ammonia may escape into the outside medium and scavenge protons there but much of the ammonia and carbon dioxide remain in the periplasm where they mediate pH homeostasis of that compartment (Fig. 8). The regulation of periplasmic pH is in contrast with other neutralophiles such as

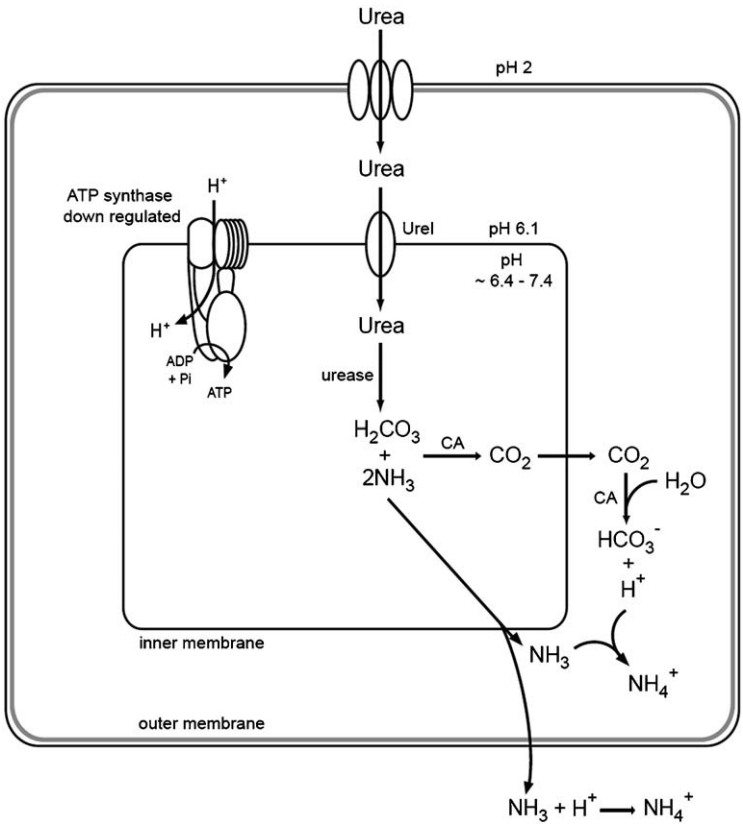


Figure 8 pH homeostasis in *H. pylori* growing in extreme acid. Urea-dependent pH homeostasis as an adaptive strategy in *H. pylori* requires passage of urea into the cytoplasm via the UreI channel and production of ammonia and carbon dioxide by the actions of cytoplasmic urease and carbonic anhydrase (CA). The abundant carbon dioxide and ammonia diffuse into the periplasm where a periplasmic carbonic anhydrase produces bicarbonate; the ammonia scavenges the protons released in the carbonic anhydrase reaction so that a ''cloud'' of buffering bicarbonate remains to support the periplasmic pH 6.1 and protect the cytoplasm within (After Sachs *et al.*, 2005). Among the strategies that are employed to limit proton uptake is the down-regulation of the ATP synthase (Wen *et al.*, 2003).

E. coli, in which the periplasmic pH appears to be continuous with that of the exterior (Wilks and Slonczewski, 2007). In *H. pylori*, a periplasmic pH $\sim$6.1 is maintained through the proton capture by ammonia and the generation of a large amount of buffering bicarbonate from the carbon dioxide by the activity of a periplasmic carbonic anhydrase. The

well-buffered periplasmic compartment thus produced adds an extra layer of protection for the cytoplasmic compartment.

Induction of urease, as well as additional ammonia-generating enzymes, and of carbonic anhydrase was observed in an *in vitro* experiment; motility and chemotaxis genes were also induced, consistent with mutational evidence that these activities are necessary for colonization (Scott *et al.*, 2007). Mutational loss of urease, the urea channel and the carbonic anhydrase all result in defective pH homeostasis (Eaton *et al.*, 1991; Mollenhauer-Rektorschek *et al.*, 2002; Marcus *et al.*, 2005; Bury-Moné *et al.*, 2008). Potassium uptake by the HpKchA channel of the RCK domain family is also necessary for gastric colonization by *H. pylori*, providing a specific biological function for a prokaryotic potassium channel (Stingl *et al.*, 2007). The ATP synthase that brings protons inward as it functions is down-regulated (Wen *et al.*, 2003). This down-regulation of the ATP synthase in *Helicobacter* is consistent with the observations of up-regulation of ATP synthase expression in several neutralophiles under alkaline challenge (Maurer *et al.*, 2005; Stolyar *et al.*, 2007; Wilks *et al.*, 2009).

3.3. Neutralophiles: pH Homeostasis in Base

In alkaline pH homeostasis, both non-respiratory and respiratory neutralophiles show a central role for cation/proton antiporters. These secondary membrane transporters use the PMF developed by primary pumps such as the proton-pumping respiratory chain components of respiring bacteria or ATP-dependent proton pumping by the hydrolytic activity of F_1F_0- or H^+-coupled P-ATPases. In order to support net proton accumulation in the cytoplasm, a Na^+/H^+ antiporter would have to transport more protons inward than the number of sodium ions transported outward during each turnover. Such an electrogenic antiport whose exchange involves a H^+:Na^+ ratio >1, and thus translocates net positive charge inward, could be energized by the $\Delta\Psi$ component of the PMF. Such an activity could thus achieve cytoplasmic proton accumulation (Booth, 1985; Macnab and Castle, 1987; Padan *et al.*, 2005). A K^+/H^+ antiporter could achieve acidification of the cytoplasm even if it functioned via a 1:1 electroneutral exchange, since it could use both the ΔpH component of the PMF and get the added energy required to reverse that gradient from the outwardly directed gradient of the efflux substrate, K^+, which exists under most physiological circumstances (Booth, 1985; Padan *et al.*, 2005). A detailed characterization of a typical bacterial complement of cation/proton antiporters (often more than 5) has yet to be reported for any species and is a

challenging task; selected examples from the bacteria that have been most completely characterized will be discussed but even these bacteria have predicted antiporters whose activities and roles are not known. It is further likely that some of the membrane proteins of unknown function will turn out to have (novel) activities that contribute to pH homeostasis.

With respect to adaptation to alkaline pH, *E. hirae* is among the most intensively studied non-respiratory neutralophiles (Kakinuma, 1998). In the presence of bicarbonate, *E. hirae* grows at pH values as high as 10.5, and exhibits a cytoplasmic pH of 7.8 at an external pH 8.4, and a cytoplasmic pH of 8.2 at an external pH 9.5 (Kakinuma, 1987a). Kakinuma (1987b) proposed that alkaline pH homeostasis is supported by a constitutive Na^+/H^+ antiporter energized by the PMF produced by the H^+-pumping F_1F_0-ATPase that is up-regulated by alkali. It was further predicted that *E. hirae* also supports sodium extrusion with an inducible sodium ATPase. This ATPase activity would also increase the inwardly directed sodium gradient available to drive sodium-coupled transport activities at high pH. Evidence was also found for the involvement of a K^+/H^+ antiporter in *E. hirae* pH homeostasis since a mutant with deficient pH homeostasis was also deficient in K^+/methylamine exchange (Kakinuma and Igarashi, 1999). Subsequently, a Na^+/H^+ antiporter from *E. hirae* was identified and characterized. It was designated NapA (Waser *et al.*, 1992) and it became one of the founding transporters of the Cation:Proton Antiporter-2 (CPA2) family in the Transporter Classification System (Saier *et al.*, 1999; Saier, 2000).

The anticipated Na^+-coupled ATPase has also been identified and studied in detail. Encoded by the *ntp* operon, the ATPase product is a V(vacuolar)-type ATPase that uses a rotary mechanism. It works only in the hydrolytic, Na^+ extruding direction and is composed of nine NTP proteins (Kakinuma, 1998; Murata *et al.*, 2005). Consistent with roles in support of alkaline pH homeostasis and Na^+-resistance, expression from the *ntp* promoter is increased by elevated pH or elevated Na^+ (Ikegami *et al.*, 1999). Another special feature of the *ntp* operon underscores the relationship between Na^+ cytotoxicity, K^+ requirements, and alkali stress. This relationship is as follows: at high pH, cells become more sensitive to growth inhibition by Na^+, but the K^+ status strongly influences cytoplasmic Na^+ toxicity. Even at very high pH, the toxicity of Na^+ is lessened if cytoplasmic K^+ is elevated (Padan *et al.,* 2005; Wei *et al.,* 2007). In *E. hirae*, induction of V-ATPase-mediated Na^+ extrusion at high pH allows the bacterium to address the increased toxicity at alkaline pH even when Na^+ concentrations are not substantially elevated. In addition, the *ntpJ* gene encodes a protein that is not part of the V-ATPase but, rather, is a subunit

of an *E. hirae* KtrII-type K^+ uptake system. Induction of the *ntp* operon, by either high pH or high sodium, addresses the threat of increased Na^+ cytotoxicity both by increasing extrusion of the toxic ion but also by lessening its cytotoxicity through increasing the ameliorating K^+ levels (Kawano *et al.,* 2000).

Some lactic acid bacteria, of which *E. hirae* is often considered a relative or member, do not grow at alkaline pH, but many members of the genus *Lactobacillus* are capable of growth up to pH 8.5 or even 8.9. These strains, as well as some of those that are less able to grow at elevated pH, acidify their cytoplasm relative to the external medium during growth (Sawatari and Yokota, 2007). One of the most efficacious strains, *L. acidophilus* JCM 1132^T exhibited greater cytoplasmic proton accumulation in the presence of Na^+ than in its absence, suggestive of Na^+/H^+ antiporter involvement.

Among the respiratory neutralophiles, alkali-adaptation mechanisms to support pH homeostasis have been most intensively studied in the model Gram-negative and -positive organisms *E. coli* and *B. subtilis*, respectively. In both of these bacteria, the properties and roles of many, but still not all, of the cation/proton antiporters predicted in the genome have been studied (Padan *et al.*, 2005). In *E. coli*, NhaB is a modestly electrogenic Na^+/H^+ antiporter (1.5 H^+:1 Na^+) that appears to have a housekeeping role (Pinner *et al.*, 1993, 1994). In contrast, NhaA has a H^+:Na^+ coupling stoichiometry of 2 (Taglicht *et al.*, 1993) and is a high-affinity, high-turnover antiporter that is highly regulated and tremendously activated by elevated pH (Padan *et al.*, 2004; Padan, 2008). Its properties suggest that it has a major role in Na^+ extrusion and proton capture at high pH. The high-resolution structure of NhaA offers insights into one of the problems of cation/proton antiporter function in alkaline pH homeostasis, that is, the problem of capturing protons from a highly alkaline bulk phase with kinetics that support an activity level that, in turn, achieves pH homeostasis. The NhaA structure contains two negatively charged funnels, one on each side of the membrane, that are proposed to foster efflux of the cytoplasmic Na^+ and entry of the periplasmic H^+ (Hunte *et al.*, 2005).

In spite of the efficacy of NhaA and supporting role of NhaB, it was clear from mutant studies that there must be additional contributors to pH homeostasis since double *nhaA*, *nhaB* deletion strains were very sensitive to Na^+ but still grew well in media at pH$>$8 if the Na^+ concentration was kept low (Pinner *et al.*, 1993). Plack and Rosen (1980) present evidence that *E. coli* has a K^+/H^+ antiporter that also has a role in pH homeostasis. A third *E. coli* antiporter, designated ChaA because of its Ca^{2+}/H^+ antiport activity as well as Na^+/H^+ (Ivey *et al.*, 1993), was shown to support not only sodium and calcium circulation (Ohyama *et al.*, 1994; Shijuku *et al.*,

2002) but also to have K^+/H^+ antiport activity. ChaA functions best as a K^+/H^+ antiporter at and just above pH 9.0, where NhaA is less efficacious (Radchenko *et al.*, 2006). There are two well-studied two-component transporters of the CPA2 family that have a role in electrophile resistance in *E. coli*, KefFC and KefGB (Booth *et al.*, 2003). These transporters have recently been shown to carry out K^+/H^+ antiport (Fujisawa *et al.*, 2007) but KefFC and KefGB have not been shown to play a role in pH homeostasis. Nor have two members of the CPA-1 family predicted in *E. coli* to have such a role. The one additional antiporter that has been shown to support alkalitolerance via low affinity $(Na^+)K^+/H^+$ antiport above the pH range for NhaA is a known multi-drug antiporter of *E. coli*, MdfA (Lewinson *et al.*, 2004). It is interesting that a drug/H^+ antiporter has cation/proton activity in both *E. coli* and *B. subtilis*. These findings may have implications with respect to pathways by which drug/H^+ antiporters evolve and also with respect to how they sometimes persist even without selective pressure of the drug (Krulwich *et al.*, 2005). The neutralophile *B. subtilis* can grow at pH values about 8.5–9.0, and in pH 8.5 media, it maintains a cytoplasmic pH $\sim$7.5 (Cheng *et al.*, 1994, 1996). Elements of the *B. subtilis* active ion-transport cycles that support pH homeostasis include a diverse array of antiporters (Fig. 7A). These antiporters include a chromosomal tetracycline–metal/proton antiporter TetL that has a major role in alkaline pH homeostasis by virtue of its additional capacities for Na^+/H^+ and K^+/H^+ antiport activities (Cheng *et al.*, 1994, 1996). However, wild-type *B. subtilis* keeps its cytoplasmic pH at 7.5 after a shift of external pH 7.5 to 8.5, if either Na^+ or K^+ is present (Table 1). However, *B. subtilis* cannot maintain a cytoplasmic pH at 7.5 in their absence or in the absence of a functional *tetL*; nor can it regulate its pH if subjected to the much greater challenge of a pH between 8.5 and 10.5 that can be handled by an alkaliphilic *Bacillus.* Although *tetL* deletion mutants are viable, these mutants exhibit a complex pattern of changes in the transcriptome that are apparent adjustments to a more sodium- and alkali-challenged state; most of these changes are not reversed upon re-introduction of *tetL* to the chromosome (Wei *et al.*, 2006). The 14-TMS drug/H^+ antiporters are hypothesized to have evolved from the more common 12-TMS bacterial antiporters that had a housekeeping function. Sequence analyses suggest that the two middle TMS were inserted late and the subsequent selection ultimately led to the final constellation of capacities (Griffith *et al.*, 1992). This is supported by the fact that when the two middle TMS of TetL were removed to create a 12-TMS TetL, this engineered transporter was still incorporated into the membrane and retained the Na^+- and K^+-related transport functions but could no longer carry out tetracycline–metal/proton antiport (Jin *et al.*, 2001).

Table 1 pH homeostasis in neutralophic *Bacillus subtilis* and alkaliphilic *Bacillus pseudofirmus* OF4: effects of mutations in transporters or a secondary cell wall polymer.

(A)			Cytoplasmic pH (10 minutes after shift)				
			pH 7.5→8.5		pH 8.5→10.5		
			Choline Cl	NaCl	KCl	NaCl	KCl
B. subtilis	WT	Malate	8.5	7.5	7.6	10.5	10.5
	Δ*telL*	*Malate*	8.5	8.5	8.5	–	–
	Δ*mrpA*	*Malate*	8.5	7.6	7.6	–	–
B. pseudofirmus	WT	Glucose	–	–	–	8.4	9.2
OF4	WT	Malate	8.5	7.5	8.4	8.2	10.5
	Δ*slpA*	Malate	–	–	–	9.1	10.5

(B)	Cytoplasmic pH (10 minutes after shift)	
	pH 8.5→10.5	
B. pseudofirmus OF4 strain	100 mM Na^+	2.5 mM Na^+
Wild type	8.46	9.03
SC34, NavBP deletion (Δ*ncbA*)	8.66	9.43
SC34R-NavBP restored (Δ*ncbA*, *ncbA* restored)	8.48	9.02
Mot6, non-motile (Δ*motPS*)	8.52	9.03
SC34/Mot6, double NavBP, MotPS mutant (ΔncbA, Δ*motPS*)	8.72	9.51

The indicated strains of neutralophilic *B. subtilis* or *B. pseudofirmus* OF4 were grown at pH 7.5 or pH 10.5, respectively in malate-containing media, washed and equilibrated at the starting pH for particular shift experiments in buffers to which no choline, sodium, or malate were added but which contained the indicated carbon source. A sudden alkaline shift was then imposed and the cytoplasmic pH was determined 10 minutes after the shift. (A) Left: Cells were equilibrated at pH 7.5 and shifted to pH 8.5 in the presence of malate in buffer containing added choline chloride, NaCl, or KCl (50 mM). Right: Cells were equilibrated at pH 8.5 and shifted to 10.5 in malate- or glucose-containing buffer with either NaCl or KCl (100 mM) (Cheng *et al.*, 1996; Gilmour *et al.*, 2000). (B) Cells of alkaliphile strains were subjected to an alkaline shift from pH 8.5 to 10.5 as described above except that the shift buffer contained no carbon source and efficacy of two different concentrations of NaCl were compared (Ito *et al.*, 2004b). The strains were: wild type; *SC34* gene encoding Na_VBP deleted; SC34R, Na_VBP gene restored; Mot6, deletion of *motPS*; SC34/Mot6, double Na_VBP and MotPS mutant.

Another important and unusual *B. subtilis* antiporter, the Mrp antiporter, was first discovered as the major Na^+/H^+ antiporter of alkaliphilic *B. halodurans* C-125 (Hamamoto *et al.*, 1994). This widespread antiporter is part of a unique type of cation/proton antiporter and hence

classified in its own family, CPA-3 family (Saier *et al.*, 1999; Saier, 2000). Mrp antiporters are encoded in operons that have six or seven genes whose products are all very hydrophobic membrane proteins and are all required for activity (Hiramatsu *et al.*, 1998; Ito *et al.*, 1999, 2000). All seven *B. subtilis* Mrp proteins form a hetero-oligomeric complex presumed to be the active form of the antiporter (Kajiyama *et al.*, 2007). The Mrp antiport is electrogenic, although the precise stoichiometry is not yet known for any Mrp system since the complexity of these systems makes purification and functional reconstitution especially challenging (Swartz *et al.*, 2007). In *B. subtilis*, Mrp only plays a significant role in pH homeostasis if *tetL* is absent or disrupted (Ito, unpublished data; see Table 1 for the absence of an effect of a *mrp* deletion in a homeostasis assay in *B. subtilis*). However, *mrp* mutants of *B. subtilis* are particularly sensitive to Na^+ inhibition (Ito *et al.*, 1999; Kosono *et al.*, 1999). Additional antiporters include two members of the NhaC antiporter family that play modest roles in pH homeostasis or growth at low PMF (Wei *et al.*, 2000) and NhaK, that exhibits $Na^+(Li^+)(K^+)/H^+$ antiport but has no established function (Fujisawa *et al.*, 2005) (Fig. 7A). Active proton uptake by the Na^+/H^+ antiporters is energized by the PMF generated by respiration and is critical for alkaline pH homeostasis in which the cytoplasmic pH is lower than the external pH. The PMF also energizes ATP synthesis and H^+-coupled solute transport systems (H^+/solute symporters) so protons also enter by these routes in support of pH homeostasis. The ongoing availability of cytoplasmic sodium to act as an efflux substrate for the Na^+/H^+ antiporters requires re-uptake routes for sodium which are comprised of Na^+-coupled solute uptake systems (Na^+/solute symporters) as well as a second motility system that uses Na^+-coupled MotPS, an alternative motility system that is particularly active at elevated pH, sodium, and viscosity (von Blohn *et al.*, 1997; Ito *et al.*, 2004a).

3.4. Alkaliphiles

Studies of non-respiratory alkaliphiles that grow well at pH values above 9 have demonstrated a variety of interesting ATPases, some of which are discussed in a later section. This group of anaerobic or facultatively anaerobic alkaliphiles exhibits pH homeostasis that, as in respiratory alkaliphiles, depends upon Na^+. For example, *Exiguobacterium aurantiacum* sustains a cytoplasmic pH that is $\sim$0.3 pH units higher than the starting cytoplasmic pH 8.1 after a shift in external pH to 9.4 (McLaggan *et al.*, 1984). The thermophilic, anaerobic alkaliphile *Clostridium paradoxum* maintains a

ΔpH (acid inside) of as much as 1.3 units during increases of pH from 7.6 to 9.8 (Cook *et al.*, 1996). At present, much less is known about the specific transporters or other factors that help sustain the cytoplasmic pH in the non-respiratory alkaliphiles but this should change as more genomic data is obtained and comparisons with extensively studied respiratory alkaliphiles can be conducted.

The extreme respiratory alkaliphiles whose pH homeostasis has been studied most are *Bacillus* species (Kitada *et al.*, 2000; Krulwich *et al.*, 2007) (Fig. 7B). Extreme alkaliphiles such as *B. halodurans* C-125 and *B. pseudofirmus* OF4 and many other distinct strains, maintain a cytoplasmic pH that is ≥ 2 pH units lower than the external medium (Hamamoto *et al.*, 1994; Goto *et al.*, 2005; Padan *et al.*, 2005; Krulwich *et al.*, 2007). For example, *B. pseudofirmus* OF4 maintains a cytoplasmic pH 8.2–8.3 when growing on malate at pH 10.5 (Guffanti and Hicks, 1991; Sturr *et al.*, 1994) (Fig. 1) and can even do so after an abrupt pH $8.5 \rightarrow 10.5$ shift (Table 1A). This remarkable ability for pH homeostasis is dependent upon Na^+, which cannot be replaced by K^+ as apparently is the case in neutralophilic *E. coli* and *B. subtilis* (Padan *et al.*, 2005; Krulwich *et al.*, 2007) and has been suggested for acidophiles (see Section 3.1). In the pH shift assays, non-fermentable malate is usually present as an energy source to support respiration and antiport. When glucose is added instead, overall homeostasis is not quite as good as with malate but there is a Na^+-independent capacity for modest pH homeostasis that is not observed with non-fermentable malate (i.e., the cytoplasmic pH is kept below 10.5 in the presence of K^+ when glucose but not malate is present during a pH $8.5 \rightarrow 10.5$ shift). This capacity is presumed to reflect metabolic acid generation in the cytoplasm when glucose is present (Table 1A).

The Mrp antiporter is critical for alkaliphily and alkaline pH homeostasis in extremely alkaliphilic respiratory *Bacillus* species. This was shown in *B. halodurans* C-125 which was viable but non-alkaliphilic when *mrp* was mutated (Hamamoto *et al.*, 1994). Thus far, attempts to make deletions in the more extremely alkaliphilic *B. pseudofirmus* OF4 have not succeeded, probably because the deletion is lethal (Ito, unpublished data). As with *B. subtilis*, *B. pseudofirmus* OF4 Mrp forms hetero-oligomeric complexes containing all 7 Mrp proteins (Morino *et al.*, 2008); such complexes include a species that is approximately the expected size of a complex containing one of each protein, similar to the species observed for *B. subtilis* Mrp by Kajiyama *et al.* (2007). In the alkaliphile preparations, there are also larger hetero-oligomers that could be a dimer of a full complex (Morino *et al.*, 2008). It has been hypothesized that the multiple Mrp proteins may have several different transport activities that share synergies and

present a large protein surface to the outside by forming a complex. The Mrp complex surface could be engineered to effectively funnel protons into the antiporter(s) of the complex from a very alkaline medium (Swartz *et al.*, 2005). If a larger size distribution of alkaliphile Mrp complexes compared to neutralophile complexes is confirmed, it could represent an adaptation that promotes adequate proton gathering in the more alkaline milieu. Like *B. subtilis*, alkaliphilic *Bacillus* species also express additional Na^+/H^+ antiporters (Fig. 7B), one of which, NhaC, has been shown to make a small but discernible contributions to pH homeostasis and sodium-resistance (Ivey *et al.*, 1991; Ito *et al.*, 1997). There is also a two-component $K^+(NH_4^+)/H^+$ whose only demonstrated physiological role is in ammonium efflux that prevents interference with pH homeostasis during growth in media with high amine contents (Wei *et al.*, 2003; Fujisawa *et al.*, 2007).

Other aspects of the intersecting proton and sodium cycles of respiratory alkaliphiles are quite distinct from those in neutralophilic *B. subtilis* (Fig. 7A). Oxidative phosphorylation is proton-coupled in all respiratory alkaliphiles in spite of the low-bulk PMF that directly results from pH homeostasis (i.e., the inside-acidic ΔpH detracts from the PMF) (Krulwich, 1995; Krulwich *et al.*, 2007). Some sort of sequestration of protons is widely thought to be used for proton-coupled alkaliphile oxidative phosphorylation, but the relative contributions of specific membrane lipids, particular acidic protein segments near the membrane surface, and specific adaptations in the proton pumps and ATP synthase still need to be defined (Mulkidjanian *et al.*, 2006; Krulwich *et al.*, 2007). All other ion-coupled bioenergetic work, solute transport, and motility are sodium coupled (Ito *et al.*, 2004a; Krulwich *et al.*, 2007; Fujinami *et al.*, 2007b). Use of sodium is observed even among alkaliphiles that live in environments with low sodium concentrations. Its use as the major coupling ion for bioenergetic work is clearly an adaptation to the low PMF that exists at high pH. Even if the sodium concentration is not high, the sodium motive force is generally much higher than the PMF at very high pH. In addition to facilitating solute uptake, the sodium that enters the cells with solutes during symport has been shown to be a major source of the cytoplasmic sodium. This is in turn required to sustain continued antiporter activity in the alkaliphile in support of pH homeostasis (Krulwich *et al.*, 1985b). In the presence of sodium and a solute that can help it cycle, there is no discernible rise in the cytoplasmic pH or growth arrest as seen with neutralophiles directly after a large alkaline shift (Wang *et al.*, 2004; Padan *et al.*, 2005).

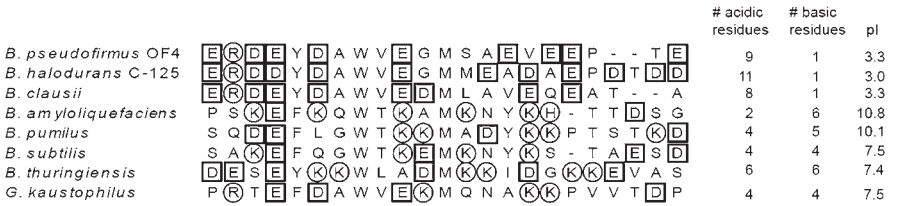
If solutes that enter *B. pseudofirmus* OF4 with sodium are largely omitted from the medium, sodium can re-enter via the sodium-coupled MotPS, in which it is the only stator-force generator for alkaliphile motility

(Ito *et al.*, 2004a), or via a voltage-gated sodium channel shown to be an alkali-activated voltage-gated sodium channel that was named NaChBac (Ren *et al.*, 2001; Ito *et al.*, 2004b) (Table 1B). Since *B. halodurans* C-125 is not genetically accessible, a deletion strain (SC34) of the *ncbA* gene that encodes the *B. pseudofirmus* OF4 homologue, Na_VBP was constructed. The *B. pseudofirmus* OF4 Na_VBP channel is required for full motility, for normal as opposed to inverse chemotaxis, and for fully normal growth at high pH (Ito *et al.*, 2004b; Fujinami *et al.*, 2007a). In pH shift experiments in the media with greatly decreased levels of substrates that enter with sodium, the Na_VBP mutant shows a deficit in pH homeostasis that increases if MotPS is also deleted (Table 1B) (Ito *et al.*, 2004b). The channel co-localizes with the polar chemotaxis receptors (MCPs) and deletion of the channel reduces the polar localization of the channel and vice versa (Fujinami *et al.*, 2007a). The mechanism and nature of the channel effect on chemotaxis will be of interest in other organisms in which channels may also interact with the chemotaxis pathway.

Finally, we note that properties of the membrane and secondary cell wall polymers (SCWPs; see Fig. 7) of alkaliphiles appear to play a role in alkaliphily that still needs more intensive investigation. Alkaliphilic *Bacillus* species have particularly high cardiolipin contents that are highest at very alkaline pH and there is also an interesting complement of neutral lipids (Clejan *et al.*, 1986). For example, it is possible that the high cardiolipin content of alkaliphile membranes fosters closeness of respiratory chain elements with the ATP synthase. Evidence for dynamic physical interaction between the terminal oxidase and ATP synthase of *B. pseudofirmus* OF4 has been shown under conditions in which the two complexes were present together in artificial phospholipid preparations (Krulwich *et al.*, 2007; Liu *et al.*, 2007).

In addition to the membrane lipids, a characteristic of the extreme alkaliphiles is that the hydrophilic loops of membrane proteins that are exposed on the outer surface have a much higher content of acidic residues than their homologues in neutralophiles, thus further increasing the negative charge near the membrane surface (Krulwich *et al.*, 2007). Figure 9 depicts a segment of the CtaC protein of caa_3-type cytochrome oxidase that is close to the outside of the membrane in proximity to the subunit, CtaD, from which pumped protons emerge. In alkaliphilic *Bacillus* species, this region is much more acidic than the same segment of neutralophile homologues (Quirk *et al.*, 1993; Hicks and Krulwich, 1995).

Secondary cell wall teichuronic acids are essential for alkaliphily in *B. halodurans* C-125 (Aono and Ohtani, 1990; Aono *et al.*, 1999). This is not the case with the S-layer polymer (SlpA) of *B. pseudofirmus* OF4. SlpA



	# acidic residues	# basic residues	pI
B. pseudofirmus OF4	9	1	3.3
B. halodurans C-125	11	1	3.0
B. clausii	8	1	3.3
B. amyloliquefaciens	2	6	10.8
B. pumilus	4	5	10.1
B. subtilis	4	4	7.5
B. thuringiensis	6	6	7.4
G. kaustophilus	4	4	7.5

Figure 9 Alkaliphile-specific sequence motif (or two) in the cytochrome oxidase. The protein sequences of subunit II of *Bacillus* sp. cytochrome caa_3 oxidases were aligned using the ClustalW program. Shown is the alignment of the region corresponding to residues 207–227 of the mature *B. pseudofirmus* OF4 protein. Acidic residues are boxed and basic residues are circled. The predicted isolectric point (pI) of the region was taken from the DS gene program. The accession number and residue numbers for each species is as follows (residues are given for the mature protein): *B. pseudofirmus* OF4, Q04441, and 207-227; *B. halodurans* C-125, NP_243481, and 206-228; *B. clausii*, YP_175889 and 205-225; *B. amyloliquefaciens*, YP_001421069 and 216-237; *B. pumilus*, YP_091295 and 216-238; *B. subtilis*, NP_389372 and 216-237; *B. thuringiensis*, YP_896312 and 209-231; and *G. kaustophilis*, YP_146935 and 215-237.

is not essential for growth of *B. pseudofirmus* OF4 at pH 10.5. However, the cells exhibit a distinctly greater lag at pH 10.5 and even more so at pH 11; a deficiency in pH homeostasis by the *slpA* mutant is also observable in pH shift experiments (Table 1A) (Gilmour *et al.*, 2000). A deletion mutant lacking the *slpA* gene grows better than the wild type at pH 7.5. Nonetheless, high levels of the major cell surface polymer are present in both pH 7.5- and 10.5-grown cells (Gilmour *et al.*, 2000). This indicates that the organism is ''hard-wired'' for alkaliphily, ready to support pH homeostasis and grow optimally if there is a sudden alkaline shift even at the expense of growth at near-neutral pH.

3.5. pH Perturbation and Recovery

When growing cells are exposed to environmental pH near the acidic or alkaline limits of their growth range, especially if the transition is abrupt, this is respectively termed ''acid shock'' or ''base shock.'' Under such conditions, the growth rate is suboptimal, presumably due to partial loss of pH homeostasis. Acid or base shock typically leads to up-regulation of gene products that enhance growth under the pH stress condition. Such shock also leads to generation of compounds that increase survival at pH values above or below the pH range for growth. Responses that increase survival

in extreme acid are known as ''acid tolerance'' or ''acid resistance'' (Foster and Hall, 1991; Lin *et al.*, 1995; Foster, 2004). Acid resistance and base resistance are discussed in Section 5.

Acidophiles, particularly extreme acidophiles (growing below pH 3) need to maintain the largest ΔpH of any known organisms. Acidophiles use this large ΔpH across the cytoplasmic membrane to generate ATP, drive proton-coupled solute transporters and, in some cases, energize motility, but re-extrusion of the protons is critical, as noted above. There are relatively few studies of acidophiles subjected to acid shock. One such study of acid-stress of *A. ferrooxidans* (Chao *et al.*, 2008) involved ''acid shock'' as defined here. In this study, an organism that grows optimally at pH 2.3 was exposed to pH 1.3. Interestingly, the acidophile exhibited up-regulation of many of the same key acid-response genes observed in neutralophiles: RpoS and other stress sigma subunits, the Fur iron-acid regulator, the EnvZ/OmpR envelope stress regulator, and a number of OMPs.

Neutralophiles exhibit the same sorts of responses in challenges that are mediated either by direct pH shifts or by mutations in transporters with important roles in pH homeostasis. The SOS response, a prototypic stress response of neutralophilic *E. coli*, is induced by cytoplasmic alkalinization in cells that lack the full complement of active pH homeostasis mechanisms (Schuldiner *et al.*, 1986) and mutants in particular two-component systems of *E. coli*, including an ArcAB deletion strain, show an alkaline-sensitive growth phenotype (Zhou *et al.*, 2003). In neutralophilic *B. subtilis*, alkali shock results in induction of a constellation of genes that significantly overlapped with the σ^W regulon that is involved in the cell wall stress response (Wiegert *et al.*, 2001; Cao *et al.*, 2002).

The actual process of pH perturbation and recovery has been observed with maximal time resolution in *E. coli* and in *Bacillus* species. In well-energized cultures of *E. coli* K-12, a rapid shift of external pH from pH 7.5 to 5.5 causes the cytoplasmic pH to decrease by more than 1.5 units (Fig. 1A, Wilks and Slonczewski, 2007) with recovery beginning within 10 seconds of acid addition. The recovery is biphasic, with a rapid recovery of most of the cytoplasmic pH value occurring within half a minute, followed by more gradual recovery approaching pH 7.4 over the next 4 minutes. By contrast, addition of 20 mM sodium benzoate permanently depresses cytoplasmic pH without recovery. Lower concentrations of benzoate allow partial, slow recovery, without the initial rapid phase. The measurement of pH recovery ultimately should provide clues as to the mechanism. So far, pH recovery rates show no correlation with flux of ions such as K^+ or Na^+ (Wilks and Slonczewski, unpublished data). An alkaline shift of *E. coli* from pH 7.2 to 8.3 results in a rapid and transient

alkalinization during which the cytoplasmic pH briefly reaches the new external pH. Growth arrests after the shift and does not resume until the cytoplasmic pH is about 7.9, about 15 minutes after the shift and complete restoration of the pre-shock cytoplasmic pH is observed about 30 minutes after the shock (Zilberstein *et al.*, 1984). In *B. subtilis*, a general stress response that depends upon σ^B is involved in acid adaptation (Hecker and Volker, 2001; Hecker *et al.*, 2007). As noted by Earl *et al.* (2008), *B. subtilis* strains survive passage through the human gastrointestinal track and acid fermentation conditions. At the alkaline side, *B. subtilis* cells have been shifted from a medium pH 6.3 to either pH 8.8 or 9.0 resulting, respectively, in a growth arrest of about an hour and 5 hours followed by restoration of growth. Shifts to pH 9.3 or higher led to growth arrest that was not reversed after 20 hours (Wiegert *et al.*, 2001).

3.6. Membrane-Permeant Organic Acids and Bases

Membrane-permeant weak acids that primarily cross the cell membrane as the hydrophobic protonated form can depress cytoplasmic pH (Kihara and Macnab, 1981; Salmond *et al.*, 1984; Russell and Diez-Gonzalez, 1998). Their net uptake is driven by ΔpH; thus, at low external pH, a high concentration of protons is released upon deprotonation of the weak acid that is internalized. This can exhaust the cell's buffering and proton export capacity that leads to depression of the internal pH. Low molecular weight organic acids with a pK_a in the range of pH 3–5 are toxic to neutralophiles, and even more so to acidophiles. These organic acids of moderately low pK_a are fully protonated at the low pH typically used for growth of acidophiles. Due to their small size and neutral charge, they easily pass the cytoplasmic membrane; hence the term "permeant weak acids." Once the permeant weak acid is inside the cell, the cytoplasmic pH is above the pK_a for the dissociation of the proton that results in acidification of the cytoplasm (Alexander *et al.*, 1987; Ciaramella *et al.*, 2005). Of the tested organic acids in *A. ferrooxidans*, propionic acid was the least effective at lowering the pH and the chloroacetic acids were the most effective; this difference corresponds with decreasing pK_a values with the time course of the drop in ΔpH over the first 10 minutes (Alexander *et al.*, 1987). A further study of *A. ferrooxidans* demonstrated that Fe^{2+} and sulfur oxidation as well as growth were inhibited by low molecular weight organic compounds including organic acids (Tuttle and Dugan, 1976). It was shown that the monocarboxylic organic acids completely inhibited growth on S^0 whereas other organic acids increased the lag phase

from 1 day in the absence of organic acid to 5–6 days during growth on S^0 but they did not completely inhibit growth (Tuttle and Dugan, 1976). However, some of the inhibitory action was due to direct inhibition of Fe^{2+} oxidation.

Compared to acidophiles, neutralophiles tolerate higher levels of permeant acids, which induce various means of adaptation. The depression of internal pH by permeant acids induces acid resistance genes in the neutralophilic Gram-negative pathogen *S. enterica* (formerly *S. typhimurium*) (Bearson *et al.*, 1998). In *E.* coli, permeant acids such as benzoic acid and acetic acid mediate pH taxis (Kihara and Macnab, 1981; Repaske and Adler, 1981). In lactococci, buildup of lactic acid limits the growth of competing organisms (Hutkins and Nannen, 1993). At low concentration, radiolabeled permeant acids serve as tools to measure internal pH (discussed above). Higher concentrations of a permeant acid that depress internal pH are useful to distinguish cytoplasmic effects from external pH (Slonczewski *et al.*, 1987; Kannan *et al.*, 2008). Exposure to a permeant acid or an uncoupler has been used to select for acid-sensitive mutants of *E. coli* (Slonczewski *et al.*, 1987), *S. enterica* (Foster and Bearson, 1994), and *Mycobacterium smegmatis* (Tran *et al.*, 2005).

The effect of permeant organic acids on cytoplasmic pH is complicated by several other kinds of effects. The most common naturally occurring organic acids are fermentation products, such as lactic, acetic, and formic acids, all of which have specific metabolic roles in cells (Russell and Diez-Gonzalez, 1998). Where ΔpH is maintained, the conjugate base accumulates in the cell in proportion to the ΔpH and specific compounds may elicit specific cellular responses. For example, in *E. coli*, acetate and formate up-regulate expression of completely different protein complements in proteome studies (Kirkpatrick *et al.*, 2001). Even a substance not metabolized by *E. coli*, such as benzoic acid, up-regulates a specific drug resistance regulon, Mar (Rosner and Slonczewski, 1994). In addition, compounds such as benzoic acid and salicylic acid are sufficiently hydrophobic to cross the membrane in the deprotonated form, thus partly collapsing $\Delta\Psi$ as well as ΔpH, and acting as uncouplers, that is, able to equilibrate protons across the membrane, resulting in depletion of the PMF. The degree of uncoupler effect will depend upon the pK_a and the membrane solubility of the compound (Salmond *et al.*, 1984).

Permeant bases cross the membrane in the deprotonated form, picking up protons inside the cell; at high concentration and high external pH, polyamines may raise cytoplasmic pH. In *E. coli*, exogenous spermine enhances bacterial survival in extreme acid, but diminishes survival in extreme base (Yohannes *et al.*, 2005). Production of polyamines can

counteract acidity (see Section 4.4). A number of commonly used biological buffers, such as tris(hydroxymethyl)aminomethane (Tris) and triethanolamine, act as permeant bases, even causing a pH-dependent chemotactic response (Repaske and Adler, 1981). The sensitivity of bacteria to inhibition by permeant bases can be used. Hsieh *et al.* (1998) noted an enormous increase in sensitivity of *Staphylococcus aureus* to cationic and weakly basic antimicrobials at alkaline pH, an effect that was synergistic with mutation of the multi-drug resistance gene *norA*. They suggested that such a strain would be useful for natural product screening.

4. pH CORRECTION MECHANISMS

If an excess of protons and hydroxide ions bypass the cell's pH homeostatic mechanisms and enter the cytoplasm, then the cell requires means by which to ameliorate the potential damage to the cell, and provide tolerance to cytoplasmic pH above or below its optimum. Some of these mechanisms are common between the neutralophiles, acidophiles, and alkaliphiles, such as cytoplasmic buffering that acts either to decrease or increase the cytoplasmic pH to regain optimum pH. Other mechanisms are found in particular species, such as catabolism that eliminates the uncoupling action of organic acids (discussed below).

4.1. Cytoplasmic Buffering

One method by which microorganisms may counteract changes in their internal pH is via the buffering capacity of the cytoplasm, in which pH-titratable cell components sequester or release protons according to the flux in pH. Cytoplasmic buffering molecules include proteins, polyamines, polyphosphates, and inorganic phosphate. Protein amino acid side chains offer potential buffering over a wide range of pH. Polyphosphates as well as inorganic phosphate have pK_a values around 7.2; thus, in principle, they could offer good buffering capacity near the optimal internal pH of neutralophiles. Polyphosphates are involved in many extreme stress adaptations (Seufferheld *et al.*, 2008) including acid exposure of *Burkholderia* (Moriarty *et al.*, 2006). Polyamines are also associated with acid resistance (Wortham *et al.*, 2007). Both polyphosphates and polyamines contribute to biofilm formation, a context in which passive buffering might provide particularly useful protection from pH shift.

Cytoplasmic buffering capacity is challenging to measure; in one example, the buffering capacity of non-growing *E. coli* cells (i.e., cells unlikely to accomplish significant enzymatic consumption of acids or bases) was estimated at 50 mM per pH unit, based on titration of intracellular pH by addition of the permeant acid sodium benzoate (Slonczewski *et al.*, 1982). Subsequently, Zychlinsky and Matin (1983) used the acid titration of unpermeabilized and permeabilized cells, as developed by others (Scholes and Mitchell, 1970; Maloney, 1979), to compare the buffering capacity of metabolically compromised *A. acidophilum* and *E. coli* giving values of 97 and 85 nmol H^+ per mg protein, respectively. Measurement of the cytoplasmic buffering capacity of an acidophilic bacterium (strain PW2) also gave a buffering capacity of 85 nmol H^+ per mg protein from a proton influx of 14.4 nmol H^+ per mg protein (Goulbourne *et al.*, 1986). This amount of proton influx had little effect on the cytoplasmic pH although the anticipated effect would have been reduction of the cytoplasmic pH to 2 in the absence of cytoplasmic buffering capacity.

In a comparative study of acidophilic, neutralophilic, and alkaliphilic *Bacillus* species conducted using the same acid titration methodology on whole and permeabilized cells, the most striking finding was that high pH-grown alkaliphiles exhibited an especially high cytoplasmic buffering capacity at pH 8–9.5, the highest pH used in the study, whereas the highest buffering capacity for *B. subtilis* was at ~pH 5 (Krulwich *et al.*, 1985a). A more refined methodology was subsequently developed by Rius *et al.* (1995) and applied to a group of Gram-negative and later to Gram-positive bacteria (Rius and Lorén, 1998); decay of an acid pulse was measured in a protocol that avoided the use of permeabilized preparations. They confirmed the high cytoplasmic buffering capacity in pH 10.5-grown cells of *Bacillus alcalophilus* and found that pH 8.5-grown cells of the alkaliphile had much lower cytoplasmic buffering, lower than that of neutralophiles *B. subtilis* and *S. aureus.* The cytoplasmic buffering capacity of diverse Gram-negative bacteria was in a comparable range to those found in other studies, in general showing little correlation with growth optima. Cytoplasmic pH buffering is a ubiquitous component of pH homeostasis in microorganisms at all pH levels although the capacity for growth at very high or very low pH does not appear to directly correlate with the level of buffering, consistent with the importance of active mechanisms. Specific buffering molecules of particular importance have not been identified in acidophiles or alkaliphiles; thus there may be many nuanced variations in the poising of cytoplasmic buffering.

Many studies have calculated cytoplasmic buffering capacity (B_i) from the difference between whole intact cell buffering (B_o) and total buffering

of permeabilized preparations (B_t). These studies consistently showed that the buffering of the cell surface is a significant proportion of the total, as expected from the presence of numerous charged components of the outer cell membrane(s), cell wall, and SCWPs; this outer buffering is also likely to be protective and changes in the total surface buffering by increased synthesis of particular polymers is a strategy that can supplement active processes, for example, as with the secondary cell wall teichuronic acid polymers of alkaliphilic *B. halodurans* C-125 (Aono and Ohtani, 1990; Aono *et al.*, 1999).

4.2. Primary Proton Pumps

A key mechanism for acid pH homeostasis is transport of protons out of the cytoplasm by primary proton pumps. Primary proton pumps of acidophile electron transport chains for which descriptions encompassing experimental evidence have been presented include pumps in: *A. ferrooxidans* (Chen and Suzuki, 2005; Ferguson and Ingledew, 2008); "*F. acidarmanus*" Fer1 (Dopson *et al.*, 2005); and a number of thermoacidophilic archaea (reviewed in Schäfer *et al.*, 1999). In addition, primary proton pumps have been identified by sequencing data in *Leptospirillium* group II (*L. ferriphilum*) and *Ferroplasma* type II (Tyson *et al.*, 2004). Based on the ΔpH decrease during anaerobiosis and the addition of a protonophore, it was concluded that the ΔpH is maintained in *B. acidocaldarius* and *Thermoplasma acidiphilum* by active PMF generation (Michels and Bakker, 1985). All of the available genomes from acidophiles that respire have elements that could be involved in PMF generation in support of pH homeostasis. Interestingly, cytochrome *c* oxidase into which the electrons from ferrous iron directly enter in *A. ferrooxidans* exhibits a deviation from the consensus that eliminates one of the two putative proton pathways found in neutralophile homologues from the cytoplasm into the hydrophobic core of the oxidase (Ferguson and Ingledew, 2008). Models have been presented to account for PMF generation in this and related acidophiles. *A. ferrooxidans* also has two putative proton-efflux P-type ATPases (Valdés *et al*., 2008) and putative proton pumps are also found in genomes of *A. caldus* and *A. thiooxidans* (Holmes, unpublished data).

As noted earlier, the F_1F_0-ATP synthase is proton-coupled in non-marine respiratory alkaliphiles in spite of the low-bulk PMF, even though much of the other bioenergetic work of the aerobic alkaliphiles is coupled to sodium (Fig. 7B). The ion-translocating complexes of the respiratory chains of these organisms are proton pumping, for example, the Complex III (bc_1 complex)

and caa_3-type cytochrome oxidase of *B. pseudofirmus* OF4 (Krulwich *et al.*, 2007). Like neutralophilic *B. subtilis*, the alkaliphilic *Bacillus* species for which there are genomic data lack a proton pumping, Complex I type NADH dehydrogenase. For electron input into the respiratory chain, they possess multiple type-II NADH dehydrogenases that carry out transfer of electrons from NADH to (mena)quinone but do not pump ions as well as additional non-pumping electron input enzymes such as succinate dehydrogenase and malate:quinone oxidoreductase (Gilmour and Krulwich, 1996; Liu *et al.*, 2008). There are several indications of a special role for the proton-pumping terminal oxidases of alkaliphiles. In *B. pseudofirmus* OF4, the caa_3-type cytochrome oxidase encoded by the *cta* operon is up-regulated at high pH (Quirk *et al.*, 1993) and disruption of *cta* results in an inability of the alkaliphile to grow non-fermentatively even though alternative terminal oxidases are retained and even up-regulated (Gilmour and Krulwich, 1997). A characteristic noted earlier, that has consistently been observed with respiratory chain components of different alkaliphilic *Bacillus* species, is that many of the species, especially of cytochrome *c*, have been found to have much lower mid-point potentials than their homologues from neutralophiles (Lewis *et al.*, 1981; Yumoto *et al.*, 1991; Hicks and Krulwich, 1995; Goto *et al.*, 2005). Goto *et al.* (2005) and Muntyan and Bloch (2008) reported that the cytochrome *a* mid-point potential in terminal oxidases of alkaliphilic *Bacillus* species is normal and thus the lower than normal mid-point potential of cytochrome *c* species that donate to the terminal oxidase may permit an unusually high capture of energy at this critical segment of the respiratory chain.

4.3. Inorganic Ion Fluxes: Co-Transport and Antiport

Acidophiles use secondary transporters such as ion-coupled co-transporters (symporters) that utilize the ΔpH to energize solute transport (Albers *et al.*, 2001; Ferguson and Ingledew, 2008). In *B. acidocaldarius*, the uptake of a lactose homologue is inhibited by uncouplers that reduce the ΔpH suggesting that lactose is coupled to proton uptake (Krulwich *et al.*, 1978). The uptake of glucose is also suggested to be coupled to proton uptake as its transport collapses the ΔpH by 20% (Matin *et al.*, 1982). The potential importance of K^+ uptake has also been noted, and is reflected in the large number of secondary cation transporters in the sequenced genomes of *Picrophilus torridus* (Fütterer *et al.*, 2004), "*F. acidarmanus*" (http://genome.ornl.gov/microbial/faci/), *S. solfataricus* (She *et al.*, 2001), and *Leptospirillium* group II (Tyson *et al.*, 2004). The *S. solfataricus* genome contains a predicted Trk-like K^+ transporter that might contribute to

production of the inside-positive $\Delta\Psi$, symporters that are expected to use the ΔpH for uptake of sugars and peptides (15 encoded proteins), and high-affinity ABC-type transporters coded by 11 operons that are probably uptake systems as they include both permeases and extra cytoplasmic-binding proteins (She *et al.*, 2001). The genome sequence of *A. ferrooxidans* also contains a large number of secondary transporters including an Na^+/H^+ antiporter, several K^+ transporters including a voltage-gated channel, K^+ uptake and efflux proteins, and two copies of an ABC K^+ import system (Valdés *et al.*, 2008). The *H. pylori* homologue of the *E. coli* NhaA Na^+/H^+ antiporter has been extensively studied and found to have lower pH range for activity than *E. coli* NhaA, a range that suggests a role for the antiporter at the upper edge of its range of pH for growth (Inoue *et al.*, 1999). Perhaps the *A. ferrooxidans* Na^+/H^+ antiporter provides protection against "alkali shock" or against the increased toxicity of cytoplasmic Na^+ when the organism finds itself above its very acidic growth range. The establishment of roles for the other cation transporters and the special contribution of K^+ in particular, requires further investigation in the acidophiles.

In respiratory alkaliphilic *Bacillus* species, the only process other than oxidative phosphorylation that requires proton uptake from the highly alkaline external *milieu* by these alkaliphiles is cytoplasmic pH homeostasis itself, since it is dependent upon active inward transport of protons by antiporters (Hamamoto *et al.*, 1994). For the alkaliphilic *Bacillus* species, use of the Mrp complex as the major antiporter has been hypothesized to provide a large protein surface on the outside that could be adapted to act as a proton-concentrating and funneling element to support antiport-based pH homeostasis (Swartz *et al.*, 2005; Morino *et al.*, 2008). However, the other ion-dependent bioenergetic work in the alkaliphilic *Bacillus* species is driven by a sodium motive force established by the vigorous efflux due to Na^+/H^+ antiport activities (see Fig. 7B). The sodium motive force is constituted by an inwardly directed Na^+ gradient and the large chemiosmotically productive $\Delta\Psi$ (inside negative); it is much larger than the PMF because it is not adversely affected by the acid-inside ΔpH. Both ion-coupled solute transport and motility of the extreme alkaliphiles is coupled to sodium, even when growth is at near-neutral pH and it has been noted that these processes of extreme alkaliphiles require higher sodium ion concentrations at near-neutral pH than at high pH (Gilmour *et al.*, 2000; Fujinami *et al.*, 2007b). This apparently reflects an optimization of the sodium-coupled processes of alkaliphiles to function at high pH. The optimization is apparently accompanied by an inhibitory effect of the increased proton concentration at near-neutral pH. Other, more moderate alkaliphiles and alkali-adaptable neutralophiles either possess distinct sodium- and

proton-coupled transporters and motility channels (see Fig. 7B) or have transporters or motility channels that can use either a sodium motive force or a PMF, depending upon the conditions (Pourcher *et al.*, 1995; Franco and Wilson, 1996; Terahara *et al.*, 2008). The pH-dependent sodium versus proton preference of a bi-functional motility channel of alkaliphilic *Bacillus clausii* can be altered in either direction by pairs of mutations in the MotB component of the channel (Terahara *et al.*, 2008).

Genomes from aerobic alkaliphiles are predicted to encode a large number of ATP-dependent transporters, especially ABC-type transporters, as well as ion-coupled transporters (Takami *et al.*, 2000). In non-respiratory alkaliphiles, several P-type ATPases have been intensively studied (Suzuki *et al.*, 2005). In view of the prediction by Niggli and Sigel (2007) that antiport is a mechanistic feature of P-type ATPases it would be interesting to probe whether P-type ATPases that pump Na^+ contribute to pH homeostasis by taking up H^+.

4.4. Production and Consumption of Acids and Bases

As noted in Section 3.3, acidophiles are especially sensitive to uncoupling by organic acids and their production during fermentation as terminal electron acceptors would be highly toxic to the cell. It is possibly due to this that no fermentative acidophiles have been identified. It is also interesting to note that the most extreme acidophiles such as *Ferroplasma* spp. are chemoorganotrophs (Dopson *et al.*, 2004) that are capable of metabolizing organic acids and thus, ameliorating their toxic effects. Due to the fact that the acidophiles are able to gain organic carbon and energy from the organic acids, it is impossible to discern if the expression of proteins for their degradation is a pH homeostasis response. Examples of genes involved in organic acid degradation are found in acidophile genomes including *P. torridus* (Angelov and Liebl, 2006) and "*F. acidarmanus*" Fer1 (http://genome.ornl.gov/microbial/faci/). The enzymes encoded include propionyl-CoA synthase, two acetyl-CoA synthetases, and lactate-2-monooxygenase that convert lactate to pyruvate (Ciaramella *et al.*, 2005).

In neutralophilic heterotrophs, a large number of different amino acid decarboxylases and related degradative enzymes are up-regulated at low pH (Table 2). These enzymes offer the opportunity to remove a proton from the cytoplasm through outward diffusion of CO_2 while retaining an alkaline product such as an amine, or exporting it through a cognate transporter (Fig. 6). In most cases, the decarboxylase is co-expressed with an antiporter that exchanges the substrate with a decarboxylated product,

Table 2 Enzymes consuming acids or bases.

Enzyme substrate	Gene(s)	Organism(s)	Reference(s)
Decarboxylases and other enzymes up-regulated at low pH			
2-Acetolactate	*alsD*	*B. subtilis*	Wilks *et al.* (2009)
Agmatine (deiminase)	*aguA1*	*Lactococcus brevis*	Lucas *et al.* (2007)
Arginine	*speA*	*E. coli*, *B. subtilis*	Gong *et al.* (2003), Stim and Bennett (1993), Wilks *et al.* (2009)
Arginine (deiminase)	*arcA*	*Streptococcus* spp., *Lactococcus* spp.	Dong *et al.* (2002), Cotter and Hill (2003)
CO_2 (carbonic anhydrase)	*aac*, *aphA*	*H. pylori*	Bury-Moné *et al.* (2008)
Glutamate	*gadA*, *B*	*E. coli, L. lactis*	Castanie-Cornet *et al.* (1999), Nomura *et al.* (1999), Richard and Foster (2004)
Glycine	*gcvPA*, *PB*	*B. subtilis*	Wilks *et al.* (2009)
Histidine		*Lactococcus* spp.	Fernández and Zúñiga (2006)
Lysine	*cadA*	*E. coli*	Auger and Bennett, 1989, Watson *et al.* (1992)
Malate	*maeA*	*B. subtilis*	Wilks *et al.* (2009)
Ornithine	*speF*	*E. coli*	Kashiwagi *et al.* (1992)
Oxalate	*yvrK*	*B. subtilis*	Tanner and Bornemann (2000)
Phosphatidylserine	*psd*	*B. subtilis*	Wilks *et al.* (2009)
Tyrosine	*tyrDC*	*Lactococcus* spp.	Fernández and Zúñiga (2006), Wolken *et al.* (2006)
Urea (urease)	*ureA*	*Helicobacter pylori, Yersinia enterocolitica*	Stingl *et al.* (2002), Young *et al.* (1996)
Deaminases up-regulated at high pH			
Arginase (removes urea)	*rocF*	*B. subtilis*	Wilks *et al.* (2009)
Serine	*sdaA*	*E. coli*	Yohannes *et al.* (2004)
Tryptophan, cysteine, and serine	*tnaA*	*E. coli*	Blankenhorn *et al.* (1999), Bordi *et al.* (2003)

under regulation of one or more regulators sensitive to external pH and anaerobiosis (Meng and Bennett, 1992a, b). The net result of these decarboxylases is release of CO_2 and an amine, with net removal of a proton from the system. For example, in the case of lysine decarboxylase,

cadAB, low external pH is detected by the regulator *cadC* (Slonczewski *et al.*, 1987; Meng and Bennett, 1992a, b; Neely *et al.*, 1994; Neely and Olson, 1996). CadC is a fused ToxR-type two-component regulator whose extracellular domain detects pH (Dell *et al.*, 1994). Through CadC, low pH induces expression of both the lysine decarboxylase (CadA) and the lysine-cadaverine antiporter (CadB). The decarboxylase converts lysine into CO_2 and cadaverine, a basic polyamine. The polyamines generated by amino acid decarboxylases have an alkalinizing effect that may raise the external pH. In addition, they block OMPs thus retarding the influx of some organic permeant acids (Pagès *et al.*, 2008).

Different species express different sets of acid-dependent decarboxylases. *E. coli* expresses the catabolic decarboxylases for lysine and arginine (Stim and Bennett, 1993), ornithine (Kashiwagi *et al.*, 1992), and glutamate (Hersh *et al.*, 1996; Castanie-Cornet *et al.*, 1999). Expression is maximal anaerobically at low pH. Lactococci express the glutamate and arginine systems (Nomura *et al.*, 1999; Cotter and Hill, 2003) as well as decarboxylases for tyrosine, histidine, and agmatine (Cotter and Hill, 2003; Fernández and Zúñiga, 2006). In *B. subtilis*, proteins up-regulated at low pH include decarboxylases for arginine, 2-acetolactate, glycine, malate, phosphatidylserine (Wilks *et al.*, 2009), and oxalate (Tanner and Bornemann, 2000).

The assessment of metabolic contributions to pH homeostasis is complicated by the fact that these systems are subject to multiple layers of regulation. For instance the lysine decarboxylase and cognate lysine-cadaverine antiporter are co-induced by acid, anaerobiosis, and lysine (Meng and Bennett, 1992a, b). The Gad system, including glutamate decarboxylase and antiporter plus an associated acid fitness island (Mates *et al.*, 2007) are induced by the stationary-phase RpoS regulon at low pH under aerobic conditions (Lin *et al.*, 1995; Masuda and Church, 2003; Foster, 2004), but RpoS also enables Gad induction at high pH under anaerobiosis (Hersh *et al.*, 1996). In *H. pylori*, the urease extreme-acid protection system is maximally expressed in the presence of urea, nickel, and low pH (Slonczewski *et al.*, 2000; van Vliet *et al.*, 2001).

In most cases the actual contribution of acid-up-regulated metabolism to pH homeostasis has been difficult to demonstrate in growing cells, because such a large number of systems are available. However, the requirement for several specific decarboxylases has been documented for pH homeostasis during non-growth survival (see Section 5.1). Other enzymes that provide protection against acid during bacterial survival at extreme low pH include urease and carbonic anhydrase that function in *H. pylori* as discussed earlier, and that are also used by

bacteria that only survive but do not colonize the acid regions of the gut (see below).

At high external pH, neutralophiles tend to catabolize amino acids and related substrates by different pathways that release ammonia rather than CO_2 (Blankenhorn *et al.*, 1999; Bordi *et al.*, 2003; Yohannes *et al.*, 2004; Hayes *et al.*, 2006). Like the decarboxylases, the amino acid deaminases show enhanced expression under anaerobiosis when the absence of oxygen limits metabolic options. The tryptophan deaminase *tnaA* is of particular interest as it catabolizes serine and cysteine as well as tryptophan. The base-up-regulated *roc* pathway of *B. subtilis* generates two or three NH_3 plus TCA cycle acids, differing markedly from the acid-up-regulated *arc* pathway of streptococci (Chen *et al.*, 2002) which generates one NH_3 plus polyamines. The alkali-adaptive activities of the *B. subtilis roc* pathway are consistent with its induction in *tetL*-deficient mutants (Wei *et al.*, 2006). Nevertheless, some ambiguous cases remain to be explained; for example, glutamate decarboxylase is also induced at high pH, particularly under anaerobiosis and stationary phase (Blankenhorn *et al.*, 1999; Hayes *et al.*, 2006). The effects of amino acid catabolism at high pH are poorly characterized.

Acid and base modulate several other pathways of anaerobic catabolism. In *Leuconostoc lactis*, low pH shunts fermentation to the neutral product acetoin (Cogan *et al.*, 1981) and in *Lactobacillus plantarum* acetoin production is associated with improved pH homeostasis (Tsau *et al.*, 1992). In *B. subtilis*, low pH favors production of lactate (Schilling *et al.*, 2007). *E. coli* limits internal acidification by producing lactate instead of acetate plus formate (Bunch *et al.*, 1997) and by conversion of formate to H_2 and CO_2 (Rossman *et al.*, 1991). Low pH up-regulates several hydrogenases, which interconvert protons with H_2 (Hayes *et al.*, 2006). Low pH appears to enhance catabolism of sugar derivatives whose fermentation minimizes acid production, including sorbitol, glucuronate, and gluconate (Hayes *et al.*, 2006). Above pH 7, the favored fermentation products are acetate and formate (Wolfe, 2005) and the favored pathways of sugar catabolism are those related to glucose (Hayes *et al.*, 2006). High pH also up-regulates amino acid deaminases that remove ammonia and direct carbon into the TCA cycle, such as tryptophan deaminase and serine deaminase (Stancik *et al.*, 2002).

5. pH HOMEOSTASIS UNDER NON-GROWTH CONDITIONS

The ability of microorganisms to remain viable during conditions not permitting growth is essential for persistence in a changing environment.

Many neutralophiles possess inducible means of maintaining limited pH homeostasis for several hours under ''extreme'' pH conditions, enabling survival below pH 3 or above pH 10. Such microorganisms are commonly said to be ''acid resistant'' or ''base resistant,'' respectively. The phenomena of extreme-acid survival in neutralophiles are highly important for microbial ecology, such as nitrogen-fixing rhizobia (Dilworth *et al.*, 1999; Tiwari *et al.*, 2004), as well as for human and animal pathogens such as *E. coli* O17:H7 (Lin *et al.*, 1996) and *Yersinia entericolitica* (de Koning-Ward and Robins-Browne, 1995). In extreme base, survival of pathogens such as *Listeria monocytogenes* is important for food treatment (Giotis *et al.*, 2008).

5.1. Extreme Acid: pH Homeostasis Without Growth

The terminology for various conditions of extreme-acid survival has shifted over the years (Gorden and Small, 1993; Slonczewski and Foster, 1996; Foster, 2004). Generally, the term ''acid survival'' or ''acid resistance'' refers to the ability of a substantial portion of cells ($>10\%$) to retain colony-forming potential at pH 7 following 2–4 hours exposure at an external pH value below pH 3. Acid resistance factors may be constitutive, such as the low H^+ conductance of membranes, or the buffering capacity of the cell, or the presence of constitutive ion transporters. Alternatively, acid resistance may be increased under various environmental conditions such as stationary phase, mediated by the RpoS regulon. Acid resistance ''inducible'' by growth in moderate acid is also called ''acid tolerance.'' The distinction between constitutive and inducible is blurred, however, as (1) ''constitutive'' acid resistance may turn out to require unidentified inducible factors; (2) ''inducible'' acid resistance of a laboratory strain may turn out to be constitutive in wild strains or clinical isolates; and (3) the transition between the ranges of growth and survival may be unclear, and may be strain-dependent.

A growing number of metabolic systems are known to help bacteria survive at pH below their growth range (Young *et al.*, 1996; Richard and Foster, 2003; Foster, 2004; Sachs *et al.*, 2005). Some of these systems are acid-independent, such as the RpoS-dependent acid tolerance response of *E. coli*, whereas others such as the Gad regulon are induced during growth at low pH and/or by other factors such as anaerobiosis. Some components help raise the pH homeostasis; whereas others help cells cope with lower pH for example by chaperones removing misfolded proteins. Here we focus

on systems that enhance pH homeostasis while maintaining a relatively large ΔpH.

In *E. coli*, several metabolic systems governed by overlapping regulons contribute to acid resistance. The stationary-phase expression of RpoS confers acid resistance through a poorly understood mechanism involving cAMP activation and the F_0F_1-ATP synthase (Castanie-Cornet *et al.*, 1999; Ma *et al.*, 2003; Richard and Foster, 2004). RpoS also enhances expression of the glutamate-dependent Gad system (Lin *et al.*, 1995; Richard and Foster, 2004). The glutamate decarboxylases (GadA and GadB) and the glutamate-2-ketoglutarate antiporter (GadC) consume protons through production of polyamines and CO_2, enabling *E. coli* to survive for many hours at pH 2.5 (Fig. 10). Survival at pH 2.5 in minimal medium requires the presence of glutamate. Analogous acid survival mechanisms have been demonstrated for arginine and lysine (Lin *et al.*, 1995; Gong *et al.*, 2003; Iyer *et al.*, 2003). Besides the polyamines that buffer pH, the CO_2 generated by decarboxylases and other metabolism enhances acid resistance by an unknown mechanism (Sun *et al.*, 2005). RpoS also amplifies the acid induction of cycopropane fatty acid synthesis, which increases survival in extreme acid (Chang and Cronan, 1999; Kim *et al.*, 2005).

The *E. coli* survival mechanism at pH 2.5 involving glutamine or arginine (and possibly lysine) requires maintenance of cytoplasmic pH at a minimum of pH 4, with the ΔΨ inverted (inside positive) (Iyer *et al.*, 2002; Richard and Foster, 2004). The source of the inside-positive ΔΨ remains unclear, although the ClC-type proton-chloride antiporter may play a role (Fig. 10). The inverted ΔΨ enables cells to sustain a ΔpH for an extended period without ongoing energy expenditure. The ClC H^+/Cl^- antiporter may also help the cell recover its inside-negative ΔΨ after the external pH recovers above a critical acid threshold, at about pH 4–5. Thus, cytoplasmic pH homeostasis in extreme acid extends strategies used during growth in moderate acid, such as the amino acid decarboxylases, while adding a mechanism found in extremophiles, the inversion of ΔΨ.

An analogous form of acid resistance is also exhibited by *H. pylori*, which can survive below its growth range of gastric acid by maintaining a cytoplasmic pH ~4.9 at an outside pH ~1.3 (Stingl *et al.*, 2001). As discussed above for growth of *H. pylori* (Section 3.2), survival below external pH 2 requires uptake of urea into the cytoplasm by UreI, cytoplasmic urease and carbonic anhydrase and a periplasmic carbonic anhydrase to maintain a periplasmic pH that is much higher than the external pH and a cytoplasmic pH that is even higher (Fig. 8) (Marcus *et al.*, 2005; Stähler *et al.*, 2005; Bury-Moné *et al.*, 2008). At pH 2, these mechanisms may support sufficiently robust pH homeostasis to ensure

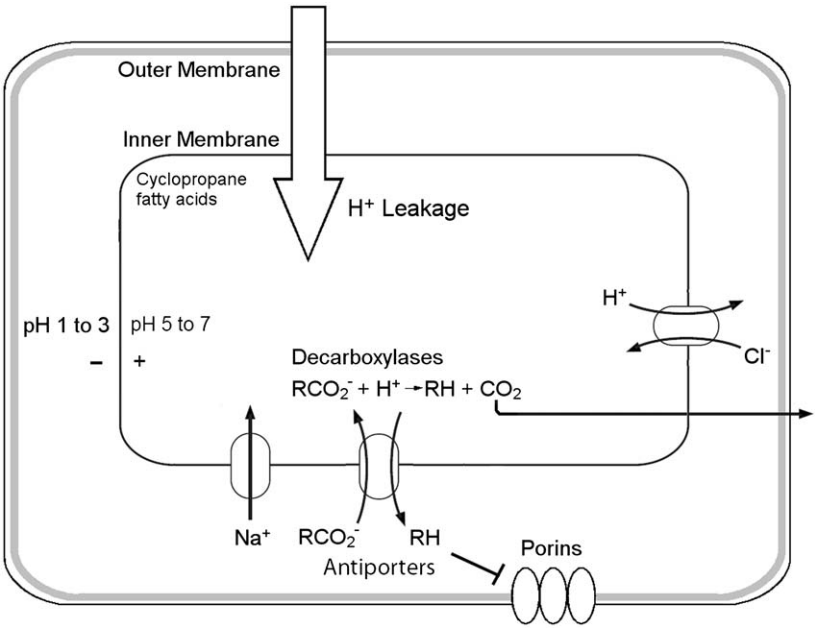


Figure 10 Acid resistance mechanisms in *E. coli* surviving at pH values below its growth range. Proton leakage into the cell is limited by the inverted $\Delta\psi$ (inside positive). The ClC chloride-proton antiporter may help cells restore the inside-negative $\Delta\psi$ following neutralization of external acid (Iyer *et al.*, 2002). Sodium transport contributes to the inside-positive charge. Decarboxylases of glutamate, arginine, and lysine consume protons, exporting amines plus CO_2 (Iyer *et al.*, 2002; Foster, 2004; Richard and Foster, 2004). CO_2 enhances acid resistance by an unknown mechanism (Sun *et al.*, 2005). CFAs in the inner membrane increase acid resistance, possibly by lowering proton conductance (Chang and Cronan, 1999).

survival with growth arrest, while they can support colonization and growth in somewhat less extremely acidic surface of the gut.

Acid-induced urease similarly enhances the acid resistance of enteric pathogens *Yersinia enterocolitica* and *Morganella morganii* (Young *et al.*, 1996). Like *H. pylori*, *Y. enterocolitica* employs urease to survive passage through the very acidic stomach; it differs from *H. pylori* in that it does not colonize a very acidic niche, but moves on to colonize the more hospitable intestine (de Koning-Ward and Robins-Browne, 1995, 1997; Young *et al.*, 1996). The cytoplasmic urease of *Y. enterocolitica* is activated 780-fold by low-pH conditions and exhibits a lower pH optimum than most ureases, in a range of 4.5–5.5 (Young *et al.*, 1996; De Koning-Ward and

Robins-Browne, 1997). This is lower than the pH optimum for the *H. pylori* urease, consistent with the *Yersinia* use of urease for survival rather than to facilitate growth (Sachs *et al.*, 2006). Mutants of *Y. enterocolitica* with a disrupted urease gene exhibited a 1,000-fold decrease in acid survival *in vitro* relative to wild type and a 10-fold reduction in viability after passage through the stomach of mice (de Koning-Ward and Robins-Browne, 1995).

Acid resistance mechanisms, both constitutive and acid-induced, are now known to be widespread among neutralophiles. Other examples not discussed above include the enteric organisms *Shigella flexneri* and *S. enterica* (Gorden and Small, 1993; Lin *et al.*, 1995); *Lactobacillus brevis*, involving agmatine deiminase and tyrosine decarboxylase (Lucas *et al.*, 2007); *L. lactis*, involving chloride transport (Sanders *et al.*, 1998) as well as mycobacteria, staphylococci, and other Gram-positive organisms (reviewed by Cotter and Hill, 2003). In some bacteria, acid resistance requires increased tolerance to metals. Adaptation for acid survival of *Lactobacillus bulgaricus* includes up-regulation of metal-transporting CPX-Type ATPases, believed to contribute to copper homeostasis (Penaud *et al.*, 2006). Similar protection from copper toxicity contributes to acid resistance of *Rhizobium leguminosarum* and *Sinorhizobium meliloti* (Reeve *et al.*, 2002).

Like the neutralophiles, even extreme acidophiles need to survive in environments at extreme pH values below their growth range, such as Iron Mountain, CA where pH values have been measured as low as pH −3.6, termed ''super acids'' (Nordstrom and Alpers, 1999). The reporting of negative pH values is controversial as by the classical definition, only pH values between 1 and 13 are possible and a new model based on sulfuric acid solutions is used (Nordstrom and Alpers, 1999). The extremely low-pH mine waters are formed due to the rock consisting of 95% pyrite, the temperature and humidity being near optimal for net proton producing acidophile catalyzed mineral dissolution, concentration of protons by evaporation, and the formation of soluble, efflorescent salts containing acidity that can be re-dissolved during periods of higher water levels whereby, the acidity is released (Nordstrom *et al.*, 2000).

Microorganisms are found in the Iron Mountain site at pH values ranging from 0 to $\approx$2.5 (Robbins *et al.*, 2000) including eukarya, bacteria, and archaea (Edwards *et al.*, 1999). Based on fluorescent *in situ* hybridization, ''*F. acidarmanus*'' Fer1 constituted up to 85% of the population with low numbers of *Leptospirillium* spp. at a pH 0.3–0.7 area of the mine suggesting the microorganisms were active and therefore, able to balance their internal pH (Bond *et al.*, 2000; Edwards *et al.*, 2000). At a second extremely acidic site between pH 0.6 and 0.8, *Ferroplasma* spp. constituted

52% of the microbial population once again with low numbers of *Leptospirillium* spp. (Espana *et al.*, 2008). "*F. acidarmanus*" Fer1 (Edwards *et al.*, 2000), *Ferroplasma thermophilum* (Zhou *et al.*, 2008), *Ferroplasma cupricumulans* (Hawkes *et al.*, 2006), and other species from the genus *Picrophilus* (Schleper *et al.*, 1995) are capable of growth at between pH 0 and 0.4. Although "*F. acidarmanus*" Fer1 cannot grow at pH −2, it has been shown to survive at this pH though it cannot survive at pH −3.6 (Edwards, personal communication). A study of the extremely acidophilic archaeon *P. oshimae* cytoplasmic pH shows that it has an internal pH around 4.6 up to an external pH 4.0, and above this value the cells rapidly lysed (van den Vossenberg *et al.*, 1998b). The ΔpH of *P. oshimae* was found to be greater than 4 pH units, rapidly declining as the external pH is raised, since internal pH remains constant (van den Vossenberg *et al.*, 1998b). Over the same external pH range, the $\Delta\Psi$ decreases from 100 to 45 mV giving a PMF of −175 mV at pH 1.0 that decreases upon increasing external pH. Therefore, the study suggests that extreme acidophiles utilize similar pH homeostatic mechanisms and, as a consequence of their low internal pH, their enzymes may show unusually low optimum pH (Golyshina *et al.*, 2006).

5.2. Extreme Base: pH Homeostasis Without Growth

Survival of neutralophiles in extreme base has been less studied than acid survival. Interestingly, in *E. coli* base survival requires RpoS, as does acid survival (Small *et al.*, 1994; Bhagwat *et al.*, 2006). Survival of an RpoS-positive strain at pH 10 requires prior growth to stationary phase at pH 8. In *L. monocytogenes*, an alkali-inducible base resistance has been demonstrated, involving glutamine and phosphate transporters, among other components (Giotis *et al.*, 2008). The role of cytoplasmic pH homeostasis in these cases has not been characterized.

5.3. Biofilms

An intriguing question for further study is the role of pH homeostasis in biofilms. In principle, biofilm formation should enhance pH homeostasis since the ratio of cytoplasmic to extracellular volume is increased by the close proximity of cells. The increased relative volume of cytoplasm should offer greater opportunities for pH maintenance, while also posing greater challenges for expulsion of excess acids and bases. Biofilms are associated with various kinds of stress protection, including acid resistance. In the oral

bacterium *Streptococcus mutans*, surface adhesion and biofilm development enhance survival at pH 3–3.5 (Zhu *et al.*, 2001; Welin *et al.*, 2003; McNeill and Hamilton, 2004; Welin-Neilands and Svensäter, 2007). In oral biofilms, arginine catabolism to polyamines may contribute to caries formation (Burne and Marquis, 2000). Maintenance of pH homeostasis is important for *S. mutans* biofilms under acid stress. Biofilm formation may also contribute to acid resistance of *H. pylori* (Stark *et al.*, 1999). Biofilms of nitrifying bacteria appear to balance acid-producing with acid-consuming forms of metabolism (Gieseke *et al.*, 2006).

In extremely acidic environments, acidophiles often exist as biofilms such as the "*F. acidarmanus*" Fer1 dominated site at Iron Mountain, CA (Edwards *et al.*, 2000) (discussed in Section 5.2) and the *Acidithiobacillus* spp. dominated snottites in the Frasassi cave system, Italy (Macalady *et al.*, 2007). Further analysis of the approximately pH 1 Iron Mountain biofilms revealed various defined stratified structures with the less acid-tolerant *Leptospirillium* group II in a dense layer at the bottom of the biofilm and the archaea in the surface areas in a mature pool pellicle biofilm (Wilmes *et al.*, 2008). However, a community proteomics analysis of an Iron Mountain biofilm did not reveal any particular pH homeostatic mechanisms (Ram *et al.*, 2005). The pH 0–1 environment in the Frasassi cave system is generated by H_2S oxidation and is extremely low for the acidithiobacilli identified that habitually inhabit environments around pH 2–3 (Macalady *et al.*, 2007). The acidic biofilms were much lower in diversity than in neutral areas of the cave system and they are one of the simplest biofilm communities known. The biofilm was unusual in that it was dominated by bacteria rather than archaea that are usually prevalent at very low-pH values and this may be reflected by the electron donor being sulfide (and the lack of Fe^{2+}) that is less commonly oxidized by the extremely acidophilic archaea. That the bacteria were growing in a biofilm may provide some degree of protection against the low pH in the cave.

6. CONCLUSIONS

The balance of protons and hydroxyl ions is of universal importance for microbial growth and for survival outside the growth range of pH. Bacteria and archaea adapted to acid maintain a cytoplasmic pH higher than that of the exterior, whereas those growing in base maintain a lower pH (Fig. 1). To maintain cytoplasmic pH at the more neutral value, cells accept a substantial energy loss through inversion of $\Delta\psi$ in extreme acid and

expenditure of $\Delta\psi$ in extreme base. Intriguingly, over the range of microbes studied, the crossover point where cytoplasmic pH equals external pH lies between pH 7–8, although not all species can actually grow at this point.

The observation and study of microbial pH is challenging for several reasons. Observation of pH within the cell requires use of probes labeled by radioactivity, fluorescence, or NMR, all of which can only be used for particular ranges of pH and culture conditions. Numerous organic and inorganic molecules within the cell can generate or consume protons. Multiple mechanisms contribute to cytoplasmic pH, often preventing isolation of mutants with a defective phenotype. Cells include various compartments such as periplasm and forespore whose pH may or may not equal that of the cytoplasm.

The mechanisms of pH homeostasis are manifold and often redundant; thus, multiple K^+ and Na^+ transporters contribute ion fluxes that balance proton flow, and multiple catabolic enzymes consume and generate protons. Membrane and envelope adaptations are most pronounced in acidophiles, although lipid and porin adaptations are also seen in neutralophiles and alkaliphiles. While diverse mechanisms predominate in particular species, such as urease/carbonic anhydrase-dependent protection from acid in *H. pylori* and Na^+/H^+ antiporters in alkaliphilic *Bacillus* species, general classes of metabolic, transport flux, and membrane-based mechanisms appear throughout the range of pH-adapted microorganisms. Thus, the study of pH homeostasis in any given species may yield clues as to mechanisms in very different species.

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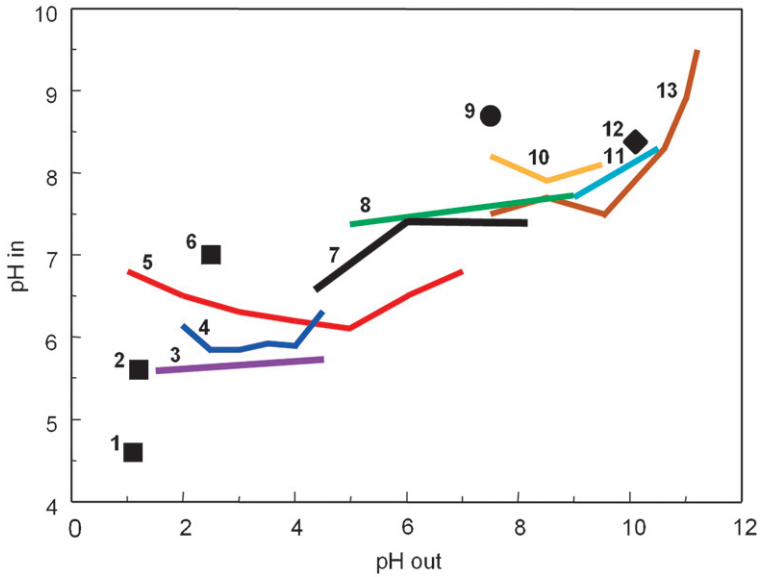

Plate 1 Cytoplasmic pH as a function of the external pH among acidophiles (■), neutralophiles (●), and alkaliphiles (♦). Acidophiles: (1) *Picrophilus torridus* (Fütterer *et al.*, 2004); (2) *Ferroplasma acidarmanus* (Baker-Austin and Dopson, 2007); (3) *Acidiphilium acidophilum* (dark purple; external pH 1–4.5; Matin *et al.*, 1982); (4) *Bacillus acidocaldarius* (dark blue; pH 2–4.5; Krulwich *et al.*, 1978); (5) *Acidithiobacillus ferrooxidans* (red; pH 1–7; Cox *et al.*, 1979); and (6) *Acidithiobacillus thiooxidans* (Baker-Austin and Dopson, 2007). Neutralophiles: (7) *Bacillus subtilis* (black; pH 4.5–8; Shioi *et al.*, 1980); (8) *Escherichia coli* (green, pH 5–9; Slonczewski *et al.*, 1981); and (9) *Bacillus licheniformis* (Hornbæk *et al.*, 2004). Alkaliphiles: (10) *Bacillus cohnii* (orange; pH 7.5–9.5; Sugiyama *et al.*, 1986); (11) *Bacillus pseudofirmus* RAB (turquoise; pH 9–10.5; Kitada *et al.*, 1982); (12) *Bacillus alcalophilus* (Hoffmann and Dimroth, 1991); and (13) *B. pseudofirmus* OF4 (brown; pH 7.5–11.2; Sturr *et al.*, 1994). (For b/w version, see page 4 in the volume.)

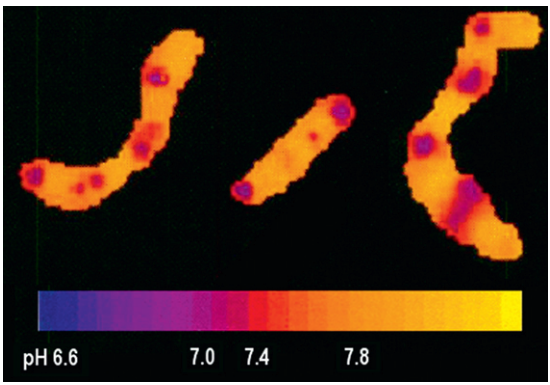

Plate 2 Fluorescent ratio image analysis of intracellular pH in sporulating *Bacillus megaterium.* Sporulating cells were loaded with BCECF-AM. Fluorescence ratios were measured and converted to pseudocolor images calibrated to pH as shown. Bacteria appear as chains of four to eight cells, in which the forespore components show lowered pH. The figure was adapted from Fig. 4 of Magill *et al.* (1994) with permission from American Society for Microbiology. (For b/w version, see page 15 in the volume.)

Physiology of Mycobacteria

Gregory M. Cook[1], Michael Berney[1], Susanne Gebhard[1], Matthias Heinemann[2], Robert A. Cox[3], Olga Danilchanka[4] and Michael Niederweis[4]

[1]*Department of Microbiology and Immunology, Otago School of Medical Sciences, University of Otago, Dunedin, New Zealand*
[2]*Institute of Molecular Systems Biology, ETH Zürich, Switzerland*
[3]*Division of Mycobacterial Research, National Institute for Medical Research, London, UK*
[4]*Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL, USA*

ABSTRACT

Mycobacterium tuberculosis is a prototrophic, metabolically flexible bacterium that has achieved a spread in the human population that is unmatched by any other bacterial pathogen. The success of *M. tuberculosis* as a pathogen can be attributed to its extraordinary stealth and capacity to adapt to environmental changes throughout the course of infection. These changes include: nutrient deprivation, hypoxia, various exogenous stress conditions and, in the case of the pathogenic species, the intraphagosomal environment. Knowledge of the physiology of *M. tuberculosis* during this process has been limited by the slow growth of the bacterium in the laboratory and other technical problems such as cell aggregation. Advances in genomics and molecular methods to analyze the *M. tuberculosis* genome have revealed that adaptive changes are mediated by complex regulatory networks and signals, resulting in temporal gene expression coupled to metabolic and energetic changes. An important goal for bacterial physiologists will be to elucidate the physiology of *M. tuberculosis* during the transition between the diverse conditions encountered by *M. tuberculosis*. This review covers the growth of the mycobacterial cell and

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how environmental stimuli are sensed by this bacterium. Adaptation to different environments is described from the viewpoint of nutrient acquisition, energy generation, and regulation. To gain quantitative understanding of mycobacterial physiology will require a systems biology approach and recent efforts in this area are discussed.

ABBREVIATIONS

ABC	ATP-binding cassette
ECF	extracytoplasmic function
EHR	enduring hypoxic response
EMP	Embden–Meyerhof–Parnas
PCR	polymerase chain reaction
GS	glutamine synthetase
SulP	sulfate permease
STPKs	serine/threonine protein kinases

It is now 100 years since the first Mycobacterium *was isolated by Hansen (1874). Somewhat ironically, this was the leprosy bacillus,* Mycobacterium leprae*, which even today is still resisting all attempts to cultivate it in the laboratory. The tubercle bacillus,* M. tuberculosis *was*

not discovered until eight years later (Koch, 1882) and this has remained an object of intensive investigation ever since. The widespread interest in the mycobacteria of course stems from the diseases they cause and, lest it be imagined that tuberculosis is a disease which has now been largely conquered and that leprosy is of relatively rare occurrence, current estimates for the number of cases of tuberculosis and leprosy in the world today are 20,000,000 and 11,000,000, respectively (Bechelli and Dominguez, 1972). The annual estimated mortality rate is equally dramatic, namely 3,000,000 (World Health Organization, 1974). Also causing unease is the continuing isolation from tubercular patients of strains already resistant to one or more chemotherapeutic agent. (C. Ratledge, 1976)

1. INTRODUCTION

The growth and nutritional requirements of mycobacteria have been intensely studied since the discovery of *Mycobacterium tuberculosis* (Koch, 1882). These studies have resulted in an overwhelming body of literature on the physiology of mycobacterial metabolism in the years before the dawn of molecular biology (Edson, 1951; Ramakrishnan *et al.*, 1972; Ratledge, 1982). This review covers growth of the mycobacterial cell and how environmental stimuli are sensed by this bacterium. Adaptation to different environments is described from the viewpoint of nutrient acquisition, energy generation and regulation. We aim to present a clear view of current knowledge of the proliferation of mycobacteria *in vitro* in order to provide a better foundation for interpreting growth during stationary phase or growth under hypoxic conditions and growth *in vivo*. Other topics such as taxonomy, molecular methods for identifying species, epidemiology, and mechanisms of drug action are considered when they are appropriate to the narrative. One topic relevant to the main theme has not been discussed: namely, detailed analysis of metabolism (e.g., fatty acids, amino acids) that have been reviewed previously by Ratledge (1976, 1982).

2. MYCOBACTERIA IN PERSPECTIVE

Our knowledge of bacterial growth has resulted from the study of relatively few strains that include *Escherichia coli* and *Bacillus subtilis*.

Both *Mycobacterium leprae* (Hansen, 1874) and *M. tuberculosis* (Koch, 1882) are amongst the first microbes to have been identified and yet our knowledge of their physiological properties has lagged behind our insights into bacterial physiology in general. Mycobacterial strains are not always easy to grow in the laboratory. For example, *M. leprae* has been harvested from infected laboratory animals and has never been grown *in vitro*. *Mycobacterium genavense* was identified by molecular techniques and has yet to be grown in culture (Böttger *et al.*, 1992; Coyle *et al.*, 1992). Other species such as *M. ulcerans* and *M. paratuberculosis* produce visible growth on a solid medium only after a month or more in culture (Wayne and Kubica, 1986). Typically, slow-growing mycobacteria produce visible colonies on a solid medium within 10–28 days. Apart from slow growth, other difficulties in studying mycobacteria include the hazards of handling pathogens, the tendency of cells to clump, and the resistance of cells to lysis.

2.1. *Mycobacterium* and its Close Relatives

Mycobacteria are Gram-positive, acid-fast and (G+C) rich (62–70%); they are aerobic and rod-shaped and mycolic acids are components of their cell walls (for review see Wayne and Kubica, 1986). Other bacteria including *Nocardia*, *Rhodococcus*, and *Corynebacterium* have related properties (see Table 1). The genera *Corynebacterium*, *Nocardia*, and *Mycobacterium* form a well-defined sub-group (CMN) of actinobacteria (Embley and Stackebrandt, 1994; Ventura *et al.*, 2007). Other genera that synthesize mycolic acids include *Dietza*, *Gordona*. *Skermania*, *Tsukamurella*, *Turicella*, and *Williamsia* (Gürtler *et al.*, 2004).

2.2. *Mycobacterium*

The genus *Mycobacterium* is now known to comprise more than 100 species which are all closely related as judged by comparison of their 16S rRNA sequences (Tortoli, 2006). This rapid expansion of the genus since 1990 is based on the invention of the polymerase chain reaction (PCR) for the amplification of DNA sequences (Saiki *et al.*, 1985) and the recognition of the value of 16S rRNA sequences as phylogenetic markers (Woese, 1987). These methods were first applied to mycobacteria less than 20 years ago (Stahl and Urbance, 1990; Rogall *et al.*, 1990a, b). Since then, mycobacteria have been found to be widely distributed in the environment. Mycobacteria

Table 1 Features of *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Corynebacterium*.

Feature	*Mycobacterium*	*Nocardia*	*Rhodococcus*	*Corynebacterium*
%G+C	62–70	64–72	63–72	51–63
Cell shape	Mainly rods, sometimes branching	Mycelium fragmenting into rods and cocci	Scanty mycelium fragmenting into rods and cocci	Pleomorphic rods
Cell envelope	Waxy coat	Waxy coat	Waxy coat	Waxy coat
Mycolic acids	60–90 carbon atoms	46–60 carbon atoms	32–66 carbon atoms	22–66 carbon atoms
Growth rate (time for visible colonies)	2–40 days	1–5 days	1–3 days	1–2 days

are opportunistic pathogens, especially in a clinical environment, and their detection is important in treatment of the infections they cause. Mycobacteria also have possible industrial applications. *M. austroafricanuum* is able to metabolize methyl tert-butyl ether, which is often added to gasoline and could be used to treat contaminated groundwater (Maciel *et al.*, 2008).

The earliest studies of mycobacterial genes concerned the rRNA operons (Bercovier *et al.*, 1986; Cox and Katoch, 1986). After a little more than a decade, the genome sequences of *M. tuberculosis* H37Rv (Cole *et al.*, 1998) and *M. leprae* (Cole *et al.*, 2001) were published. Knowledge of these sequences has facilitated very rapid progress in mycobacterial research. The number of published genome sequences has now reached seventeen.

3. PROPERTIES OF MYCOBACTERIAL CELLS

3.1. Population-Average Cells

Measurements based on cell cultures provide information about population-average cells (Schaechter *et al.*, 1958) and the picture that emerges is that of a virtual cell. A bacterial culture comprises cells of all ages ranging from newborn cells of age $a = 0$ to cells about to divide (age $a = 1$). The age range of cells comprising a culture remains constant as the number of cells increase (Powell, 1956) during both exponential growth in batch culture and growth in continuous culture (a chemostat). Thus, the virtual cell is representative of the entire age range. DNA, RNA, and protein fractions isolated from cell cultures provide information about population-average cells. Suppose, that D, R, and P refer to the mass of DNA, RNA, and protein, respectively, per ml of culture and N_c is the number of cells per ml of culture, then $m_{DNA(av)}$, $m_{RNA(av)}$, and $m_{p(av)}$ that are, respectively, the masses of DNA, RNA, and protein per population-average cell are defined in Equations (1)–(3).

$$m_{DNA(av)} = \frac{D}{N_c} \tag{1}$$

$$m_{RNA(av)} = \frac{R}{N_c} \tag{2}$$

$$m_{\mathrm{p(av)}} = \frac{P}{N_{\mathrm{c}}} \tag{3}$$

Furthermore, the synthesis rates such as $\omega_{\mathrm{p(av)}}$ the specific protein synthesis rate [see Equation (4)]

$$\omega_{\mathrm{p(av)}} = \mu \cdot m_{\mathrm{p(av)}} \tag{4}$$

and $\omega_{\mathrm{RNA(av)}}$ the specific RNA synthesis rate [see Equation (5)]

$$\omega_{\mathrm{RNA(av)}} = \mu \cdot m_{\mathrm{RNA(av)}} \tag{5}$$

describe the population-average cell.

A schematic view of a population-average cell of *M. tuberculosis* was devised to provide an instantaneous view of the macromolecular composition ($m_{\mathrm{DNA(av)}}$, $m_{\mathrm{RNA(av)}}$, and $m_{\mathrm{p(av)}}$) and of protein synthesis and other features (Cox and Cook, 2007).

3.2. Cell Size and Shape

Mycobacterial cells are irregular rods 0.3–0.5 μm in diameter and of variable length (Wayne and Kubica, 1986). The images of cells of *M. smegmatis* shown in Fig. 1 show that the increase in cell size is reflected in an increase in length rather than the diameter of the rods (compare left and right panels). For example, any changes in the diameter are small whereas length ranges from 1.5 to 4.0 μm according to the period of culture. This mode of cell growth provides the cell with a high ratio of surface area to mass, which is favorable for cells that are relatively impervious to aqueous solvents; that is, the number of porin channels per unit surface area is low compared with *E. coli*. It has been shown that nutrients are taken up by the cell by passive diffusion through the permeability barrier and then actively transported across the cytoplasmic membrane (Nikaido and Rosenberg, 1981; Stephan *et al.*, 2005; please see Section 8). Hence, *in vitro* the rate of passive diffusion can be increased by increasing the concentration of nutrients in the growth medium to allow the optimal growth rate to be attained. It is not known if this situation (occasions of "feast") is often encountered in the environment.

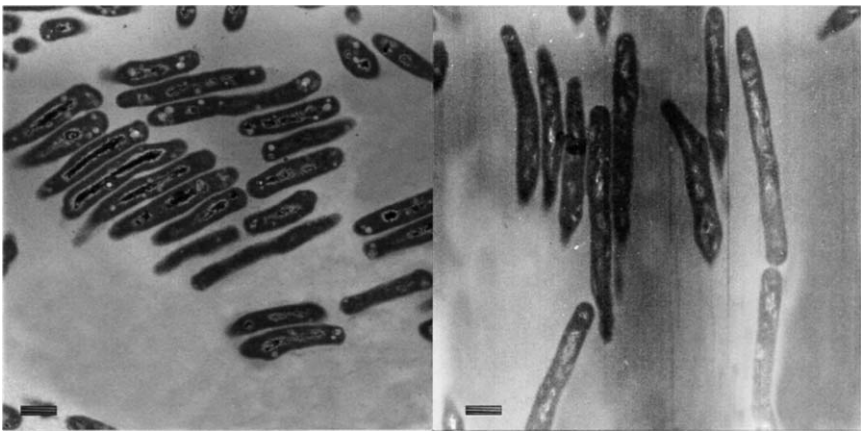

Figure 1 Electron micrograph of *M. smegmatis* mc^2155. Cells were grown at 37 °C with shaking (200 rev/min) in Kohn-Harris succinate medium plus 1% Tween-80. Cells were grown without (left panel, exponential phase) or with (right panel, late exponential phase/early stationary) a spring (10 cm long × 1.3 cm diameter) to increase agitation; in each case $\mu = 0.24$ per hour. For electron microscopy (Jeol JEM-100 Transmission Electron Microscope) cells were first fixed with 2.5% glutaraldehyde and sections were prepared. In each panel the bar represents 0.5 μm (Zaragoza-Contreras et al., 2005).

3.3. Macromolecular Composition

Measurements of amounts of DNA, RNA, and proteins per population-average cell at ''defined'' specific growth rates provide valuable insights into cell metabolism. Ninety percent of the energy needed for macromolecular synthesis is devoted to protein synthesis, which accounts for approximately one half of the energy needed for cell growth. The incorporation of each amino acid into a polypeptide chain is achieved at the expense of four high-energy phosphate bonds (for review see Cox and Arnstein, 2007). Cell growth requires protein synthesis, which, in turn requires ribosome synthesis. These aspects of cell proliferation are reflected in the ratios DNA:RNA:protein. However, few data are available for mycobacteria largely because of the difficulties encountered in lysing cells. Available data for *M. tuberculosis* H37Rv and *M. bovis* BCG are summarized in Table 2. There is little agreement between the three sets of data. The observed differences are probably too large to be accounted for by differences in growth conditions. Technical difficulties are more likely to be responsible for the wide range of reported values. For example, Youmans and Youmans (1968) used the orcinol method to measure RNA

Table 2 DNA, RNA, and protein contents reported for *M. bovis* BCG and *M. tuberculosis* H37Ra.

Property	*M. tuberculosis* H37Rv[a]	*M. bovis* BCG[b] (Glaxo)	*M. bovis* BCG[c]	
Generation time (hours)	2.40	2.40	23.10	69.30
DNA (fg/cell)	6.90[d]	6.90[d]	6.73[e]	5.35[e]
RNA (fg/cell)	232	13.3	9.96	2.24
Protein (fg/cell)	294	156.00	72.10	37.70

[a]Youmans and Youmans (1968). Cells were grown as a pellicle.
[b]Winder and Rooney (1970). Cells were grown in liquid culture.
[c]Beste *et al.* (2005). Cells were grown in a chemostat at two growth rates.
[d]Values DNA, RNA, and protein were calculated on the basis of one genome equal to 4.79 fg and 1.45 genome per population.
[e]Values DNA, RNA, and protein were calculated on the basis of 1.40 and 1.10 genomes per population-average cell, respectively, for faster and slower growth rates.

content and this reagent also reacts with polysaccharides. Beste *et al.* (2005) used different methods to lyse cells for measurements of DNA, RNA, and protein and failed to use a secondary standard to measure the extents of cell lysis. The data reported by Winder and Rooney (1970) was shown to be consistent with an equation derived by Bremer (1975) relating the growth rate with parameters affecting the rates of synthesis of ribosomes and RNA polymerase in bacteria (Colston and Cox, 1999). Thus, there is a dearth of information about the DNA, RNA, and protein contents of mycobacteria.

3.4. Does Genome Size Influence the Specific Growth Rate?

The genome sizes of mycobacterial species obtained by DNA sequencing range from 3,268,203 base pairs (bp) (*M. leprae*) to 6,988,209 bp (*M. smegmatis* mc^2155). Optimum specific growth rates have been reported for *M. tuberculosis* H37Rv (Wayne, 1994), *M. marinum* (Clark and Shepard, 1963), and *M. smegmatis* mc^2155 (Sander *et al.*, 1996). The range in genome sizes poses the question: ''How can specific growth rates be related to genome sizes''? This problem was approached in the following way. *E. coli* was chosen as the reference species (The Eco_model) because it is widely studied and an extensive database is available (see Bremer and Dennis, 1996). The properties of *E. coli* B/r grown at 37 °C in a minimal salts medium with succinate as the carbon source were used as its reference state because, under these conditions of growth, the genome is replicated once only during the cell division cycle (Bremer and Dennis, 1996).

Previously, it was found that equations developed to relate the specific growth rates of *E. coli* B/r with its macromolecular composition (Bremer, 1975) also applied to *M. bovis* BCG (Colston and Cox, 1999). Quantitative relationships relating specific growth rates and macromolecular composition of *M. tuberculosis*, *Streptomyces coelicolor* A3(2) and *E. coli* B/r were used to develop an instantaneous view of macromolecular synthesis (Cox, 2004). Thus, *E. coli* grown in succinate medium is a suitable model for the other strains considered in this review, namely mycobacteria, corynebacteria, and streptomyces, which also replicate their genomes once only during the growth cycle. The model is based on a generation time t_D of 1.67 hours, an interval of $t_{G_1} = 0.05$ hours between cell division and the start of DNA replication (S-phase) which continues for $\tau_g = 1.17$ hours (the genome is replicated at a rate of $\varepsilon_{DNA} = 2.08 \times 10^6$ bp per hour per replication fork); S-phase is followed by the period $t_{G_2} = 0.5$ hours before cell division takes place. It is supposed that if the species under scrutiny were as metabolically active as *E. coli* B/r growing in succinate medium at 37 °C then t_{G_1}, t_{G_2}, and ε_{DNA} would have the same values as the *E. coli* B/r model. However, in this model τ_g the period of DNA synthesis is directly proportional to l_g base pairs, the genome size of the species of interest. The generation time t_D^* of the Eco_model is given by Equation (6) where *E. coli* values are shown by the superscript ♯ sign.

$$t_D^* = t_{G_1}^{\#} + \left(\frac{l_g}{2\varepsilon_{DNA}^{\#}}\right) + t_{G_2}^{\#} \tag{6}$$

Hence, μ* the specific growth rate of the Eco_model may be evaluated. The Eco_index, which is the ratio of the observed and Eco_model derived specific growth rates, provides a measure of the relative metabolic activities of the subject species growing optimally in an appropriate medium and *E. coli* B/r growing in succinate medium at 37 °C.

The model was applied to three mycobacterial species, which have well-characterized optimal specific growth rates, and two other species, namely *S. coelicolor* A3(2) and *Corynebacterium glutamicum* ATCC 13032 that have genomes of 8,667,507 and 3,309,401 bp, respectively. The results are shown in Table 3. The Eco_index was found to vary from 0.1 (*M. tuberculosis* H37Rv) to 1.15 (*S. coelicolor* A3(2)). Three species grow optimally at 30 °C; namely *M. marinum* strain M, *C. glutamicum*, and *S. coelicolor* A3(2). No correction was applied to allow for growth at different temperatures. The results indicate that both *M. smegmatis* mc^2155 and *M. marinum* strain M have specific growth rates that are close (74 and 80%, respectively) to the value predicted for the Eco_model. The model

Table 3 Comparisons of observed and Eco_model derived specific growth rates of mycobacteria and other strains.

Species	Specific growth rate (per hour)		Eco_index
	Observed (μ)	Eco_model[a]	
E. coli B/r	0.42 (a)	0.42	1.00
M. tuberculosis H37Rv	0.043 (b)	0.43	0.10
M. marinum strain M	0.17[b] (c)	0.32	0.53[b]
M. smegmatis mc^2155	0.23 (d)	0.31	0.74
C. glutamicum ATCC13032	0.40[b] (e)	0.52	0.77[b]
S. coelicolor A3(2)	0.30[a] (f)	0.26	1.15[b]

(a) Bremer and Dennis (1996); (b) Wayne (1994); (c) Clark and Shepard (1963); (d) Sander *et al.* (1996); (e) Frunzke *et al.* (2008); (f) Shahab *et al.* (1996). See also Cox (2004).
[a]The Eco_model is defined in the text (see Equation (1)).
[b]The subject strain was grown at 30 °C.

for *M. smegmatis* mc^2155 is based on a DNA replication rate of $\varepsilon_{DNA} = 2.08 \times 10^6$ bp per hour per replication fork. The value of $\varepsilon_{DNA} = 2.0 \times 10^6$ bp per hour per replication fork can be deduced from an observed S-phase of 1.75 hours (Hiriyanna and Ramakrishnan, 1986), which is very close to the rate observed for *E. coli* B/r grown in succinate medium.

In contrast, the Eco_index of 0.1 derived for *M. tuberculosis* H37Rv indicates a metabolic activity of 10% of the *E. coli* B/r model. This expectation is in accord with the observed value of $\varepsilon_{DNA} = 0.21 \times 10^6$ bp per hour per replication fork deduced from the reported S-phase of 10.33 hours (Hiriyanna and Ramakrishnan, 1986), which is close to one tenth of the rate observed for *E. coli* B/r. To a first approximation, the Eco_index reflects the relative rates of DNA replication, which provides a guide to the relative metabolic activities of the species concerned. If an allowance was made for growth at 30 °C, then the Eco_index of *M. marinum*, *C. glutamicum*, and *S. coelicolor* A3(2) would be closer to or even exceed 1.0.

3.5. Mycobacterial *rrn* Operons

Classical pathogenic slow growers such as *M. tuberculosis* were shown to have a single *rrn* operon per genome whereas classical fast growers such as *M. smegmatis* and *M. phlei* were found to have two (Bercovier *et al.*, 1986; Domenech *et al.*, 1994). The generalization that mycobacteria have either one or two *rrn* operons per genome has proved to be correct. All the mycobacteria studied to date were found to have one operon (*rrnA*)

located downstream from *murA* (Gonzalez-y-Merchand *et al.*, 1996, 1997; Rivera-Gutiérrez *et al.*, 2003; Helguera-Repetto *et al.*, 2004): the second operon (*rrnB*), if present, was found downstream from *tyrS* (Gonzalez-y-Merchand *et al.*, 1996; Predich *et al.*, 1995; Menendez *et al.*, 2002). A phylogenetic tree based on 16S rRNA sequences was related to the numbers of *rrn* operons per genome. The combined data supports the view that the ancestral *Mycobacterium* possessed one *rrnA* and one *rrnB* operon and that on two separate occasions an *rrnB* operon was lost giving rise to two clusters of mycobacteria possessing a single *rrn* operon (*rrnA*) per genome (Stadthagen-Gomez *et al.*, 2008). One cluster includes the classical pathogens *M. leprae* and *M. tuberculosis* and the other includes *M. abscessus* and *M. chelonae*.

3.5.1. *The Number of* rrn *Operons per Genome is not a Reliable Guide to Growth Rate*

The number of operons per genome was found to be an unreliable guide to growth rate. For example, *M. chelonae* and *M. abscessus* are classified as fast growers and yet each has a single *rrn* (*rrnA*) operon per genome (Domenech *et al.*, 1994). In contrast, two slow growers, *M. terrae* (Ninet *et al.*, 1996) and *M. celatum* (Reischl *et al.*, 1998) were each found to have two *rrn* operons per genome. The fish pathogen *M. marinum* which has a single *rrn* (*rrnA*) operon per genome is classified as a slow grower that is closely related to *M. tuberculosis* (Goodfellow and Magee, 1998; Devulder *et al.*, 2005) and grows optimally at 30 °C with a generation time of 4 hours (Clark and Shepard, 1963; Cosma *et al.*, 2003) compared with a generation time of 16 hours or more at 37 °C for *M. tuberculosis*. The explanation for the poor correlation found between the number of *rrn* operons and specific growth rate was provided by Sander *et al.* (1996) who showed that the specific growth rate of *M. smegmatis* mc^2155 was unaltered by inactivation of either one or the other of its two *rrn* operons: in other words, the second *rrn* operon is redundant. The replication of *rrn* operons is discussed in the following section.

4. DNA REPLICATION

Our knowledge of the replication of the genomes of mycobacteria is limited to a single study of the duration of S-phases of *M. smegmatis* and *M. tuberculosis* H37Rv (Hiriyanna and Ramakrishnan, 1986). However, it is assumed that the current view of the replication of a circular bacterial

genome applies. Replication is initiated at a single site, *oriC*, and proceeds in both clockwise and counterclockwise directions until the terminus (*ter*) is reached (Prescott and Kuempel, 1972; Higgins, 2007). The genome is replicated once only during the growth cycle (see, e.g., Boye *et al.*, 2000; Zakrzewska-Czerwinska *et al.*, 2007). DNA replication occurs during S-phase at an average rate of ε_{DNA} bp per hour per replication fork. S-phase is preceded by G_1-phase that is needed to prepare the enzymes for DNA synthesis and is followed by G_2-phase during which the newly synthesized genomes are segregated and septum formation takes place.

4.1. Replication of Individual ORFs

The replication of a particular ORF occupies only a small fraction of S-phase. An ORF ($ORF_{(0)}$) located close to *oriC* is replicated at the beginning of S-phase when two copies become available for the rest of the cell's lifetime. In principle, it can benefit the cell if an ORF, whose gene product is in high demand, is located near to *oriC*. Then the newly replicated ORF becomes available at the earliest opportunity, namely, the end of G_1-phase. In contrast, an ORF ($ORF_{(t)}$) located near to *ter* provides the cell with a newly replicated copy for no longer than the G_2-period.

The time during S-phase when $ORF_{(i)}$ is replicated depends on the position of $ORF_{(i)}$ within the genome. The positions of *rrn* operons are variable and the replication coefficient facilitates comparisons between mycobacteria. The replication coefficient γ is defined as the shortest distance (base pairs) of an $ORF_{(i)}$ from *oriC* measured either clockwise or counter clockwise divided by one half the length (base pairs) of the genome. The replication coefficients of mycobacterial *rrn* operons are >0.5 revealing that they are replicated in the second half of S-phase. In contrast, the replication coefficients of the 10 *rrn* operons of *B. subtilis* subsp. *subtilis* are close to 0 showing they are replicated very early in S-phase (Kunst *et al.*, 1997). The replication coefficient $\gamma_{ORF_{(i)}}$ is related to the time $\tau_{ORF_{(i)}}$ when $ORF_{(i)}$ is replicated in S-phase. In turn, $\tau_{ORF_{(i)}}$ plus the G_1 period is equal to the time $t_{ORF_{(i)}}$ during the cell division cycle when $ORF_{(i)}$ is replicated. The age $a_{(i)\bullet R}$ of a cell when $ORF_{(i)}$ is replicated is obtained by dividing $t_{ORF_{(i)}}$ by t_D the generation time. Once $a_{(i)\bullet R}$ is known the number of copies of $ORF_{(i)}$ per population-average cell can be calculated (see appendix). The limited data available for *M. tuberculosis* H37Rv shows that the *rrnA* operon ($\gamma = 0.67$) is replicated in the second half of S-phase, leading to 1.36 copies of *rrnA* per population-average cell. In contrast,

Table 4 Replication of *rrn* operons of *M. smegmatis* mc^2155.

Property	Strain		
	Wild type	*rrnA* deletion	*rrnB* deletion
Generation time (hours)	3	3	3
Duration of S-phase (hours)	1.75	(1.75)	(1.75)
Duration of G_1-phase (hours)	0.67	(0.67)	(0.67)
Functional *rrn* operons	*rrnA*, *rrnB*	*rrnB*	*rrnA*
Cell age when replicated	0.56, 0.70	0.70	0.56
Number of copies/ population-average cell	2.54	1.18	1.36

$ORF_{(0)}$ ($\gamma_{rep} = 0$), the number of copies of $ORF_{(i)}$ range from 1.14 copies of $ORF_{(0)}$ to 1.70 copies of $ORF_{(t)}$.

$ORF_{(0)}$ located close to *oriC* is present as 1.72 copies per population-average cell, whereas there are 1.20 copies of $ORF_{(t)}$ located near to *ter*.

The available data for *M. smegmatis* mc^2155 show that this bacterium can grow at its optimum rate with a single *rrnB* operon; this operon, which has a replication coefficient of 0.90, is replicated late in S-phase. Hence, this mutant has 1.18 copies of *rrnB* per population-average cell. Thus, a single *rrn* operon that is replicated late in S-phase is sufficient to support mycobacterial growth of 3 hours generation time, a rate that is 74% of the Eco_model (see Table 4). Moreover, when the *M. tuberculosis rrnA* operon was expressed in *M. smegmatis*, it was competitive with the *rrnA* and *rrnB* operons of the host (Verma *et al.*, 1999). By analogy, it is safe to conclude that the single *rrnA* operon of *M. tuberculosis*, which is present at 1.36 copies per population-average cell (see above) is also sufficient to support a generation time of 3 hours. In other words, the possession of a single *rrn* operon per genome is not related to the slow growth of the tubercle bacillus. This view is reinforced by the demonstration that *E. coli* can grow with a specific growth rate of $\mu = 0.6$ per hour even after inactivation of six of its seven *rrn* operons or even after inactivation of all seven operons with a functional operon provided by a plasmid (Asai *et al.*, 1999). Fluorescence spectroscopy revealed that deletion of six or seven *rrn* operons changed the size and shape of the cells from short and fat to long and thin, as in mycobacteria.

5. MYCOBACTERIAL RIBOSOMES

Ribosomes of the *M. tuberculosis* complex were shown to have sedimentation properties similar to those of ribosomes of *E. coli* (Trnka *et al.*, 1968;

Worcel *et al.*, 1968), with 16S, 23S, and 5S rRNA components (Worcel *et al.*, 1968). The ribosome fraction was shown to be active in cell-free protein synthesis (Shaila *et al.*, 1973).

5.1. Components of Mycobacterial Ribosomes

The RNA (rRNA) moiety of ribosomes is encoded by classical rRNA (*rrn*) operons in the order 5′ 16S rRNA, 23S rRNA, and 5S rRNA 3′ (Suzuki *et al.*, 1988; Liesack *et al.*, 1990, 1991; Kempsell *et al.*, 1992). The information available for ribosomal proteins (r-proteins) is largely derived from genomic sequences. For example, r-proteins of *M. tuberculosis* are orthologues of *E. coli* r-proteins. However, four r-proteins: namely, S14, S18, L28, and L33 (see Table 5) are each encoded by two non-identical genes. Not all of the available mycobacterial genomic sequences were found to have these extra genes. When present, the four genes were located as a group encoding r-proteins in the order 5′ L28, L33, S14, S18 3′. This cluster of genes is absent from *M. leprae*, *Mycobacterium species* JLS, *Mycobacterium species* KMS, and *Mycobacterium* species *MCS.* The extra set of genes encode four proteins which have different amino acid sequences from their standard orthologues; for example, two proteins with non-identical amino acid sequences are identified as orthologues of *E. coli* L28. The non-identical orthologues of each of the four r-proteins raises the question of the homogeneity of the ribosome fraction. If ribosomes are heterogeneous with respect to their complement of r-proteins is ribosome function affected? Microarray measurements reveal that the additional set

Table 5 Ribosomal proteins S14, S18, L28, and L33 of *M. tuberculosis* H37Rv are each encoded by two genes.

Gene tag	Gene locus	*E. coli* equivalent	Essential gene	Size of protein (amino acids)	% similarities of orthologous proteins
Rv0717	*rpsN1*	S14		61	36
Rv2056c	*rpsN2*	S14	No	101	36
Rv0055	*rpsR1*	S18		84	54
Rv2055c	*rpsR2*	S18	No	88	54
Rv0105c	*rpmB1*	L28	No	94	53
Rv2058c	*rpmB2*	L28	No	78	53
Rv2057c	*rpmG1*	L33	No	54	30
Rv0634B	*rpmG2*	L33		55	30

Table 6 Changes in the expression ratios of the genes encoding S14 with the period of cell culture of *M. tuberculosis*[a].

Gene tag	Encoded protein	Expression ratio		
		Period (days) of cell culture		
		0	8	14
Rv0717	*rps*N1 (S14)[b]	1.02	0.65	0.28
Rv2056C	*rps*N2 (S14)	1.15	7.8	10.4

[a]Calculated from supplementary data of Voskuil *et al.* (2004).
[b]The expression ratios observed are typical of the majority of r-proteins.

of r-proteins are regulated by growth rate in a manner that differs from that of the other r-proteins. Genes encoding r-proteins are usually down-regulated as the specific growth rate is decreased. In contrast, genes encoding the additional proteins are up-regulated, as shown in Table 6 for S14 of *M. tuberculosis* grown in batch culture. A comparable result was obtained when *M. bovis* BCG was grown in continuous culture. A threefold change (from 1 day to 3 days) in the specific growth rate led to expression ratios of 0.61 for *rps* N1 and 6.0 for *rps* N2 (calculated from supplementary data of Beste *et al.*, 2007b).

5.2. Growth Rate Control of Ribosome Synthesis

Except at very low growth rates, the number of ribosomes per cell is dependent on growth rate (growth rate control of ribosome synthesis). This conclusion is based on the finding that the RNA content of cells varies with growth rate (Schaechter *et al.*, 1958; Verma *et al.*, 1999; Paul *et al.*, 2004b). As expected, the r-protein content is also dependent on growth rate. The results of microarray experiments obtained for *M. tuberculosis* and its close relative *M. bovis* BCG are presented in Fig. 2. Panel (a) provides an essential control in which the two labeled cDNA were prepared from the same RNA fraction isolated from mid-exponential cells of *M. tuberculosis* and the distribution of the expression ratios of the standard 50 r-proteins was examined. The mean value of $r_{(50)} = 1.00 \pm 0.10$ was obtained, which is a measure of the accuracy of the expression ratios. The second panel refers to cDNA prepared from mutant (*dosR* minus) and wild-type *M. tuberculosis*; both mutant and wild type were found to grow at the same rate so that the ribosome contents of the two strains would be expected to be identical. In this experiment, the mean value of the expression ratios of

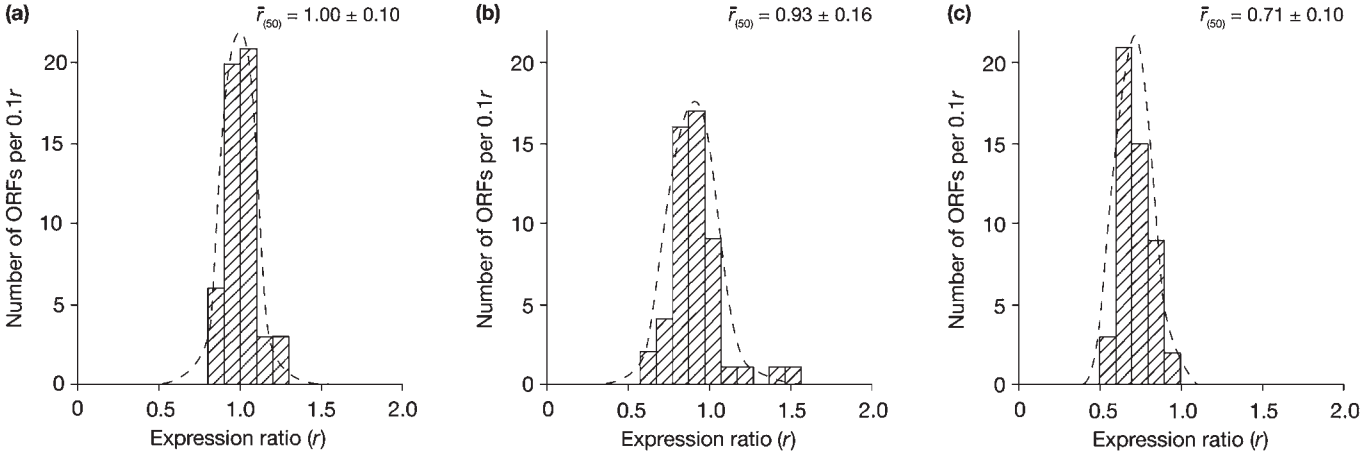

Figure 2 Comparison of the expression of r-protein genes of *M. tuberculosis* and *M. bovis* BCG grown under different conditions. In each panel, the broken line denotes the values expected for a normal distribution according to the mean value and standard deviation cited. (a) Comparison of mid-exponential phase cells (optical density 0.15) when samples of the same cDNA preparation were labeled with fluorofors f' and f'' (Voskuil *et al.*, 2004); expression ratios were obtained from Voskuil *et al.* (2004) for a culture of *M. tuberculosis* clinical isolate 1,254 grown at 37 °C in 7H9 medium (supplemented with bovine serum albumin, NaCl, glucose, and glycerol) shaking at 90 rpm. The expression ratio at this time point was obtained by using the RNA fraction to prepare one sample of cDNA labeled with fluorofor f' and another labeled with fluorofor f''. (b) Comparison of *dosR* minus mutant with wild-type *M. tuberculosis* (Kendall *et al.*, 2004); both wild type and mutant were found to grow at the same rate. The profile was computed from supplementary data. (c) Comparison of ribosomal gene expression for *M. bovis* BCG grown in a chemostat at two different specific growth rates; namely, 0.01 per hour (f'') and 0.03 per hour (f'); computed from the supplementary data of Beste *et al.* (2007b).

the standard set of r-proteins was found to be $r_{(50)} = 0.93 \pm 0.16$ which is similar to the control shown in panel (a). The third panel refers to *M. bovis* BCG grown in continuous culture; the specific growth rates of reference and experimental strains were 0.03 and 0.01 per hour, respectively. In this experiment, the expression ratios were found to have a mean value of $r_{(50)} = 0.71 \pm 0.01$, which indicates a lower level of r-protein mRNAs per microgram cDNA in the slower growing cells and, therefore, a lower level of r-proteins (Cox, 2007). This conclusion is reinforced by the comparison shown in Fig. 3, which shows both the expression profiles of all 3,475 ORFs

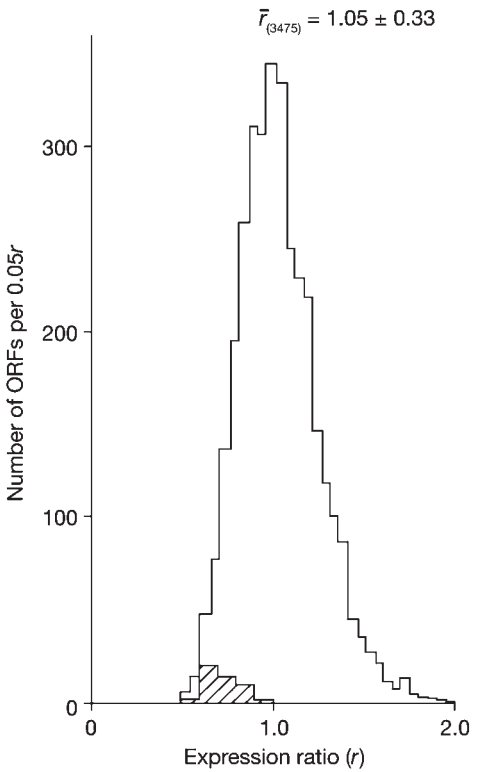

Figure 3 Comparison of the effects of slow growth on gene expression of *M. bovis* BCG: comparison of the expression ratios of all genes with r-protein genes. *M. bovis* BCG was grown in continuous culture at growth rates of 0.01 per hour (r'') and 0.03 per hour (r'), see Beste *et al.* (2007b). Supplementary data were used to calculate the distribution values of all 3,475 ORFs investigated. The hatched section indicates the profile of r-protein genes.

investigated and the ORFs encoding the set of 50 r-proteins. The group of 50 r-protein genes form a subset that is down-regulated.

6. rRNA SYNTHESIS

6.1. Organization; Transcription of *rrn* Operons

The organization of the two *rrn* operons of mycobacteria are shown in Fig. 4. The *rrnA* is present in all mycobacteria studied thus far. The characteristic features of *rrnA* include the numbers and locations of its promoters (Verma *et al.*, 1994; Gonzalez-y-Merchand *et al.*, 1997). This operon has a minimum of two promoters; one promoter (P1) is located within the coding region of the *murA* gene near to its 3′-end and the other (PCL1) is located near to a conserved sequence motif (CL1). Up to three additional promoters have been found, separated by 80–100 bp, in some species; the *rrnA* operon of *M. smegmatis* has three promoters (P1, P2, and PCL1), *M. fortuitum* has four promoters (P1, P2, P3, and PCL1) and *M. chelonae* and *M. abscessus* each have five (P1, P2, P3, P4, and PCL1). Moreover, transcripts of *murA* continue their progress to transcribe the *rrn* operon (Gonzalez-y-Merchand *et al.*, 1999). The contributions of individual promoters to precursor-rRNA synthesis was found to vary according to growth rate (Gonzalez-y-Merchand *et al.*, 1998; Verma *et al.*, 1999). In brief, a single promoter tends to be dominant at high growth rates.

In contrast with *rrnA*, the *rrnB* operon is regulated by a single (P1) promoter. Transcription of *tyrS* was found to continue to progress to transcribe the *rrnB* operon (Gonzalez-y-Merchand *et al.*, 1999). The mechanism of upstream activation of the *rrnB* operon was investigated (Arnvig *et al.*, 2005). The results were found to follow the model reported for *B. subtilis* rather than the model for *E. coli* (Krásný and Gourse, 2004). *B. subtilis* rRNA promoters are much less dependent on UP elements and do not seem to use upstream-binding factors analogous to Fis. When the genome of a species encodes both *rrnA* and *rrnB*, the sequences corresponding to precursor-rRNA are very similar (see, e.g., Sander *et al.*, 1996), but not always identical (Ninet *et al.*, 1996; Reischl *et al.*, 1998). The relatively few differences that may be present are unlikely to affect the principle features of pre-rRNA secondary structure that is processed by RNase to yield 16S rRNA, 23S rRNA, and 5S rRNA. Consensus secondary structures were derived for pre-16S rRNA (Ji *et al.*, 1994a) and pre-23S

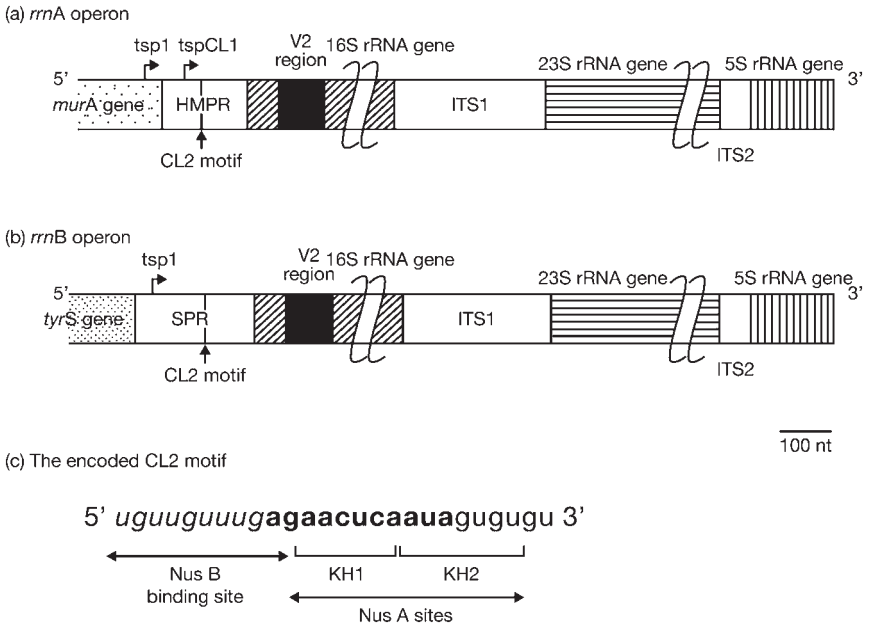

Figure 4 Organization of the *rrnA* and *rrnB* operons of mycobacteria. The operons are defined by the identities of their upstream genes. (a) *tsp*, transcription starting point; CL1, conserved leader sequence 1; HMPR, hypervariable mature promoter region. The number of promoters in this region ranges from 1 to 4, each promoter is separated by 80–100 bp; diagonal hatching, 16S rRNA coding region; ITS1, internal transcribed spacer region 1; V2 region (black shading), variable region of 16S rDNA which serves to identify the species; horizontal hatching, 23S rRNA coding region; ITS2, internal transcribed spacer region 2; vertical hatching, 5S rRNA gene. (b) SPR, single promoter region. All other abbreviations and shadings are defined in (a). (c) Nucleotide sequence of the conserved CL2 motif and its NusA and NusB binding sites. The locations of this motif are shown in (a) and (b). Two regions of NusA (KH1 and KH2) are involved in binding to sequence shown in bold (Beuth *et al.*, 2005). The NusB component of the NusB. NusE (S10) dimer is thought to bind to the BoxA motif shown in italics (Das *et al.*, 2008).

rRNA and pre-5S rRNA (Ji *et al.*, 1994b, c) based on comparisons of mycobacterial sequences all of which are closely related.

6.2. The Stringent Response

rRNA synthesis is the rate-limiting step in ribosome synthesis (Paul *et al.*, 2004a) and bacteria have devised mechanisms that ensure that ribosome

synthesis (and hence rRNA synthesis) is efficiently controlled to meet the cell's need for protein biosynthesis, including the need to survive under starvation conditions (the stringent response, for review see Cashel *et al.*, 1996). Much of our knowledge of the control of rRNA synthesis has come from studies of *E. coli*. Two small molecules are important regulators of rRNA synthesis in *E. coli*: nucleotide triphosphates, especially the NTP incorporated at the 5′-ends of the precursor-rRNA transcripts (Barker and Gourse, 2001) and guanosine 5′-diphosphate 3′-diphosphate (ppGpp) and its precursor pppGpp. Two proteins encoded by *relA* and *spoT* are implicated in the synthesis of ppGpp, which is synthesized by RelA in response to uncharged tRNAs at the A-site of the ribosome (Hogg *et al.*, 2004). It is thought that ppGpp may bind directly to RNAP (for review see Paul *et al.*, 2004b). A protein DksA is essential for regulation of rRNA synthesis by NTPs and ppGpp (Paul *et al.*, 2004a). The stringent response has been studied in *M. tuberculosis* H37Rv (Primm *et al.*, 2000). Whereas *E. coli* ppGpp is synthesized through the activities of RelA and SpoT proteins, the tubercle bacillus has a single protein, Rel_{Mtb}, to achieve the same effect; this single protein produces ppGpp in response to nutrient starvation. A knockout mutant (Δrel_{Mtb}) was produced and was used to show that the Rel_{Mtb} protein is needed for the long-term survival of *M. tuberculosis* under starvation conditions.

6.3. Anti-Termination of *rrn* Transcription

The size of the pre-rRNA transcript of the *rrnA* operon of *M. tuberculosis* extending from the start site of the PCL1 promoter to the intrinsic termination site is 5,550 nucleotides. As transcription proceeds, the nascent pre-rRNA transcript will recruit r-proteins and undergo processing by nucleases. Synthesis of rRNA is the rate-limiting step in ribosome synthesis. Thus, the cell is required to synthesize long pre-rRNA transcripts when ribosomes are needed. The strategy employed is to modify the RNAP complex with anti-termination factors to prevent premature transcription of the *rrn* operon. A feedback mechanism is achieved if a r-protein plays a role in anti-termination. Particular sequence motifs first identified in phage-λ were recognized as elements in the upstream regions of *rrn* operons so that related nomenclatures (Nus – N utilizing substances – proteins and *nut* sequences) are used for both phage-λ and *rrn* operons. Transcription of the *rrn* operon requires the formation of a stable RNAP elongation complex that is able to safely ignore transcription termination signals. The properties of the anti-termination complex identified for the N protein

system of bacteriophage-λ (Mogridge *et al.*, 1995) provides the model for the composition of the anti-termination complex needed to obtain full-length transcripts of precursor-rRNA. The proteins identified in the phage-λ system are N, NusA, NusB, NusE (which is identical with r-protein S10), and NusG. With one exception, the same proteins are needed to obtain full-length bacterial precursor-rRNA transcripts; all the Nus proteins are needed, but the cellular equivalent of the N protein has not yet been identified (Condon *et al.*, 1995).

The *nut* sequence elements are found in both the leader and ITS1 regions of rDNA. This duplication of *nut* sequences suggests that the anti-termination complex is formed at the leader region, dispersed after the 16S rRNA gene is transcribed by competition for *nut* sequences by a complementary strand of RNA located downstream from the 3′-end of the 16S rRNA gene and the complex is reformed before the 23S rRNA gene is transcribed and dispersed after the transcription of the 23S rRNA gene is completed (Ji *et al.*, 1994c). The mycobacterial *nut* site within the leader region of rDNA comprises a 26 nucleotide sequence (the CL2 motif), which is present in all the mycobacteria examined so far (Gonzalez-y-Merchand *et al.*, 1997; Menendez *et al.*, 2002); the rRNA sequence is 5′-UGUUGUUUGAGAACUCAAUAGUGUGU-3′ [see Fig. 4(c)]. A similar, but not identical sequence is found in the ITS1 region.

Insights into the mechanisms of *rrn* anti-termination have been advanced through physico-chemical and structural studies. A high-affinity interaction between the CL2 region of *M. tuberculosis* and its cognate NusA was reported (Arnvig *et al.*, 2004). The crystal structure of the NusA component was determined (Gopal *et al.*, 2001a). The X-ray structure of the NusA–RNA complex (Beuth *et al.*, 2005) revealed that the RNA sequence bound by NusA was 5′-AGAACUCAAUA-3′ region of the CL2 sequence [see Fig. 4(c)]. Nodwell and Greenblatt (1993) first proposed that a complex NusB and NusE (S10) interacted with the *nut* sequence element BoxA. The mycobacterial BoxA sequence occurs at the 5′-end of the CL2 motif [see Fig. 4(c)].

The *M. tuberculosis* NusB structure was determined by X-ray crystallography (Gopal *et al.*, 2000) and interaction between NusB and S10 was also demonstrated (Gopal *et al.*, 2001b). The structure of *E. coli* NusB in solution was determined by Altieri *et al.* (2000). NusB binds to BoxA sequence directly, but its affinity for BoxA is increased by interaction with NusE (S10), as shown by Das *et al.* (2008). This study strengthens the view that the ternary complex of BoxA RNA/NusB/NusE (S10) interacts with the RNA polymerase complex and stabilizes it to overcome termination signals.

7. PROTEIN BIOSYNTHESIS

7.1. *In Vitro* Cell-Free Systems

Translation of mRNA into protein is a complex process requiring many of the cell's resources including initiation, elongation, termination factors, and the consumption of energy; the formation of each peptide bond requires the hydrolysis of four high-energy phosphate bonds. Studies of mycobacterial protein synthesis were neglected until Böttger and co-workers reconstituted an *in vitro* protein synthesis system comprising *M. smegmatis* ribosomes and the complete range of factors synthesized *in vitro* from the parent genes. Fully functional hybrid ribosomes were constructed by modifying *M. smegmatis* 16S rRNA to form bacterial eukaryotic hybrids. The hybrids were formed by replacing the bacterial decoding site (helix 44) with a eukaryotic counterpart. The drug sensitivities of the hybrid ribosomes were dependent on the origin of the eukaryotic sequence (Hobbie *et al.*, 2007). The *M. smegmatis in vitro* protein synthesizing system was then used to compare the rates of reaction of individual steps in protein synthesis with the homologous reaction in *E. coli*. It was found that the elemental reaction rates of initiation and elongation were remarkably similar with *M. smegmatis* and *E. coli* components. The reconstituted translation system from individual purified *M. smegmatis* components is an alternative to that from *E. coli* to study mechanisms of translation and to test the action of antibiotics against Gram-positive bacteria (Bruell *et al.*, 2008).

7.2. Post-Translational Modification of Proteins

A number of proteins possess an unusual feature termed an intein. The primary transcript contains an insertion sequence that is translated. The amino acid sequence corresponding to the intron sequence is termed an intein. ''Inteins are self-splicing elements that exist as in-frame protein fusions with two flanking sequences called exteins'' (Perler *et al.*, 1994). The intein splices itself out of primary translational product leaving the two exteins, which constitute the mature protein. The first mycobacterial intein recognized was found to be RecA of *M. tuberculosis* (Davis *et al.*, 1991). Since then inteins were identified in three other proteins; namely, DnaB, GyrA, and Pps1. Fourteen mycobacterial species are now known to have one or more intein-containing proteins (see http://bioinfo.weizmann.ac.il/~pietro/inteins/Inteins_table/html). The precise function of inteins is still a

matter for speculation. However, the properties of inteins have been widely examined, including the use of X-ray crystallographic and mutational studies (Van Roey *et al.*, 2007).

8. THE CELL ENVELOPE OF MYCOBACTERIA

8.1. Permeability Barriers in Mycobacterial Cell Envelopes

Nutrient uptake mechanisms obviously depend on the permeability barriers imposed by the cell envelope. Therefore, it is necessary to review the current status of the research about the cell envelope of mycobacteria. The terms are defined as follows (Graham *et al.*, 1991; Beveridge, 1995; Beveridge and Kadurugamuwa, 1996): The *cell wall* consists of the periplasm, the peptidoglycan layer and, for Gram-negative bacteria, the outer membrane. The *periplasmic space* represents an extracytoplasmic compartment confined between the plasma membrane and an outer structure (outer membrane vs. a peptidoglycan–teichoic acid–protein network for Gram-negative and -positive bacteria, respectively). The periplasm is composed mostly of soluble components. The *cell envelope* comprises the inner membrane and the cell wall. The components of the cell envelopes of both Gram-positive and -negative bacteria have recently been visualized by cryo-electron microscopy (Matias *et al.*, 2003; Matias and Beveridge, 2005; Matias and Beveridge, 2006).

In microbiology textbooks, mycobacteria are classified as Gram-positive bacteria. However, it is well-documented that mycobacteria, unlike other Gram-positive bacteria, have evolved a very complex cell wall, comprising a peptidoglycan–arabinogalactan polymer with covalently bound mycolic acids of considerable size (up to 90 carbon atoms), a large variety of extractable lipids (Barry *et al.*, 1998; Daffé and Draper, 1998) and pore-forming proteins (Niederweis, 2003). Most of the mycobacterial lipids are constituents of the cell envelope, which provides an extraordinarily efficient permeability barrier to noxious compounds, rendering mycobacteria intrinsically resistant to many drugs (Brennan and Nikaido, 1995). Due to the paramount medical importance of *M. tuberculosis*, the ultrastructure of mycobacterial cell envelopes has been intensively studied for decades by electron microscopy. To account for the extraordinary efficiency of the mycobacterial cell wall as a permeability barrier, Minnikin (1982) originally proposed a model in which the mycolic acids are covalently bound to the peptidoglycan–arabinogalactan co-polymer and form the inner leaflet of an

asymmetrical bilayer. Other lipids extractable by organic solvents were thought to form the outer leaflet of this outer bilayer. X-ray diffraction studies of mycobacterial cell walls showed that the mycolic acids are oriented parallel to each other and perpendicular to the plane of the cell envelope (Nikaido *et al.*, 1993). This provided experimental support for some fundamental aspects of the Minnikin model. Mutants and treatments affecting mycolic acid biosynthesis and the production of extractable lipids showed an increase of cell wall permeability and a drastic decrease of virulence, underlining the importance of the integrity of the cell wall for intracellular survival of *M. tuberculosis* (Barry *et al.*, 1998). These indirect structural, biochemical, and genetic data are consistent with the existence of an outer lipid bilayer as proposed by Minnikin (1982). However, this model faced criticism mainly because electron microscopy of mycobacteria, in particular thin sections thereof, never showed evidence for an additional, outer lipid bilayer (Daffé and Draper, 1998; Draper, 1998). Recently, we have visualized the envelope of intact *M. smegmatis* and *M. bovis* BCG cells by cryo-electron tomography and have provided, for the first time, direct, and final evidence for the existence of this structure (Hoffmann *et al.*, 2007, 2008). An important result was that cell envelopes of *M. smegmatis* and *M. bovis* BCG are very similar both in appearance and in dimensions. Thus, it makes sense to name this unusual structure "mycobacterial outer membrane" (Hoffmann *et al.*, 2008). Cryo-electron tomography and cryo-electron microscopy of ultrathin sections clearly showed that the mycobacterial outer membrane is a bilayer structure. A mycolic acid-deficient mutant of *C. glutamicum*, a close relative of mycobacteria, did not produce an outer membrane demonstrating that mycolic acids are an essential component of the outer membrane in these bacteria (Hoffmann *et al.*, 2008). The results of this study were subsequently confirmed (Zuber *et al.*, 2008) and are summarized in Fig. 5.

8.2. Transport Across Mycobacterial Outer Membranes

The utilization of many solutes such as carbohydrates, alcohols, carboxy acids, fatty acids, and amino acids by mycobacteria was originally examined as early as 1951 (Edson, 1951). It is, therefore, striking that the identity of many transporters for essential nutrients of *M. tuberculosis* and other mycobacteria are still unknown despite considerable progress in the development of genetic methods for mycobacteria (Kana and Mizrahi, 2004; Machowski *et al.*, 2005). Recently, significant progress has been made to understand the uptake of some nutrients both in *M. smegmatis* and

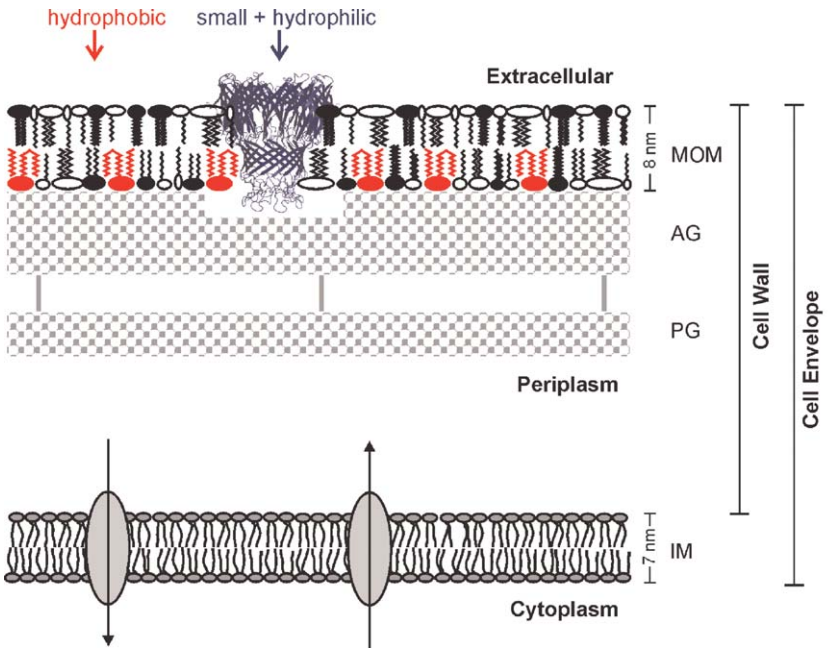

Figure 5 Transport processes across the mycobacterial cell envelope. Schematic representation of the mycobacterial cell envelope consisting of the inner membrane (IM) and the cell wall. This representation is based on cryo-electron micrographs (Hoffmann *et al.*, 2007, 2008). Mycolic acids are covalently linked to the arabinogalactan (AG)–peptidoglycan (PG) co-polymer and are an essential component of the inner leaflet of the outer membrane (OM). Extractable lipids are represented in black. The porin MspA mediates the uptake of small and hydrophilic nutrients such as sugars (Stephan *et al.*, 2005) and phosphates (Wolschendorf *et al.*, 2007) across the OM of *M. smegmatis*. The MspA channel is 9.6 nm long (Faller *et al.*, 2004). Approximately, 7 nm of the MspA surface are inaccessible to hydrophilic reagents (Mahfoud *et al.*, 2006). Hydrophobic compounds are assumed to diffuse directly across the OM. The dimensions are approximately to scale. (See plate 3 in the color plate section.)

M. tuberculosis. For example, proteins were identified and characterized that are involved in uptake of several nutrients such as phosphate (Vyas *et al.*, 2003; Webb, 2003; Peirs *et al.*, 2005; Gebhard *et al.*, 2006; Wolschendorf *et al.*, 2007), sulfate (Wooff *et al.*, 2002), and some amino acids (Seth and Connell, 2000; Talaue *et al.*, 2006). The current knowledge about nutrient uptake across both inner and outer membranes in mycobacteria was recently summarized (Niederweis, 2008a, b) and is updated here. A putative link between outer membrane transport and growth rate of mycobacteria has recently been discussed (Cox and Cook, 2007).

8.3. Porin-Mediated Diffusion of Hydrophilic Solutes

Porins are defined as non-specific protein channels in bacterial outer membranes which enable the influx of hydrophilic solutes (Nikaido, 2003). Channel-forming proteins that are functionally similar to porins of Gram-negative bacteria have been observed in many mycobacteria (Niederweis, 2003) and other closely related genera such as corynebacteria. MspA was discovered as the first porin of *M. smegmatis* (Niederweis *et al.*, 1999). Deletion of *mspA* reduced the outer membrane permeability of *M. smegmatis* toward cephaloridine and glucose ninefold and fourfold, respectively (Stahl *et al.*, 2001). These results show that MspA is the major general diffusion pathway for hydrophilic solutes in *M. smegmatis*. Consecutive deletions of the two porin genes *mspA* and *mspC* reduced the number of pores by 15-fold compared to wild-type *M. smegmatis*. The loss of porins lowered the permeability for glucose by 75-fold and, concomitantly, the growth rate of *M. smegmatis* on plates and in liquid medium dropped drastically (Stephan *et al.*, 2005). This showed for the first time that the porin-mediated influx of hydrophilic nutrients limited the growth rate of porin mutants. However, it is unknown which and how many nutrients are in low supply in the *M. smegmatis* porin mutants. Since MspA could not be expressed in *M. smegmatis* above wild-type levels (Stephan *et al.*, 2005), it is also not clear whether the influx of nutrients really limits the growth rate of wild-type mycobacteria as suggested earlier (Jarlier and Nikaido, 1990). The fact that the lack of the MspA and MspC porins also caused a reduced uptake of phosphates and slower growth on low-phosphate plates (Wolschendorf *et al.*, 2007) indeed suggested that the slow uptake of essential hydrophilic nutrients other than the carbon source may also contribute to the slow growth of *M. smegmatis* porin mutants.

The crystal structure of MspA represents the first crystal structure of any mycobacterial outer membrane protein and revealed an octameric goblet-like conformation with a single central channel of 10 nm in length (Faller *et al.*, 2004). This structure is different from that of the trimeric porins of Gram-negative bacteria, which have one pore per monomer and are approximately 5 nm long (Koebnik *et al.*, 2000). Its structural features define MspA as the founding member of a new class of outer membrane proteins. The crystal structure also revealed that the constriction zone of MspA consists of 16 aspartates (D90/D91). Thus, the zone of MspA with the smallest diameter is highly negatively charged (Faller *et al.*, 2004). This probably explains the previously observed preference of MspA for cations (Kartmann *et al.*, 1999). The MspA pore provides an example of how outer

membrane transport proteins can contribute to the selectivity of mycobacteria toward particular nutrients.

The existence of channel-forming proteins in *M. tuberculosis* and in *M. bovis* BCG has been demonstrated (Senaratne *et al.*, 1998; Kartmann *et al.*, 1999; Lichtinger *et al.*, 1999). Uptake of serine, but not of glycine, was reduced in the *ompATb* mutant compared to wild-type *M. tuberculosis.* This was interpreted as proof that OmpATb is a porin consistent with its apparent channel-forming activity *in vitro* (Raynaud *et al.*, 2002b). However, the overall permeability of the outer membrane of *M. tuberculosis* was reduced at pH 5.5 compared to pH 7.2, although the levels of OmpATb in the outer membrane were strongly increased (Raynaud *et al.*, 2002b). Considering these contradicting results, it is doubtful that OmpATb has significant porin function in *M. tuberculosis* (Niederweis, 2003). The observation that a central domain of approximately 150 amino acids is sufficient for the channel activity of OmpATb *in vitro* does not contribute to the understanding of its biological functions (Molle *et al.*, 2006).

Rv1698 was annotated as a protein of unknown function and was predicted as an outer membrane protein of *M. tuberculosis* (Song *et al.*, 2008). Lipid bilayer experiments of purified protein and uptake experiments in a porin mutant of *M. smegmatis* demonstrated that Rv1698 is a channel-forming membrane protein (Siroy *et al.*, 2008). Its surface accessibility was shown by protease experiments in whole cells (Song *et al.*, 2008). A homologue of Rv1698 exists in all mycolic acid-containing bacteria. Hence, Rv1698 represents the first member of a new class of channel proteins specific for mycolic acid-containing outer membranes.

8.4. Direct Diffusion of Hydrophobic Solutes Through the Cell Membranes

Hydrophobic molecules, in particular non-electrolytes, can easily diffuse through phospholipid bilayers. However, the lipopolysaccharide-containing outer membrane of Gram-negative bacteria constitutes a considerable permeability barrier which does not allow the penetration of even extremely hydrophobic β-lactam antibiotics (Nikaido *et al.*, 1983). The lipids in mycobacterial cell walls are likely to be organized in a very unusual, asymmetric bilayer (Nikaido *et al.*, 1993). Differential scanning calorimetry showed that the lipids in mycobacterial cell walls have very high phase transition temperatures in the range of 60–70 °C. This is suggestive of a lipid domain of extremely low fluidity (Liu *et al.*, 1995). Isolated cell walls of corynebacteria, which contain much shorter

corynemycolic acids, displayed a much lower temperature transition, suggesting that the fluidity of this lipid bilayer is mainly determined by the mycolic acids. Since direct diffusion of a hydrophobic molecule through a lipid membrane requires that it is dissolved in the lipid phase, the permeability of a particular membrane is directly correlated with its fluidity. This has been demonstrated directly by Nikaido and co-workers (Andersson *et al.*, 1996). It is concluded that the mycobacterial outer membrane presents a strong permeability barrier for hydrophobic molecules. On the other hand, there is emerging evidence that fatty acids rather than carbohydrates might be the dominant carbon source of *M. tuberculosis* after the onset of the immune response. This includes the requirement for isocitrate lyase for growth and persistence of *M. tuberculosis* in macrophages and in mice (McKinney *et al.*, 2000), and the induction of expression of genes encoding enzymes involved in the β-oxidation of fatty acids in macrophages (Schnappinger *et al.*, 2003) and mice (Dubnau *et al.*, 2005; Timm *et al.*, 2003). However, it is unknown how fatty acids are transported across the outer membrane of mycobacteria.

9. TRANSPORT ACROSS THE INNER MEMBRANE

9.1. Transporters of Carbohydrates

It has been widely documented that *M. smegmatis* can grow on many carbon sources such as polyols, pentoses, and hexoses (Edson, 1951; Franke and Schillinger, 1944; Izumori *et al.*, 1976). A recent comprehensive analysis of carbohydrate uptake systems revealed that the soil bacterium *M. smegmatis* has 28 putative carbohydrate transporters (Titgemeyer *et al.*, 2007). The majority of sugar transport systems (19/28) in *M. smegmatis* belongs to the ATP-binding cassette (ABC) transporter family (Fig. 6A). *M. smegmatis* further possesses one putative glycerol facilitator of the major intrinsic protein (MIP) family, four sugar permeases of the major facilitator super family (MFS) of which one was assigned as a glucose transporter, and one galactose permease of the sodium solute super family (SSS). Thus, inner membrane transport systems for polyols, pentoses, and hexoses are predicted to exist in *M. smegmatis* (Fig. 6A). This bioinformatic prediction was proven recently by the discovery of glucose permease GlcP (Msmeg_4182) from *M. smegmatis* mc^2155. Upon overexpression of this protein in a glucose-negative mutant of *E. coli*, it was shown that the strain was capable of glucose fermentation in addition to increased uptake of

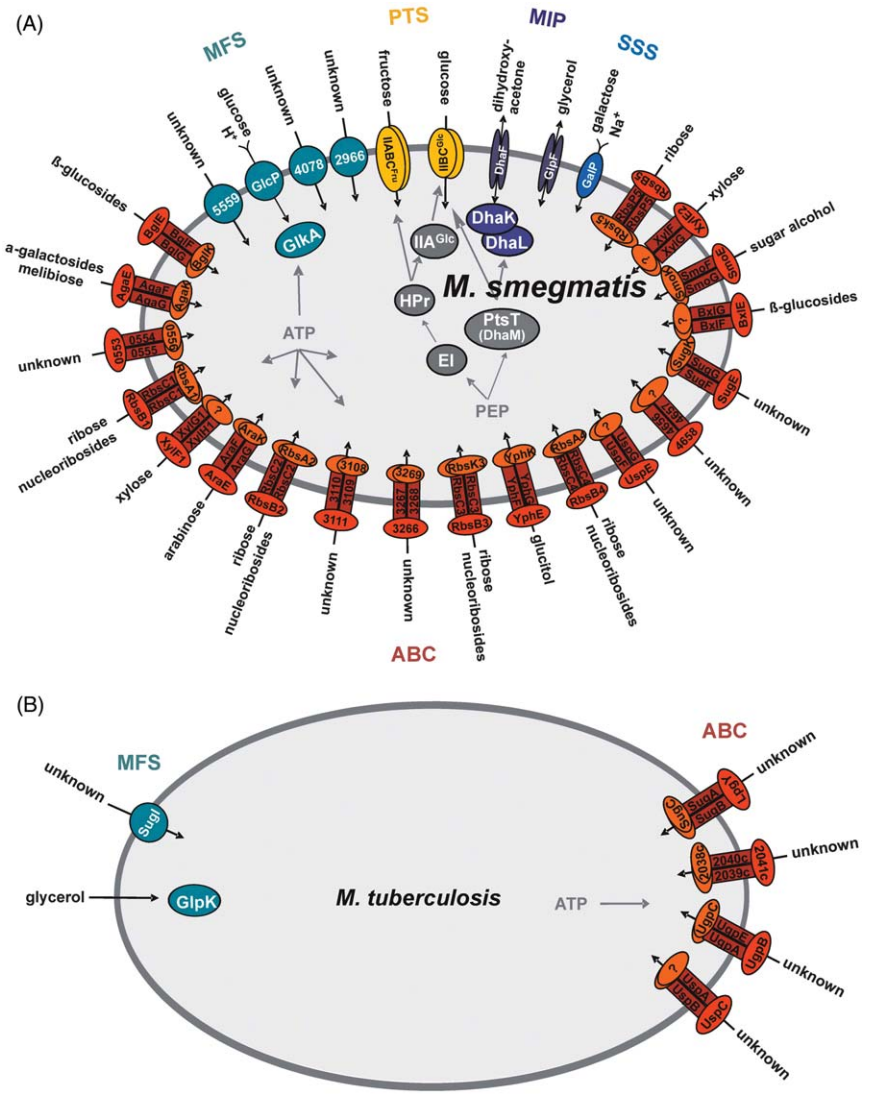

Figure 6 Transporters for uptake of carbohydrates across the inner membrane of *M. smegmatis* and *M. tuberculosis.* Shown are the transport proteins of the ATP-binding cassette (ABC, red), phosphotransferase system (PTS, yellow), major facilitator super family (MFS, green), major intrinsic protein family (MIP, dark blue), and the sodium solute super family (SSS, blue). The derived putative substrates are inferred from *in silico* analyses in combination with experimental data (Titgemeyer *et al.*, 2007). Systems are denoted by the protein name instead of their locus tags (MSMEG_XXXX) if the substrates were experimentally verified or were predicted with a high likelihood. Note that the OMs of *M. smegmatis* and *M. tuberculosis* were omitted for clarity reasons. The figure was taken from Niederweis (2008b) with permission from SGM. (See plate 4 in the color plate section.)

glucose (Pimentel-Schmitt *et al.*, 2008). Therefore, GlcP is the first known example of a high-affinity sugar permease (K_m of 19 μM) found in mycobacteria.

M. smegmatis does not grow on lactose, maltose, and sucrose as a sole carbon source (Titgemeyer *et al.*, 2007). Franke and Schillinger (1944) obtained the same result for lactose and maltose, but observed respiration of *M. smegmatis* in the presence of sucrose. *M. smegmatis* has at least three inner membrane transport systems with significant similarities to other bacterial disaccharide transporters (Niederweis, 2008b). However, the substrate specificities of the transporters encoded by *msmeg0501–0508* and *msmeg0509–0517* are not known. Growth of bacteria on disaccharides as sole carbon sources requires enzymes, which cleave the disaccharide and release the monosaccharides for further utilization. The absence of proteins similar to known bacterial β-D-galactosidases (LacZ of *E. coli*, BgaB of *B. circulans*, MbgA of *B. megaterium*, LacA of *S. coelicolor*) provides a molecular explanation for the inability of *M. smegmatis* to utilize lactose as a sole carbon source. By contrast, *M. smegmatis* has six homologues (MSMEG3191, 3577, 4901, 4902, 4685, 6477) of MalL of *B. subtilis*, which hydrolyzes maltose, longer maltodextrines up to maltohexose, isomaltose, and sucrose (Schönert *et al.*, 1999), and of the cytoplasmic trehalase TreC of *E. coli*, which cleaves trehalose-6-phosphate (Rimmele and Boos, 1994). It is conceivable that these enzymes are used in trehalose metabolism considering the unusual importance of trehalose in mycobacteria (Woodruff *et al.*, 2004; Murphy *et al.*, 2005) and the observation that trehalose was the only disaccharide which was used by *M. smegmatis* as a sole carbon source. However, it cannot be discounted that some of the enzymes with similarities to TreC and MalF may have roles in pathways distinct from trehalose metabolism.

Bioinformatic analysis of the genome of *M. tuberculosis* H37Rv revealed four ABC-type transporters and one permease of the MFS class for carbohydrates (Titgemeyer *et al.*, 2007) (Fig. 6B). These ABC transporters have been described earlier in a global analysis of the *M. tuberculosis* genome (Braibant *et al.*, 2000; Content *et al.*, 2005). It is obvious that *M. tuberculosis* is poorly equipped with carbohydrate transport systems in comparison to *M. smegmatis*. Two of the operons, the *lpgY sugABC* and the *uspABC* operons, are highly conserved between the two species. The proteins of the ABC^{Sug} and of the ABC^{Usp} systems share between 62 and 80% similar amino acids, compared to only 25–30% similar amino acids for the ABC^{Ugp} and the Rv2038c/Rv2039c/Rv2040c/Rv2041c systems. The similarities of all four ABC systems to known transporters outside the genus *Mycobacterium* is so low (<25%) that substrates of these transporters cannot be predicted (Titgemeyer *et al.*, 2007).

The ABC^{Sug} sugar transport system was predicted to be essential for virulence of *M. tuberculosis* in mice based on transposon site hybridization (TraSH) experiments (Sassetti *et al.*, 2003). Previously, it was suggested that this permease may transport maltose or maltodextrins (Borich *et al.*, 2000; Braibant *et al.*, 2000). However, both the similarities of ABC^{Sug} and of the corresponding substrate binding protein LpgY to the maltose transporters and periplasmic maltose binding proteins MalE of *E. coli* and *S. coelicolor* are very low (<25%). Thus, it is questionable whether maltose is the substrate of ABC^{Sug}. This conclusion is supported by the fact that neither *M. smegmatis*, which has a highly similar ABC^{Sug} system, nor *M. tuberculosis* (Edson, 1951) grow on maltose as a sole carbon source. It has to be noted that similar uncertainties exist about the substrate specificities of the four other carbohydrate uptake system of *M. tuberculosis* including the ABC^{Usp} transporter which was proposed to transport sn-glycerol-3-phosphate based on low protein similarities (Braibant *et al.*, 2000; Content *et al.*, 2005). The SugI transporter of the MFS class shows distant sequence similarity to the glucose permease GlcP (28%) of *S. coelicolor* and to the galactose (GalP, 24%) and arabinose (AraE, 24%) transporters of *E. coli*. Thus, the SugI system may transport a monosaccharide.

Glycerol is used as the standard carbon source to grow *M. tuberculosis*; however, no uptake system is known or apparent by sequence similarity (Titgemeyer *et al.*, 2007). Since *M. tuberculosis* grows with a generation time of 24 hours and it has been shown that glycerol can directly diffuse through lipid membranes both *in vitro* (Paula *et al.*, 1996) and *in vivo* (Eze and McElhaney, 1981), it is conceivable that the rate of glycerol intake by passive diffusion may be sufficient for growth. Incoming glycerol would then be converted by glycerol kinase (GlpK) to glycerol-3-phosphate to enter the route of central carbon metabolism (Fig. 6B). *M. tuberculosis* has one putative glycerol kinase that shows a high similarity to the two glycerol kinases of *M. smegmatis* (MSMEG_6759, 77% and MSMEG_6756, 57%) and to the two glycerol kinases from *S. coelicolor* SCO0509 (75%) and SCO1660 (59%).

9.2. Comparison of Inner Membrane Sugar Transporters of *M. smegmatis* and *M. tuberculosis*

The analysis of the carbohydrate uptake proteins in the genomes of *M. smegmatis* and *M. tuberculosis* confirms the very early phenotypical observations that the saprophytic mycobacteria have a much broader

spectrum of substrates, which they can use as sole carbon and energy sources (Edson, 1951). It is striking that the genome of *M. tuberculosis* has only five permeases for carbohydrate uptake compared to 28 of *M. smegmatis*. This suggests that the phagosome does not provide an environment rich in diverse sugars. The tantalizing conclusion is that an experimental analysis of the substrate specificity of the inner membrane carbohydrate transporters of *M. tuberculosis* may reveal the carbon sources available in the phagosome of human macrophages and/or in other cellular hideouts of *M. tuberculosis* inside the human body.

9.3. Transporters of Lipids

Several lines of evidence strongly suggest that *M. tuberculosis* switches from a carbohydrate to a fat diet after the onset of the adaptive immune response. (i) Biochemical studies suggest that, in chronically infected lung tissues, fatty acids might be a major source of carbon and energy for *M. tuberculosis* (Wheeler *et al.*, 1990). (ii) During the first 10 days of infection of mice, *M. tuberculosis* requires the sugar transporter SugAB for survival (Sassetti *et al.*, 2003). Thereafter, enzymes such as isocitrate lyase and malate synthases are essential for virulence (McKinney *et al.*, 2000). This indicates that lipids are the major carbon and energy source of *M. tuberculosis* because the glyoxylate shunt is required for running the citric acid cycle on acetate, which is produced by degradation of lipids through β-oxidation. (iii) *M. tuberculosis* possesses four genes encoding putative phospholipases C, *plcA*, *plcB*, *plcC*, and *plcD*. These genes are required for virulence of *M. tuberculosis* in mice (Raynaud *et al.*, 2002a). The fact that the phospholipases C are attached to the cell wall by lipid anchors argues for a role for these enzymes in the controlled release of fatty acids from phospholipids of the phagosomal membrane. However, while several proteins have been identified in *M. tuberculosis* that are involved in the transport of lipids from the cytoplasm to the outer membrane (Jackson *et al.*, 2007), the proteins involved in the transport of lipids across the outer membrane are yet unknown. By contrast, the mechanisms that govern the uptake of exogenous fatty acids are well-established in *E. coli* (Dirusso and Black, 2004). When the cell encounters long-chain fatty acids in the environment, these ligands bind to outer membrane protein FadL and via a ligand-induced conformational shift within the protein are transported into the periplasmic space. The more acidified environment of the periplasmic space promotes the formation of uncharged fatty acid molecules, which partition into and flip across the

inner membrane. Within the cytosol, the acyl-CoA synthase FadD partitions into the inner membrane, where it functions in the vectorial esterification of the long-chain fatty acids (Dirusso and Black, 2004). Consistent with the importance of lipid uptake, *M. tuberculosis* possesses numerous homologues of FadD proteins (Trivedi *et al.*, 2004). Moreover, an 82-gene cluster (Rv3492c–Rv3574) responsible for the catabolism of cholesterol including at least one set of genes that encode all enzymes necessary to perform the full cycle of β-oxidation and a multicomponent cholesterol uptake system consisting of *mce4ABCDEF* and an ABC-transporter *supAB* has been found in *M. tuberculosis* (Van der Geize *et al.*, 2007). It was shown that Mce4 forms the major cholesterol import system of *M. tuberculosis*, which can be used by the cell for both carbon and energy production as a primary carbon source (Pandey and Sassetti, 2008). Furthermore, mutation in *mce4* operon leads to attenuation during chronic phage of infection in mouse spleen and lungs and in activated, but not resting, macrophages suggesting that uptake of lipids function as a major carbon source at the later stage of *M. tuberculosis* infection (Sassetti *et al.*, 2003; Joshi *et al.*, 2006; Pandey and Sassetti, 2008).

9.4. Transporters of Phosphorus-Containing Solutes

Phosphorus is indispensable for energy supply, for the biosynthesis of nucleic acids and phospholipids, and many other cellular processes. While inorganic phosphate is the preferred source of phosphorous, many bacteria can also take up organic phosphates and release phosphate by the action of periplasmic phosphatases such as PhoA. Gram-negative bacteria employ sophisticated transport mechanisms to acquire phosphorus-containing nutrients from the environment. *E. coli* uses four phosphate transport systems Pst, Pit, GlpT, and UhpT to translocate inorganic phosphate across the inner membrane (van Veen, 1997). Part of the Pst system is the periplasmic protein PstS, which binds and transfers phosphate to the transmembrane components PstA and PstC. PstB hydrolyzes ATP and delivers energy for phosphate translocation across the inner membrane by PstA/PstC. Pst systems from Gram-negative bacteria bind and transport phosphate with binding constants and apparent transport K_m values in the submicromolar range. *M. tuberculosis* contains several copies of the genes encoding the Pst system (Braibant *et al.*, 1996). Two Pst components, PstS1 and PstS2, have been shown to be virulence factors in *M. tuberculosis* (Sassetti *et al.*, 2003; Peirs *et al.*, 2005). Further, *M. tuberculosis* contains two genes, *pitA* and *pitB*, which encode putative constitutive inorganic

phosphate transporters (Content *et al.*, 2005). The physiological role of the Pit transporters is unclear.

The single *pstSCAB* operon of the fast-growing *M. smegmatis* encodes a high-affinity Pst system with an apparent K_m value of 40 μM phosphate. A second high-affinity phosphate uptake system of *M. smegmatis* is encoded by the *phnDCE* operon (Gebhard *et al.*, 2006). However, even a *phnD pstS* double mutant did not show a reduced phosphate uptake suggesting the presence of a third high-affinity phosphate uptake system of *M. smegmatis* (Gebhard *et al.*, 2006). Considering the presence of three high-affinity phosphate uptake systems, which are inducible at low phosphate concentration in *M. smegmatis*, it is unclear why a *pstB* mutant showed a reduced phosphate transport (Bhatt *et al.*, 2000). Taken together, these results underline the importance of phosphate uptake for mycobacteria. The transcriptional profiles of *M. tuberculosis* and *Salmonella enterica* in infected macrophages revealed that the proteins involved in inorganic phosphate transport are up-regulated (Eriksson *et al.*, 2003; Schnappinger *et al.*, 2003) indicating that phosphate levels inside phagosomes of macrophages are indeed limited. Consistent with this conclusion, genes encoding efficient phosphate transport systems were found to be essential for the survival of *M. tuberculosis* in macrophages and mice (Sassetti *et al.*, 2003; Rengarajan *et al.*, 2005). However, it is unknown how inorganic or organic phosphates cross the outer membrane of *M. tuberculosis.* Since direct diffusion of phosphates through model lipid membranes is extremely slow (permeability coefficient of the monoanion: 5×10^{-12} cm/s; Chakrabarti and Deamer, 1992), it appears likely that slowly growing mycobacteria also use outer membrane pore proteins for uptake of phosphate. Indeed, the existence of a porin with anion specificity has been demonstrated (Lichtinger *et al.*, 1999). This porin still awaits discovery.

9.5. Transporters of Sulfur-Containing Solutes

Sulfur is essential in cells for biological activities such as translation, initiation, and maintenance of the redox potential. Transposon insertions in the *cysA* and *subI* genes of *M. bovis* BCG yielded methionine auxotrophs. These mutants were resistant to chromate and did not take up sulfate. These results identified the products of the genes *cysTWA* and *subI* as components of a sulfate permease and indicated that this transporter is the sole sulfate transporter of *M. bovis* BCG (McAdam *et al.*, 1995; Wooff *et al.*, 2002). The sensitivity of sulfate uptake to azide and 1,3-dicyclohexylcarbodiimide are characteristic of ABC transporters.

Survival of the *cysA* and *subI* mutants in mice was not different from wild-type *M. bovis* BCG. This indicated that, in the host, methionine may be a more important sulfur source than sulfate for growth of the *M. tuberculosis* complex. This may also explain how *M. leprae* remains a pathogen, despite being a natural methionine auxotroph because of its loss of *cysTWA* for sulfate transport (Wood, 1995). An alternative explanation may be that other putative sulfate transporters, such as the predicted sulfate permease (SulP) of *M. tuberculosis*, are induced *in vivo* and compensate for the loss of the CysTWA transporter (Content *et al.*, 2005). To this end, it was shown that overexpression of SulP protein Rv1739c in *E. coli* leads to increased uptake of sulfate by intact cells with its maximum at pH 6.0 (Zolotarev *et al.*, 2008). However, to prove the function of this protein as a sulfate transporter of *M. tuberculosis*, construction of knockout mutant and sulfate uptake experiments are required.

9.6. Transporters of Nitrogen-Containing Solutes

Nitrogen is an essential component of nearly all complex macromolecules in a bacterial cell, such as proteins, nucleic acids, and cell wall components. Ammonium is the preferred nitrogen source of many bacteria. In enteric bacteria, diffusion of uncharged ammonia (NH_3) through the cytoplasmic membrane into the cell is sufficient to support growth in the presence of high amounts of ammonium (NH_4^+) in the growth medium. Only when diffusion across the cell envelope becomes limiting for growth is the ammonium transporter AmtB synthesized. As in enteric bacteria, mycobacteria possess homologues of AmtB (Nolden *et al.*, 2001). However, no biochemical data are available for ammonium uptake by mycobacteria and the role of AmtB in this process. Two ammonium transporters, AmtB (Msmeg_2425) and Amt1 (Msmeg_6259), have been proposed to take up ammonium in *M. smegmatis* based on their transcriptional regulation by GlnR, a key regulator of nitrogen control in mycobacteria, and RT-PCR analysis under nitrogen limited conditions (Amon *et al.*, 2008). However, direct evidence on involvement of *amtB* and *amt1* into transport of ammonium in *M. smegmatis* is missing.

Nitric oxide (NO) is generated in large amounts within the macrophages and restricts the growth of *M. tuberculosis.* Nitrate can be produced by oxidation of nitric oxide and is an alternative source of nitrogen for bacteria within the human host. Early work in *E. coli* had suggested that *narK* was involved only in nitrite export (Rowe *et al.*, 1994), and so the homologous *narK2* in *M. tuberculosis* was annotated as a "nitrite extrusion protein."

More recent work with an *E. coli narK narU* double mutant indicated that the two proteins could transport nitrate into and nitrite out of the cell (Clegg *et al.*, 2002; Jia and Cole, 2005). In *M. tuberculosis*, four genes, *narK1* through *narK3* and *narU* are homologous to *narK* and *narU*. Since *M. tuberculosis* is unable to reduce nitrite, which could accumulate to toxic levels, it must be exported out of the cell. The *M. tuberculosis narK2* was shown to complement the *E. coli narK narU* mutant, supporting a role for *narK2* in nitrate reduction by coding for a transporter of nitrate into and nitrite out of the cell (Sohaskey and Wayne, 2003). Nitrate reduction by *M. tuberculosis* is regulated by control of nitrate transport into the cell by NarK2. It is proposed that NarK2 senses the redox state of the cell, possibly by monitoring the flow of electrons to cytochrome oxidase, and adjusts its activity so that nitrate is transported under reducing, but not under oxidizing, conditions (Sohaskey, 2005). Inhibition of nitrate transport by oxygen has been documented in other bacteria (Moir and Wood, 2001). It is intriguing that *M. tuberculosis*, classified as an obligate aerobe, should have such intricate control of an anaerobic enzyme system. Transcription of *narK2* is controlled by DosR/DevR, which responds to oxygen (O_2), nitric oxide (NO), and carbon monoxide (CO) (Ohno *et al.*, 2003; Voskuil *et al.*, 2003; Kumar *et al.*, 2008). Both the transcription of the *narK2* gene and the activity of NarK2 are controlled by similar signals (Sohaskey, 2005).

9.7. Transporters of Amino Acids

Many microorganisms use amino acids as a source of energy and/or nitrogen, and also for biosynthetic purposes. It was shown early in seminal papers by Yabu that D-amino acids are taken up rapidly by mycobacteria while the L-forms are transported at a much lower rate (Yabu, 1967, 1970, 1971). These results can be attributed to the specificity of the inner membrane transporters for the natural form of amino acids. It was also found early that *M. tuberculosis* cannot utilize amino acids to support metabolism in contrast to saprophytic mycobacteria (Edson, 1951). Nevertheless, some amino acids are taken up by *M. tuberculosis* and *M. bovis* BCG (Seth and Connell, 2000). In particular, uptake of arginine was examined because arginine also plays an important role in the cellular immune response as the substrate of the inducible nitric oxide synthase (iNOS) which generates nitric oxide to kill bacterial and parasitic pathogens in macrophages (Nathan and Shiloh, 2000; Chan *et al.*, 2001). Thus, competition between the pathogen and macrophages for arginine has been suggested to contribute to the outcome of infection (Mills, 2001).

Not surprisingly, *M. tuberculosis* has several genes encoding putative L-arginine uptake transporters: Rv0522, Rv1979c, Rv1999c, Rv2320c, and Rv3253c (Cole *et al.*, 1998). Transport of L-arginine, but not of L-lysine and L-ornithine, was reduced by 70% in a mutant of *M. bovis* BCG lacking the gene homologous to *rv0522*. This identified Rv0522 (GabP) as an arginine transporter of *M. tuberculosis* (Seth and Connell, 2000). The remaining 30% of L-arginine transport activity and the uptake of other cationic amino acids by the mutant are probably mediated by other amino acid permeases.

Some amino acids such as proline and glycine betaine are important in regulating the osmotic pressure in bacterial cells (Wood *et al.*, 2001). Recently, it was demonstrated that the ABC transporter ProXVWZ imports glycine betaine and protects *M. tuberculosis* against osmotic stress and thereby provides a growth advantage both *in vitro* and in macrophages (Price *et al.*, 2008).

9.8. Transporters of Inorganic Cations

Metal ions such as Fe^{2+}/Fe^{3+}, Cu^{+}/Cu^{2+}, and Zn^{2+} play structural and catalytic roles in metalloenzymes. Genome analysis of *M. tuberculosis* revealed 28 genes encoding a broad repertoire of putative metal ion transporters. They comprise eight families of secondary active transporters and three families of primary active transporters, including 12 "P" type ATPases, and represent approximately one quarter of all transporters in this organism. Potential metal ion specificities include K^{+}, Na^{+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+}, $Fe^{2+/3+}$, Hg^{2+}, AsO_2^{-}, and AsO_4^{3-}. Seventeen of these transporters are also encoded as complete open reading frames in *M. leprae*, suggesting a role in intracellular survival. The properties of these transporters, including the NRAMP orthologue MntH which transports manganese ions in other bacteria, has been reviewed recently (Agranoff and Krishna, 2004; Content *et al.*, 2005). Here we summarize only the new findings for the uptake of iron by *M. tuberculosis.*

Throughout the living world, iron is contained in the active centers of most redox enzymes. Because iron occurs in the insoluble Fe^{3+} form under oxic conditions (10^{-9} M Fe^{3+} in soil and water) (Ratledge and Dover, 2000), proteins and siderophores with high binding affinity are required to make Fe^{3+} biologically available. *M. tuberculosis* produces salicylate-containing siderophores named mycobactins. The more polar form (carboxymycobactin) is released into the medium, whereas the less polar form (mycobactin) remains cell-associated (Ratledge and Dover, 2000). Upon binding by siderophores, Fe^{3+} is transported into the bacterium and

released from the siderophore, possibly by reduction. In most bacteria, Fe^{3+}–siderophore complexes bind to specific receptor proteins on the cell surface and are actively transported into the cytoplasm by specialized proteins that belong to the family of ABC transporters (Braun and Killmann, 1999). It is suggested that three proteins, IrtA, IrtB, and Rv2895c, are mainly involved in iron homeostasis in *M. tuberculosis* (Farhana *et al.*, 2008). The ABC transporter IrtAB is required by *M. tuberculosis* to replicate in iron-deficient medium and to use carboxymycobactin as an iron source, indicating that IrtAB is involved in the transport of this compound (Rodriguez, 2006). Further evidence suggested that IrtA is a carboxymycobactin exporter based on *in vitro* reconstitution experiments, while the two-component IrtB-Rv2895c system functions as importer of Fe^{3+}-carboxymycobactin. A knockout mutant of *msmeg_6554*, the *irtA* homologue in *M. smegmatis*, displayed an impaired siderophore export that is restored upon complementation with *M. tuberculosis irtA* (Farhana *et al.*, 2008). Moreover, deletion of the *irtAB* genes in *M. tuberculosis* reduced its ability to survive in macrophages and in the lungs of mice. However, the lack of *irtAB* does not completely eliminate replication of *M. tuberculosis* in iron-deficient conditions, which indicates that other transporters can partially compensate for the lack of IrtAB (Rodriguez and Smith, 2006). Given the importance of iron, this is not surprising because pathogenic bacteria often have multiple pathways for iron acquisition. The genome of *M. tuberculosis* does not reveal other obvious siderophore transporters (Rodriguez, 2006). However, there are numerous ABC transporters, the substrates of which are unknown; one or more of these could contribute to iron uptake.

10. SENSORY PERCEPTION

Mycobacteria possess an extraordinary ability to adapt to and survive under adverse conditions, including nutrient deprivation, hypoxia, various exogenous stress conditions and, in the case of the pathogenic species, the intraphagosomal environment. A key requirement for this resilience is sensory perception of the surrounding conditions and the composition of the extracellular milieu. The genome of *M. tuberculosis* contains an array of signal transduction systems, including 11 complete two-component regulatory systems, 14 eukaryotic type serine/threonine protein kinases (STPKs) and phosphatases, and 10 extracytoplasmic function (ECF) sigma factors (Cole *et al.*, 1998), which have been the subject of several recent

reviews (Manganelli *et al.*, 2004; Tyagi and Sharma, 2004; Tucker *et al.*, 2007; Wehenkel *et al.*, 2008), as well as an unusually high number of nucleotide cyclases (McCue *et al.*, 2000). To date, only a few of these systems have been assigned a signal they respond to, reflecting the fact that very little is known about stimulus perception in these bacteria. The numbers of regulatory systems vary between species of mycobacteria, for example, with *M. smegmatis* containing only 6 of the 11 STPKs of *M. tuberculosis*, but up to 23 ECF sigma factors (Tyagi and Saini, 2004; Tyagi and Sharma, 2004; Waagmeester *et al.*, 2005) and *M. leprae* containing only 4 STPKs and 3 ECF sigma factors (Cole *et al.*, 2001; Tyagi and Saini, 2004; Tyagi and Sharma, 2004). These differences do not always correlate with differences in genome size, but may rather reflect specific adaptations to lifestyle, that is, pathogenic or environmental, and the various conditions encountered in the respective environments. The mechanisms by which mycobacteria sense different aspects of their environment are discussed below.

10.1. Sensing Nutrient Availability

10.1.1. Carbon Source

Mycobacteria can use a wide variety of carbon sources, such as carbohydrates, fatty acids, and lipids or the carbon backbones of amino acids (Wheeler and Blanchard, 2005). Fatty acids are thought to constitute the main source of carbon for mycobacteria within the host, and the switch from carbohydrate utilization to fatty acid utilization is crucial for the successful establishment of persistent infection (McKinney *et al.*, 2000; Höner zu Bentrup and Russell, 2001; Schnappinger *et al.*, 2003). Despite the importance of this adaptation, little is known about how mycobacteria sense the availability and adjust their metabolism to the utilization of the available carbon source. Microarray analysis showed several genes involved in fatty acid metabolism, for example, *icl* and *fadB2*, to be up-regulated after exposure of *M. tuberculosis* to acidic pH (Fisher *et al.*, 2002). The authors proposed that a drop in pH, which the bacteria will encounter upon entry into the phagosome, may constitute an indirect signal that the cell has now entered the host and will need to switch its metabolism to the utilization of fatty acids (Fisher *et al.*, 2002). However, Hampshire *et al.* (2004) found genes for fatty acid metabolism up-regulated during late-stationary phase of an *M. tuberculosis* culture grown on glucose and

glycerol. These experiments were done with careful control of temperature, oxygen-tension and pH, and therefore the signal that led to induction of fatty acid metabolism genes was most likely directly related to the depletion of carbohydrates. In another study, 6 of the 18 genes for fatty acid degradation, which were found to be induced in *M. tuberculosis* grown within murine bone-marrow macrophages, were also found to be induced *in vitro* during growth on palmitic acid (Schnappinger *et al.*, 2003), again suggesting a direct perception of the carbon source available for growth. Furthermore, utilization of fatty acids as carbon sources is not restricted to pathogenic species of mycobacteria, and it is therefore unlikely that an indirect signal associated with entry into the phagosome provides the sole stimulus for the appropriate change in gene expression.

A strain of *M. tuberculosis* in which expression of the membrane-bound STPK PknF had been knocked down using antisense RNA displayed an increase in glucose uptake, but no difference in uptake of other carbon sources such as leucine or oleic acid (Deol *et al.*, 2005). The authors, therefore, proposed an involvement of PknF in the repression of sugar transport. However, PknF was not found to be differentially expressed in a nutrient starvation model of *M. tuberculosis*, where key enzymes of glycolysis and the TCA cycle were down-regulated (Betts *et al.*, 2002), suggesting that alternative mechanisms for the regulation of carbon source utilization must exist. An adenylyl cyclase of *M. tuberculosis*, Rv2212, has been shown to respond to pH, and this response is stimulated by the presence of fatty acids (Abdel Motaal *et al.*, 2006). It is likely that this enzyme integrates these two signals and produces cAMP as the output signal, which then triggers an appropriate cellular response (Abdel Motaal *et al.*, 2006). A second adenylyl cyclase, Rv1264, is closely related to Rv2212 and was shown to bind oleic acid, and while the role of this fatty acid appears to be structural rather than regulatory, it cannot be excluded that it also serves as a functional signal in response to a changing lipid environment (Findeisen *et al.*, 2007). Further investigations into the sensing mechanisms involved in adaptation to different carbon sources are required to gain a better understanding of this crucial process in the physiology of mycobacteria.

10.1.2. Nitrogen Source

The preferred nitrogen source for mycobacteria are amino acids, particularly asparagine, glutamate, and aspartate, but they are also able to use inorganic sources such as ammonium salts (Ratledge, 1976). The uptake system for glutamate was found to be inducible by the presence

of glutamate in the growth medium in *M. tuberculosis* and *M. smegmatis*, but constitutive in *M. fortuitum* and *M. phlei* (Lyon *et al.*, 1967). This suggests that a sensory mechanism exists in some species of mycobacteria to detect glutamate, but its nature has not been identified to date.

In the absence of other nitrogen sources, mycobacteria can assimilate ammonium, a process that requires the enzymes glutamine synthetase (GS) and glutamate synthase. Uptake of ammonium in actinomycetes is via the AmtB transport system, and regulation of this transporter occurs post-translationally via GlnK, which in turn is regulated through reversible adenylation by GlnD in response to nitrogen levels. This process has been studied in detail in *C. glutamicum* (Strosser *et al.*, 2004), but not yet in mycobacteria. The gene organization as an *amtB–glnK–glnD* operon is conserved within the Actinomycetales, and it is therefore likely that the mechanism of regulation is also conserved (Harper *et al.*, 2008).

In addition to the ammonium uptake system, the activity of GS is also subject to nitrogen-dependent regulation. In *S. coelicolor*, post-translational control of GS activity is via reversible adenylation by GlnE (Fink *et al.*, 1999), and because GlnE is also present in the genome of *M. tuberculosis* (Cole *et al.*, 1998) regulation of GS activity in this bacterium is thought to involve the same mechanism (Harper *et al.*, 2008).

Transcriptional regulation of the genes involved in nitrogen metabolism, including *amtB* and *glnA* (encoding GS) in *S. coelicolor* requires the global regulator of nitrogen metabolism, GlnR (Tiffert *et al.*, 2008). Some evidence exists that this mechanism of regulation is conserved in many actinomycetes, including *M. tuberculosis* (Tiffert *et al.*, 2008). The primary sensor for the nitrogen status has not yet been identified in actinomycetes, nor is it known which molecule constitutes the signal, although intracellular concentrations of 2-oxoglutarate or ammonium ions appear to be likely candidates (Harper *et al.*, 2008).

10.1.3. Phosphate and Sulfate (Inorganic Anions)

Inorganic phosphate is the main source of phosphorus to mycobacteria, and genes involved in its uptake have been implicated in virulence of *M. tuberculosis* (Collins *et al.*, 2003; Peirs *et al.*, 2005). The two-component regulatory system responsible for phosphate limitation induced expression of high-affinity phosphate uptake systems and other genes involved in acquisition of phosphate was recently identified in *M. smegmatis* (Glover *et al.*, 2007). SenX3 is a membrane-bound histidine kinase that responds to low extracellular concentrations of phosphate by phosphorylation of the cognate response regulator, RegX3, leading to activation of gene

expression. Expression of RegX3-dependent genes in *M. smegmatis* is deregulated in mutants lacking the high-affinity phosphate transport system Pst (Kriakov *et al.*, 2003; Gebhard and Cook, 2008), and it has been proposed that the Pst transporter constitutes the actual sensor of phosphate availability. Similar to the situation in *E. coli* (Wanner, 1996), mycobacterial SenX3 lacks an obvious extracellular loop and is probably unable to bind phosphate itself, but obtains this information indirectly via the Pst system (Glover *et al.*, 2007). A further protein, PhnF, is involved in regulation of phosphate transport in *M. smegmatis*. PhnF is a cytoplasmic protein and predicted to respond to a small soluble molecule present in the cell under phosphate-limited conditions, but the nature of this ligand has not been identified (Gebhard and Cook, 2008).

Expression of mycobacterial genes involved in assimilation and metabolism of sulfate is dependent on environmental signals such as sulfur limitation and oxidative stress as reviewed in Bhave *et al.* (2007) and Schelle and Bertozzi (2006). This regulation has been studied in some detail for key genes of sulfate assimilation, *cysD* and *cysNC*, of *M. tuberculosis* (Pinto *et al.*, 2004), but the sensing or regulatory mechanism involved remains unknown. One candidate for the control of sulfur-metabolism genes in response to environmental stimuli is the ECF sigma factor SigH of *M. tuberculosis*. Manganelli *et al.* (2002) were able to show that induction of several genes of cysteine biosynthesis in response to oxidative stress by diamide treatment was abolished in a SigH deletion mutant.

10.1.4. Metals and Trace Elements (Inorganic Cations)

Potassium is an important intracellular ion, largely due to its role in counteracting osmotic pressure. The high-affinity potassium transport system KdpFABC is, therefore, carefully regulated in bacteria in response to extracellular potassium concentration and osmotic pressure (Gassel and Altendorf, 2001). The *kdpFABC* operons of *M. tuberculosis* and *M. smegmatis* were found to be induced by low potassium concentrations, and the regulatory mechanisms have been studied (Steyn *et al.*, 2003). Expression of *M. tuberculosis kdpFABC* is controlled by the KdpDE two-component system. The KdpD sensor kinase interacts with two accessory membrane proteins, LprF and LprJ, and because KdpD does not contain an obvious extracellular sensing domain, it is thought that these proteins modulate KdpD activity, presumably in response to the extracellular K^+ concentration (Steyn *et al.*, 2003).

Iron is an essential component of several metabolic enzymes, but excess iron in the cell is harmful, due to the release of oxidative agents by the

Fenton reaction. Its intracellular concentration, therefore, has to be very tightly controlled. This is reflected in the large number of genes (a total of 153 genes) differentially expressed in response to iron availability in *M. tuberculosis* (Rodriguez *et al.*, 2002). Several genes involved in iron acquisition have also been found to be induced by growth of *M. tuberculosis* in macrophages, suggesting the bacteria encounter conditions of iron limitation during infection (Gold *et al.*, 2001; Schnappinger *et al.*, 2003). In mycobacteria, iron-dependent gene expression is controlled by the DtxR homologue IdeR. DtxR-like proteins act as repressors of transcription when in complex with Fe(II), and such a mechanism has been shown for IdeR of *M. tuberculosis* (Schmitt *et al.*, 1995; Pohl *et al.*, 1999). Thus IdeR acts as a direct sensor of intracellular iron concentrations in mycobacteria, inducing proteins for iron storage and repressing proteins for iron acquisition under conditions of excess iron (Gold *et al.*, 2001; Rodriguez *et al.*, 2002). Curiously, *ideR* of *M. tuberculosis* is an essential gene (Rodriguez *et al.*, 2002), while that of *M. smegmatis* is dispensable (Dussurget *et al.*, 1996). A second DtxR homologue, SirR, exists in the genome of *M. tuberculosis* (Cole *et al.*, 1998), but this protein contains a sequence with similarity to manganese binding sites and has been proposed to play a role in manganese-responsive gene regulation (Rodriguez, 2006).

Copper is another metal that is required for the activity of some enzymes, yet can cause oxidative damage when present as free Cu(I) within the cell. Liu *et al.* (2007) identified an operon, the *cso* operon, in *M. tuberculosis*, which is induced at high extracellular copper concentrations . One of the genes in this operon, *ctpV*, encodes a putative copper transporter and is thought to be required for detoxification of intracellular copper. It was shown that expression of the *cso* operon is regulated by the copper-sensor CsoR, encoded by the first gene of the *cso* operon. CsoR acts as a repressor in the absence of copper by binding to the *cso*-promoter. CsoR detects intracellular copper directly by binding Cu(I) ions, and in its Cu(I)-bound form is unable to bind to DNA, and repression of the *cso* operon is relieved (Liu *et al.*, 2007).

Regulation of zinc uptake in environmental and pathogenic mycobacteria occurs via yet another metal responsive transcriptional regulator, Zur (also named FurB). It is encoded in the Rv2358-*furB* operon in *M. smegmatis* and *M. tuberculosis*, and induced in response to high zinc concentrations (Milano *et al.*, 2004). This induction occurs via a second regulatory protein, encoded by Rv2358, which acts as a repressor in the absence of zinc and then repression is relieved when zinc is bound (Canneva *et al.*, 2005). Zur (FurB) itself also acts as a transcriptional repressor, but binds to the promoters of its target genes in the presence of

Zn(II) ions (Lucarelli *et al.*, 2007; Maciag *et al.*, 2007). Microarray analysis of a *zur* mutant of *M. tuberculosis* together with DNA-binding assays revealed eight promoters (24 genes) that are directly controlled through Zur. Among these are several genes with putative functions in zinc uptake and it has been proposed that Zur is involved in regulation of zinc transport (Maciag *et al.*, 2007). Rv2358 and Zur probably constitute a zinc-responsive regulatory network of mycobacteria to ensure sufficient zinc uptake while avoiding the accumulation of toxic intracellular concentrations. At low extracellular zinc concentrations, transcription of Rv2358-*furB* is repressed by Rv2358 and any Zur proteins present in the cell are inactive, leading to expression of zinc-uptake systems. At high extracellular zinc concentrations, repression of *furB* transcription is relieved, Zur binds intracellular Zn(II) ions, represses transcription of zinc uptake and therefore prevents excess uptake.

10.2. Oxygen and Redox State

Mycobacteria are obligate aerobes and as such have to possess mechanisms to detect the ambient oxygen tension to enable them to adapt to changes in oxygen availability by adjusting their metabolism accordingly. Furthermore, gradual depletion of oxygen has been implicated in entry of *M. tuberculosis* into non-replicating persistence and latency (Wayne and Sohaskey, 2001). The mechanisms by which mycobacteria sense oxygen have been extensively studied, and the two major sensory systems known to date, the DosT/DosS/DosR system and WhiB3, are discussed below.

10.2.1. The Dos System

The dormancy survival, or Dos, system consists of the three proteins DosR (or DevR), DosS (or DevS), and DosT. DosR has been shown to act as a transcriptional regulator, which is responsible for the induction of nearly all hypoxia-induced genes of *M. tuberculosis* (Park *et al.*, 2003). The activity of DosR is regulated by the two sensor kinases, DosS and DosT, which phosphorylate DosR under conditions of low oxygen tension or in the presence of NO or CO (Roberts *et al.*, 2004; Kumar *et al.*, 2007; Sousa *et al.*, 2007). DosS and DosT both appear to contribute about equally to the activation of DosR in response to hypoxia. Single deletions of either *dosS* or *dosT* in *M. tuberculosis* H37Rv caused about a 45% decrease in activation of the hypoxia reporter gene *hspX*, while in a *dosS*/*dosT* double mutant induction of the *hspX* promoter was abolished completely

(Roberts *et al.*, 2004). The Dos system is not restricted to pathogenic mycobacteria, because homologues of DosR and DosS were identified in *M. smegmatis* (Mayuri *et al.*, 2002), and an *M. smegmatis dosR* deletion mutant was impaired in survival of oxygen limited stationary phase (O'Toole *et al.*, 2003).

Two independent studies recently revealed the mechanism of activation of both DosT and DosS in response to oxygen, NO, and CO. Both proteins contain one heme as a prosthetic group, and both proteins can bind oxygen, NO, and CO (Kumar *et al.*, 2007; Sousa *et al.*, 2007). While DosT is relatively resistant to oxidation to the ferric form (Sousa *et al.*, 2007), DosS is rapidly converted to the met form (Fe^{3+}) when exposed to oxygen (Kumar *et al.*, 2007). The autokinase activity of DosS is drastically reduced when oxidized to met-DosS, and this finding led to the proposal that DosS acts as a redox sensor (Kumar *et al.*, 2007). This is consistent with the findings of Sousa *et al.* (2007), who found only a sixfold difference in autokinase activity between oxy-DosS and deoxy-DosS. In contrast, the activity of DosT is regulated solely by its oxygenation state, where deoxy-DosT has about a 50 times higher autokinase activity than oxy-DosT (Sousa *et al.*, 2007). DosT is, therefore, thought to be an oxygen sensor, which is active in the absence of oxygen and loses its activity in the presence of oxygen (Kumar *et al.*, 2007; Sousa *et al.*, 2007). In addition to hypoxia, the Dos-regulon has also be shown to be activated by exposure of *M. tuberculosis* to NO or CO (Voskuil *et al.*, 2003; Kumar *et al.*, 2008; Shiloh *et al.*, 2008). An elegant study of the binding kinetics of oxygen, NO, and CO to DosT and DosS have shown that this is most likely due to competition for binding between the ligands. The equilibrium dissociation constants (K_d) of NO and CO binding to DosT are 5,000 and 30 times lower, respectively, than that of oxygen binding (Sousa *et al.*, 2007). NO and CO, unlike oxygen, do not "switch off" DosT activity, but because of their much stronger binding displace oxygen from its binding site, thus simulating low oxygen tension and activating DosT autophosphorylation activity (Sousa *et al.*, 2007). A similar phenomenon was observed for DosS, where NO and CO appear to stabilize DosS in its active, ferrous form (Fe^{2+}), thus maintaining the active form of the protein even in the presence of oxygen (Kumar *et al.*, 2007).

10.2.2. WhiB3

Another protein, which has been implicated in sensing of oxygen and redox state by mycobacteria, is WhiB3. The *whiB3* gene was found to be strongly induced in *M. tuberculosis* during acute infection of mouse lungs and also

during growth in resting bone marrow-derived macrophages, but repressed after INF-γ activation of the macrophages (Banaiee *et al.*, 2006). The authors hypothesized that this might be due to production of NO by activated macrophages, but were unable to identify an *in vitro* stimulus (NO exposure, oxidative stress, hypoxia, or other stresses) leading to *whiB3* induction (Banaiee *et al.*, 2006). More recently, Singh *et al.* (2007) were able to show that WhiB3 contains a 4Fe–4S cluster, which can bind NO. Furthermore, in the presence of oxygen, the WhiB3 $[4Fe–4S]^{2+}$ cluster is degraded first to a $[3Fe–4S]^{+}$ cluster, then a $[2Fe–2S]^{2+}$ and subsequently lost altogether, in a mechanism reminiscent of the one found in the *E. coli* oxygen sensor FNR (Crack *et al.*, 2004; Singh *et al.*, 2007). Apo-WhiB3 was shown to have protein disulfide reductase activity, and it has been proposed that loss of the Fe–S cluster is required to gain this activity (Suhail Alam and Agrawal, 2008). WhiB3-mediated response to the presence of oxygen, therefore, may occur through direct control of the activity of metabolic proteins or through modification of transcriptional regulators (Suhail Alam and Agrawal, 2008). A role for WhiB3 in regulation of the transcriptional machinery in mycobacteria may be supported by the finding that WhiB3 interacts with the major sigma factor, SigA (RpoV) (Steyn *et al.*, 2002), but the effect of this interaction on SigA activity is not known, and further study is required to understand the precise role of WhiB3 in mediating any adaptation of mycobacteria to changes in oxygen tension.

10.2.3. Additional Systems

While the Dos system and WhiB3 are the most studied regarding the perception of oxygen tension by mycobacteria, they are probably not the only systems used by these bacteria. For example, DosR of *M. tuberculosis* H37Rv was shown not to be strictly required for survival of hypoxia *in vitro* (Rustad *et al.*, 2008). The authors further found that expression of Dos regulon genes in response to hypoxia was transient, with expression of about half of the 50 DosR-dependent genes returning to baseline levels within 24 hours of hypoxia. In contrast, a set of 230 genes was significantly up-regulated at 4 and 7 days of hypoxia, but not initially, and were termed the enduring hypoxic response (EHR) (Rustad *et al.*, 2008). Induction of EHR was independent of DosR, suggesting that other sensory and regulatory mechanisms must exist to signal prolonged exposure to hypoxia. Strikingly, the EHR genes contained an unusually high number of regulatory genes (FurA, FurB, PhoP, three WhiB family members and two ECF sigma factors, SigH and SigE), but it is not yet known which of

these, if any, are involved in entry into EHR (Rustad *et al.*, 2008). A study into the effect of addition of cAMP to growing cultures or *M. bovis* BCG or *M. tuberculosis* H37Rv found that the number of genes affected by cAMP was larger under low oxygen, CO_2 enriched conditions than under ambient air (Gazdik and McDonough, 2005). The authors proposed that cAMP may be used by mycobacteria as a signaling molecule in response to hypoxic conditions. However, the signal leading to cAMP synthesis, or which adenylyl cyclase might catalyze this reaction under hypoxia, remains unknown.

10.3. Exogenous Stress Conditions

10.3.1. Oxidative Stress

As obligate aerobes, mycobacteria are exposed to oxidative stress. Furthermore, the killing mechanisms of macrophages include production of reactive oxygen species, and the ability of pathogenic mycobacteria to respond to oxidative stress is thus essential for establishing an infection. In enteric bacteria, the oxidative-stress response is mediated through the transcriptional regulator OxyR (Christman *et al.*, 1985; Tartaglia *et al.*, 1989). In most mycobacteria, with the exception of *M. leprae* and *M. avium*, the *oxyR* orthologous genes are inactivated by several mutations (Deretic *et al.*, 1995; Sherman *et al.*, 1995), and induction of an oxidative-stress response must, therefore, be via a different mechanism. There appear to be considerable differences between saprophytic and pathogenic species, because *M. smegmatis* was found to mount a protective oxidative-stress response, while *M. tuberculosis* and *M. avium* could not (Sherman *et al.*, 1995). Several studies have focused on how mycobacteria perceive and respond to reactive oxygen species, and a plethora of sensory and regulatory systems have been identified, confirming the importance of this stress condition to mycobacteria.

A mutant of *M. smegmatis* in the iron-responsive regulator IdeR had an increased sensitivity to oxidative stress (Dussurget *et al.*, 1996), and this was shown to be due to reduced expression of catalase-peroxidase (KatG) and iron-dependent superoxide dismutase (SodA) (Dussurget *et al.*, 1998). However, the molecular mechanism of *katG* and *sodA* regulation by IdeR in *M. smegmatis* remain to be discovered. Although an *ideR* mutant of *M. tuberculosis* H37Rv also showed an increase in sensitivity to oxidative

stress, neither expression of *katG* and *sodA* nor the activities of the respective proteins was affected by the mutation (Rodriguez *et al.*, 2002). The role of IdeR in the oxidative-stress response of *M. tuberculosis*, therefore, appears to differ from that in *M. smegmatis*. A further example of coupling the response to oxidative stress with iron metabolism in mycobacteria is regulation of *katG* by FurA, which is annotated as a ferric uptake regulator (Cole *et al.*, 1998), although a definite role for this protein in control of iron uptake in mycobacteria could not be shown (Pym *et al.*, 2001; Zahrt and Deretic, 2001). FurA is encoded by a gene immediately upstream of *katG* in all mycobacterial species tested, and was shown to be involved in the repression of *katG* but not *katE* or *ahpC* in *M. smegmatis* (Zahrt and Deretic, 2001). Similar results were obtained for *M. tuberculosis*, where it was shown that expression of the *furA–katG* operon, but not of *sodA* or *ahpC*, was repressed by FurA (Pym *et al.*, 2001). The mechanism by which FurA detects oxidative stress, as well as how it represses transcription of *katG* and which other genes might be under its control, is not yet known.

The protein WhiB4 of *M. tuberculosis* has also been implicated to play a role during oxidative stress (Alam *et al.*, 2007). Like WhiB3 discussed above, WhiB4 contains a [4Fe–4S] cluster, and this cluster is converted to a [2Fe–2S] cluster and subsequently lost from the protein upon exposure to oxygen or hydrogen peroxide. Upon loss of the Fe–S cluster, two intramolecular disulfide bonds are formed and WhiB4 gains protein disulfide reductase activity. This gain of activity under oxidative conditions led to the proposal that WhiB4, and possibly other WhiB proteins, play an important role in redox signaling during oxidative stress in *M. tuberculosis*. The effect of WhiB4 may be direct through reactivation of cellular proteins which have formed non-specific disulfides, or indirect through modification of the activity of transcriptional regulators (Alam *et al.*, 2007).

Several alternative and ECF sigma factors have been shown to play a role in resistance of mycobacteria to oxidative stress. These include SigH (Raman *et al.*, 2001; Manganelli *et al.*, 2002) and SigJ (Hu *et al.*, 2004) in *M. tuberculosis*, and SigH (Fernandes *et al.*, 1999) and SigF (Gebhard *et al.*, 2008) in *M. smegmatis*. Of these, the role of *M. tuberculosis* SigH has been studied in most detail and it was shown that activity of the sigma factor is controlled by a redox-responsive anti-sigma factor, RshA (Song *et al.*, 2003). The gene arrangement of *sigH–rshA* is conserved in *M. tuberculosis*, *M. smegmatis*, and *M. avium*. Binding of *M. tuberculosis* RshA to SigH occurs only under reducing conditions, with SigH being released and therefore active during oxidative stress (Song *et al.*, 2003).

10.3.2. Extracellular pH

Maintenance of a near-neutral internal pH despite changes in external pH is essential for all bacteria. Environmental mycobacteria are likely to encounter acidic pH in their soil habitat (Iivanainen *et al.*, 1999), and pathogenic mycobacteria are exposed to a drop in pH upon entry into the phagosome (Amer and Swanson, 2002). A reduction in external pH was shown to cause differential expression of a large number of genes in *M. tuberculosis* (Fisher *et al.*, 2002; Saviola *et al.*, 2003), and *M. smegmatis* and *M. bovis* BCG exhibited intracellular pH homeostasis over a large range of external pH values (Rao *et al.*, 2001). These data imply that mycobacteria are able to perceive the pH of their environment, and two adenylyl cyclases have been suggested to fulfill that role in *M. tuberculosis*. Rv1264 has a 40-fold increased adenylyl cyclase activity at pH 6 as compared to pH 8, and this pH-dependent activation is mediated by a pH-responsive catalytic domain (Tews *et al.*, 2005). In response to pH, the protein undergoes major conformational rearrangements and these may involve an unsaturated fatty acid as a structural element (Tews *et al.*, 2005; Findeisen *et al.*, 2007). In the closely related adenylyl cyclase Rv2212, pH-sensing activity is strongly induced by binding of unsaturated fatty acids, but the mechanism of this effect is not fully understood (Abdel Motaal *et al.*, 2006). While both Rv1264 and Rv2212 have increased activity at acidic pH, the mechanism for activation appears to be different between the two proteins, because the pH response of Rv1264 is largely due to an increase of V_{max} (Tews *et al.*, 2005), while that of Rv2212 is due to an increase in substrate affinity for ATP (Abdel Motaal *et al.*, 2006).

10.3.3. Heat Shock

The heat-shock response of *M. tuberculosis* involves the up-regulation of over 100 genes, including those encoding the ECF sigma factors SigH and SigE, the general stress sigma factor SigB, and conserved heat-shock proteins such as the Hsp60/GroE family and Hsp70 (DnaK) (Stewart *et al.*, 2002). Control of the heat-shock response involves a network of overlapping regulatory circuits. SigH and SigE up-regulate expression of many genes in response to elevated temperatures (Fernandes *et al.*, 1999; Manganelli *et al.*, 2001, 2002; Raman *et al.*, 2001), while HspR and HrcA act as transcriptional repressors (Stewart *et al.*, 2002). Furthermore, heat inducible expression of SigE is dependent on SigH, and expression of SigB depends on both SigE and SigH (Raman *et al.*, 2001). The mode of signal (i.e., heat) recognition by these regulators is not well-understood,

but evidence exists that the interaction between SigH and its anti-sigma factor RshA is disrupted by elevated temperatures (45–55 °C), leading to activation of SigH (Song *et al.*, 2003).

Curiously, *M. leprae*, which has a reduced ability to survive temperatures above 33 °C, appears to lack a heat-shock response. The key heat-shock genes of *M. tuberculosis* are not induced by a shift from 33 to 37 °C or 45 °C in *M. leprae*, although functional copies of the heat-shock regulators SigE, HspR, and HcrA are present (Williams *et al.*, 2007). However, *sigH* of *M. leprae* is a pseudogene, and it is therefore likely that the lack of heat-shock response in this bacterium is due to a lack of SigH-dependent induction of SigE and SigB (Williams *et al.*, 2007), further confirming the central role of this ECF sigma factor in the orchestration of a heat-shock response in mycobacteria.

10.3.4. Entry into Phagosome/Macrophage

Due to the importance of this process for the virulence of *M. tuberculosis*, the reaction of the bacterium to entry into the phagosome has been the focus of many studies. At the same time, it is extraordinarily difficult to dissect the various responses occurring because there is no one stimulus associated with the transition to the intracellular environment. The bacteria will be exposed to a drop in pH, elevated temperatures, reactive oxygen, and nitrogen species as well as a change in nutrient availability (Amer and Swanson, 2002; Schnappinger *et al.*, 2003). Several two-component regulatory systems have been implicated in being important for the adaptation to intracellular growth based on attenuation of corresponding mutants. However, it is difficult to ascertain whether the attenuation is due to a specific requirement of the regulatory system in the intraphagosomal environment or simply reflects a decrease in the bacteria's ability to cope with adverse conditions.

One of these two-component systems is MtrAB. The gene encoding the response regulator, *mtrA*, is essential in *M. tuberculosis* (Zahrt and Deretic, 2000). A transcriptional fusion of *M. tuberculosis mtrA* to *gfp* is up-regulated during growth of *M. bovis* BCG in murine macrophages as compared to *in vitro* growth (Via *et al.*, 1996), but constitutively expressed in *M. tuberculosis* grown *in vitro* or murine or human macrophages (Zahrt and Deretic, 2000), suggesting differences in *mtrA* expression between virulent and avirulent strains. Overexpression of MtrA in *M. tuberculosis* caused attenuation for growth in macrophages, mouse spleens and lungs and an impaired ability to block phagosome–lysosome fusion. This depended on the phosphorylation-competence of MtrA, suggesting that

maintenance of a certain MtrA to phospho-MtrA ratio within the cell was essential for proliferation of *M. tuberculosis* (Asensio *et al.*, 2006). The expression of the essential replication initiation gene, *dnaA*, is controlled by MtrA, and it has been proposed that overexpression of *dnaA* by excess phospho-MtrA during infection could lead to the observed growth defect (Fol *et al.*, 2006). MtrB of *M. avium* has been proposed to play a role in regulation of cell surface proteins and permeability of the cell envelope (Cangelosi *et al.*, 2006). However, neither the environmental signal detected by MtrAB nor the complete MtrAB regulon have been identified to date.

The two-component system MprAB was identified as being required for virulence during the persistent stage of infection, and mutants in the response regulator gene, *mprA*, had altered growth characteristics in murine and human macrophages (Zahrt and Deretic, 2001; Zahrt *et al.*, 2003). As for MtrAB, there were distinct differences between virulent and avirulent strains, supporting a role for this system during infection: in *M. tuberculosis* H37Rv, *mprA* expression was not detected during growth in resting macrophages and an *mprA* mutant had increased intracellular survival compared to the wild type (Zahrt and Deretic, 2001), whereas in *M. bovis* BCG, *mprA* was induced during intracellular growth and an *mprA* mutant was impaired in survival (Zahrt and Deretic, 2001; Zahrt *et al.*, 2003). Microarray analysis showed that the MprAB system regulates several sets of genes under different growth conditions, including normal growth and detergent stress (He *et al.*, 2006). Two of the genes under direct control of MprAB and induced by detergent stress are the general stress sigma factors, SigE and SigB (He *et al.*, 2006; Pang *et al.*, 2007), indicating the existence of a complex regulatory network, because expression of *sigB* and of *mprAB* itself is also controlled by SigE under these conditions (Manganelli *et al.*, 2001).

Mutants of *M. tuberculosis* in a further two-component regulatory system, the PhoPR system, were impaired in growth within macrophages and attenuated in virulence in mice (Perez *et al.*, 2001; Walters *et al.*, 2006). PhoP was shown to control expression of several genes involved in synthesis of complex cell wall lipids, some of which had previously been identified as virulence genes (Walters *et al.*, 2006). Because of its sequence similarity to the Mg^{2+}-responsive system PhoPQ of *Salmonella* (Lejona *et al.*, 2003), *M. tuberculosis* PhoPR was also thought to respond to Mg^{2+} limitation, and the *phoP* mutant indeed displayed an increased requirement for Mg^{2+} ions for growth (Walters *et al.*, 2006). However, no clear evidence is available that Mg^{2+} limitation constitutes the signal for PhoP-dependent gene expression, and it is rather thought that the structural alterations in

the cell envelope necessitate Mg^{2+} ions for the stabilization of the mutants' cell wall (Walters *et al.*, 2006). The physiological signal for PhoPR therefore remains to be discovered.

The PrrAB system of *M. tuberculosis* was shown to be transiently required during early stages of infection in murine macrophages and this period coincided with induction of the *prrA*-promoter (Ewann *et al.*, 2002). Other systems that have been shown to affect the intracellular growth of *M. tuberculosis* include DosRS (Malhotra *et al.*, 2004), and SenX3-RegX3 (Parish *et al.*, 2003), which are discussed above. Further study is required to elucidate the role of these and other sensory systems during growth of mycobacteria within the host, and to establish precisely what the signal perceived by each of these systems is.

11. ENERGETICS OF MYCOBACTERIAL GROWTH

M. tuberculosis is a metabolically versatile bacterium able to oxidize a variety of carbon sources, including carbohydrates, fatty acids, TCA cycle intermediates, and host lipids (e.g., cholesterol). A striking feature of *M. tuberculosis* metabolism is the ability of the bacterium to remain viable in the absence of cell growth and adapt to changing environments in the host. The mechanisms responsible for these adaptations are poorly understood.

11.1. Mycobacterial Metabolism *In Vitro*

A large body of detailed literature is available on the physiology of mycobacterial metabolism (Edson, 1951; Ramakrishnan *et al.*, 1972; Ratledge, 1976, 1982; Wheeler and Blanchard, 2005). During *in vitro* growth of *M. tuberculosis*, various carbon and energy sources feed into central metabolic pathways where they are oxidized to pyruvate and ultimately CO_2. In mycobacteria, the primary route of glucose conversion to pyruvate is the Embden–Meyerhof–Parnas (EMP) pathway. Approximately 70% of the glucose is directed down the EMP pathway and the remaining glucose (30%) flows into the pentose phosphate pathway to generate C_5 and C_4 sugars and reducing power in the form of NADPH (Ratledge, 1976). No other pathways exist for sugar catabolism in pathogenic mycobacteria. The preferred carbon source for mycobacteria is glycerol and the metabolic route for its degradation is well-established in

these bacteria (for reviews see Ratledge, 1976; Wheeler and Blanchard, 2005). The metabolism of glycerol involves the phosphorylation of glycerol to form glycerol-3-phosphate via glycerol kinase, followed by the oxidation of this intermediate to dihydroxyacetone phosphate via glycerol-3-phosphate dehydrogenase. This triose phosphate is further metabolized via the EMP pathway and the Krebs cycle. *Mycobacterium bovis* lacks the ability to utilize glycerol unless pyruvate is provided, and this defect has been shown to be due to a single nucleotide polymorphism in the gene *pykA* encoding for pyruvate kinase (Keating *et al.*, 2005). The Krebs cycle in *M. tuberculosis* appears atypical because α-ketoglutarate dehydrogenase is not present and is replaced by the following reaction scheme: α-ketoglutarate is first converted to succinyl semialdehyde by a α-ketoglutarate decarboxylase and then oxidized to succinate by a succinyl semialdehyde dehydrogenase coupled to NADP reduction (Tian *et al.*, 2005).

In general, the complete oxidation of 1 mole of glycerol yields 7 moles of reduced pyridine nucleotide (NADH) or equivalent ($FADH_2$). Six moles of NADH are generated by glycerol-3-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate dehydrogenase, isocitrate dehydrogenase, succinyl semialdehyde dehydrogenase, and malate dehydrogenase and 1 mole of $FADH_2$ (via succinate dehydrogenase). At present, it has not been experimentally determined how much energy (e.g., moles of ATP) is produced per mole of glycerol utilized by mycobacterial species. Hypothetical calculations based on a mycobacterial respiratory chain that has three proton translocating sites when NADH is used as the endogenous electron donor (Prasada Reddy *et al.*, 1975), and assuming 1 mole ATP is synthesized per three protons translocated by the ATP synthase, would suggest that the oxidation of 1 mole NADH leads to the synthesis of 2 moles of ATP. Oxidation of 1 mole of $FADH_2$ would lead to the synthesis of approximately 1 mole of ATP. In addition, 3 moles of ATP (or an energetically equivalent nucleotide) are produced via substrate level phosphorylation (via phosphoglycerate kinase, pyruvate kinase, and succinyl-CoA synthetase) and one of these is consumed by glycerol kinase. Based on these calculations, the theoretical ATP yield would be 15 moles of ATP produced per mole of glycerol consumed. In *E. coli* and *Bacillus megaterium*, the complete oxidation of 1 mole of glycerol yields 14 moles of ATP (Downs and Jones, 1975; Farmer and Jones, 1976).

The energetics of mycobacterial growth has been studied in glycerol-limited continuous culture (Beste *et al.*, 2005). In the slow-growing *M. bovis* BCG, at both low (0.01 per hour) and high (0.03 per hour) dilution rates, the cell yield or $Y_{glycerol}$ was 27 g [dry weight] cells/mol glycerol utilized and

33 g [dry weight] cells/mol glycerol utilized, respectively (Beste *et al.*, 2005). Under similar growth conditions in continuous culture, the fast-growing non-pathogenic saprophyte *M. smegmatis* exhibits a $Y_{glycerol}$ of 30–40 g [dry weight] cells/mol glycerol utilized over the dilution rate range (0.02–0.15 per hour) (Tran and Cook, unpublished data). The glycerol consumption rate for maintenance functions ($m_{glycerol}$) is 0.28 mmoles of glycerol/h/g [dry weight] cells (Tran and Cook, unpublished data), a value that is comparable with *E. coli*. Beste *et al.* (2007b) have studied the effect of growth rate on gene expression of *M. bovis* BCG in carbon-limited continuous culture at dilution rates of 0.01 per hour ($t_d = 69$ hours) and 0.03 per hour ($t_d = 23$ hours). At low growth rate, there was a down-regulation of genes involved in aerobic respiration, TCA cycle enzymes and the ATP synthase, despite the oxygen tension being maintained at 70–100%, but consistent with a decreased oxygen demand due to a low growth rate. The authors reported that there was some overlap with the transcriptional profile of *M. bovis* BCG to growth rate and both Wayne's model of persistence (Muttucumaru *et al.*, 2004) and macrophage infection (Schnappinger *et al.*, 2003), but little correlation with the response of *M. tuberculosis* to oxygen limitation in chemostat culture (Bacon *et al.*, 2004). These data suggest that adaptation to slow growth rate in carbon-limited conditions is an important trigger for gene expression in both the Wayne model of persistence and growth of *M. tuberculosis* in macrophages (Beste *et al.*, 2007b).

11.2. Mycobacterial Metabolism *In Vivo*

The now widely accepted view for *in vivo* metabolism of *M. tuberculosis* is that the bacterium switches its intermediary metabolism from carbon sources such as glucose and glycerol to fatty acids and host lipids during the course of infection (Schnappinger *et al.*, 2003; Munoz-Elias and McKinney, 2005, 2006; Munoz-Elias *et al.*, 2005, 2006; Pandey and Sassetti, 2008). This concept was first supported by the sequencing of the *M. tuberculosis* genome, which revealed a large repertoire of genes for lipid degradation (Cole *et al.*, 1998). A central role for fatty acid metabolism is also implied by the considerable duplication of genes involved in lipid metabolism in mycobacterial genomes (Cole *et al.*, 1998, 2001; Fleischmann *et al.*, 2002; Garnier *et al.*, 2003). Further studies have shown an induction of genes in the phagosomal environment that encode for enzymes that are required for the biochemical activation and β-oxidation of fatty acids (Schnappinger *et al.*, 2003). Breakdown products of fatty acids are further metabolized via the Krebs cycle and the carbon conserving (no loss of CO_2) glyoxylate

shunt (McKinney *et al.*, 2000; Schnappinger *et al.*, 2003). *M. tuberculosis* has two copies of the gene (*icl1* and *icl2*) that encodes the enzyme isocitrate lyase, a key enzyme in the glyoxylate shunt that converts isocitrate to succinate and glyoxylate. Recent work has unequivocally shown that a double knockout mutant ($\Delta icl1 \times \Delta icl2$) of *M. tuberculosis* is unable to establish an infection in mice due to its inability to metabolize host-derived lipids (Munoz-Elias and McKinney, 2005). The methylcitrate cycle is important for the metabolism of propionyl-CoA in *M. tuberculosis*, an intermediate of odd chain number fatty acid breakdown, but paradoxically is not essential for persistence in mice (Munoz-Elias *et al.*, 2006).

Two recent studies have shown that cholesterol, a major component of host cell membranes, is essential for *M. tuberculosis* persistence in the lungs of chronically infected animals and for growth within the IFN-γ-activated macrophages that predominate at this stage of infection (Gatfield and Pieters, 2000; Pandey and Sassetti, 2008). Pandey and Sassetti (2008) demonstrate that both *M. tuberculosis* and *M. smegmatis* utilize cholesterol, but the mechanism of energy generation during growth on cholesterol is unknown. Kendall *et al.* (2007) have identified a transcriptional repressor, KstR, that controls the expression of a significant number of genes involved in lipid metabolism in both *M. tuberculosis* and *M. smegmatis*.

11.3. Recycling of Reducing Equivalents and Electron Transport

A crucial feature in the adaptation of any bacterium to alternative energy sources and changing environmental parameters (e.g., oxygen tension) is the balance of oxidative and reductive reactions in the metabolic scheme. During mycobacterial growth, both *in vitro* and *in vivo*, substrate oxidation leads to the formation of reduced cytoplasmic electron carriers, for example, NADH, $FADH_2$, or ferredoxin. The oxidation of NADH or equivalent by aerobic bacteria is critical for continuous metabolic flux, and in the absence of a fermentative metabolism, NADH oxidation will be carried out primarily by membrane-bound NADH dehydrogenases. NADH dehydrogenase is the first component of the respiratory chain and transfers electrons from NADH to quinones (e.g., ubiquinone or menaquinone). Weinstein *et al.* (2005) have identified genes for two classes of NADH:menaquinone oxidoreductases in the genome of *M. tuberculosis*. NDH-1 is encoded by the *nuoABCDEFGHIJKLMN* operon and as this dehydrogenase transfers electrons to menaquinone, it conserves energy by translocating protons across the membrane to generate a proton-motive

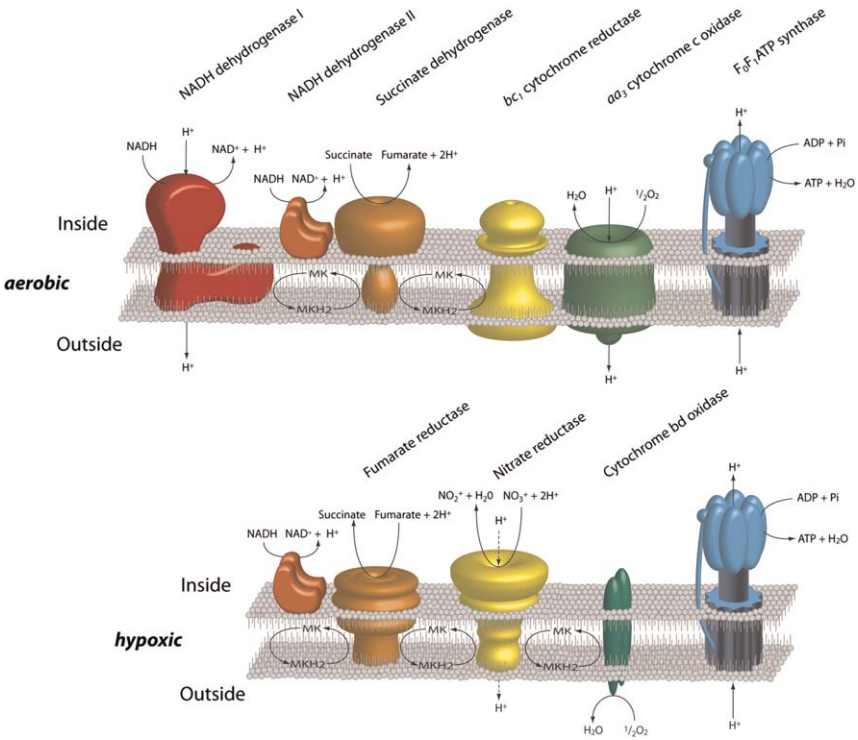

Figure 7 Schematic diagram outlining the electron transport chain components and ATP synthase of *Mycobacterium tuberculosis* growing under aerobic (top panel) and hypoxic conditions (bottom panel). MK, menaquinone; MKH_2, menaquinol.

force ($\Delta\mu_{H^+}$) (Fig. 7). The second class is NDH-2, a non-proton translocating NADH dehydrogenase that does not conserve energy and is present in two copies (*ndh* and *ndhA*) in *M. tuberculosis* (Weinstein *et al.*, 2005). Mutagenesis studies have established that both NDH-1 and *ndhA* are dispensable for growth *in vitro* (Sassetti *et al.*, 2003), but the lack of a viable strain with a disrupted or deleted *ndh* suggests that it is essential for growth despite the presence of *ndhA* (McAdam *et al.*, 2002; Sassetti *et al.*, 2003; Weinstein *et al.*, 2005). Furthermore, deleterious point mutations in *ndh* of *M. smegmatis* are pleiotropic, conferring temperature-sensitive growth arrest and multiple amino acid auxotrophy. Some mutants also have a 25-fold reduced NADH dehydrogenase activity, implying NDH-2 is the primary enzyme responsible for NADH oxidation and is essential for the viability of *M. smegmatis* (Miesel *et al.*, 1998; Vilcheze *et al.*, 2005).

In *M. tuberculosis*, no energetic role for the non-essential type I NADH:menaquinone oxidoreductases has been established and the persistence of *M. tuberculosis* in an *in vitro* Wayne model was shown not to be compromised in a *nuo* operon deletion mutant (Rao *et al.*, 2008). Velmurugan *et al.* (2007) have demonstrated a role for *nuo* (i.e., the NuoG subunit) in the ability of *M. tuberculosis* to inhibit macrophage apoptosis. The *nuo* operon has been lost from the genome of *M. leprae* with a single *nuoN* pseudogene remaining (Cole *et al.*, 2001). *M. smegmatis* also contains genes for a type I NADH:menaquinone oxidoreductases (viz. *nuoA-N*), but enzyme assays for NDH-I activity in *M. smegmatis* failed to detect the enzyme, suggesting it is not expressed (Miesel *et al.*, 1998). Several studies have reported that *nuo* is down-regulated in *M. tuberculosis* during mouse lung infection (Shi *et al.*, 2005), survival in macrophages (Schnappinger *et al.*, 2003), in both NRP-1 (1% oxygen saturation) and NRP-2 (0.06% oxygen saturation) relative to aerated mid-log growth (Shi *et al.*, 2005), and upon starvation *in vitro* (Betts *et al.*, 2002). The transcription of *ndh* is also down-regulated in *M. tuberculosis* during mouse lung infection, but transcript levels for *ndh* peak (are induced) during NRP-2 *in vitro* demonstrating that the pattern of *ndh* regulation is different between *in vivo* and *in vitro* conditions (Schnappinger *et al.*, 2003). These data are in contrast to *E. coli* where NuoA-N (NDH-1) is usually associated with anaerobic respiratory pathways (e.g., fumarate) and non-coupling dehydrogenases such as NDH-2 are synthesized aerobically (Unden and Bongaerts, 1997). Moreover, *E. coli* mutants lacking NDH-I have a competitive disadvantage in stationary phase (Zambrano and Kolter, 1993). Some interesting questions arise from these observations. The first is ''why do mycobacteria use type II NADH dehydrogenases to recycle NADH when they could continue to use the energy conserving ($\Delta\mu_{H^+}$ generating) NDH-1?'' One potential explanation is that because NDH-2 are non-proton translocating, they will not be impeded by a high $\Delta\mu_{H^+}$ which could ultimately slow down glycolytic flux due to back pressure on the system. This mechanism is akin to a ''relief valve'' that would allow for a higher metabolic flux and ultimately higher rates of ATP synthesis at the expenses of low energetic efficiency of the respiratory chain. Secondly, why is *ndh* an essential gene when mycobacteria could also use *ndh2* or *nuo*? The fact that *ndh* is essential implies that mycobacteria do not have another mechanism to recycle NADH during normal aerobic growth. Alternatively, this is the only NADH dehydrogenase that is operating under these growth conditions and the activity of this enzyme and subsequent electron transfer (and proton translocation) to oxygen is what fuels the essential F_1F_0-ATP synthase of mycobacteria (Tran and Cook, 2005).

The mechanisms used by mycobacteria to recycle NADH under either hypoxia or anaerobic conditions remain unknown. Compounds that target either the F_1F_0-ATP synthase or NDH-2 are bactericidal toward hypoxic non-replicating *M. tuberculosis* suggesting that the respiratory chain is essential for the recycling of NADH under these conditions (Rao *et al.*, 2008).

11.4. Aerobic Respiratory Pathways in Mycobacteria

During aerobic respiration, electrons from the oxidation of either NADH (via NADH:menaquinone oxidoreductases) or succinate (via succinate dehydrogenase *sdhABCD*) flow into the menaquinone$_{(oxid)}$–menaquinol$_{(red)}$ pool (Fig. 7). In mycobacteria, menaquinol can transfer electrons either directly to a cytochrome *bd*-type menaquinol oxidase (encoded by *cydABCD*) or through a cytochrome *c* pathway (Kana *et al.*, 2001; Boshoff and Barry, 2005; Matsoso *et al.*, 2005). The cytochrome *bd* branch is the bioenergetically less efficient branch (non-proton translocating) and is synthesized at low oxygen tensions (Kana *et al.*, 2001). The cytochrome *c* pathway consists of a menaquinol–cytochrome *c* oxidoreductase termed the bc_1 complex (encoded by the *qcrCAB* operon) and an aa_3-type cytochrome *c* oxidase (encoded by *ctaBCDE*) belonging to the heme-copper respiratory oxidase family (Boshoff and Barry, 2005; Matsoso *et al.*, 2005). The cytochrome *c* oxidase functions as a proton pump and may form a ''supercomplex'' with menaquinol cytochrome *c* oxidoreductase (Matsoso *et al.*, 2005). Support for this hypothesis comes from the observation that such supercomplexes have been reported in other actinomycetes such as *C. glutamicum* (Niebisch and Bott, 2003). In addition to cytochrome *bd* and an aa_3-type cytochrome *c* oxidase, the *M. smegmatis* respiratory chain has been proposed to contain a third possible respiratory branch terminating in the YthAB (*bd*-type) menaquinol oxidase (Kana *et al.*, 2001). The bc_1–aa_3 pathway is the major respiratory route in mycobacteria under standard aerobic culturing conditions (Matsoso *et al.*, 2005). Matsoso *et al.* (2005) have demonstrated that disruption of this pathway in *M. smegmatis* is accompanied by a constitutive up-regulation of the cytochrome *bd*-type menaquinol oxidase. In *M. tuberculosis*, the bc_1–aa_3 pathway is essential for growth suggesting an inability of this bacterium to adapt in a manner analogous to *M. smegmatis*. The aa_3 branch is also proposed to contain two *ctaD* alleles in *M. smegmatis* versus the one in *M. tuberculosis*, suggesting alternative isoforms of cytochrome *c* oxidase in *M. smegmatis* (Kana *et al.*, 2001).

11.5. Generation of an Electrochemical Gradient of Protons ($\Delta\mu_{H^+}$) in Mycobacteria

When growing aerobically at an external pH of 7.0, *M. smegmatis* and *M. bovis* BCG generate an $\Delta\mu_{H^+}$ of approximately −180 mV which appears to be of a significant magnitude to drive proton-coupled bioenergetic processes (e.g., ATP synthesis, solute transport, etc.) (Rao *et al.*, 2001). Growth of mycobacteria is sensitive to the electrogenic proton translocator carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) demonstrating that a $\Delta\mu_{H^+}$ is indispensable for normal growth. Moreover, growth is inhibited by the F-type ATP synthase inhibitor *N*,*N*′-dicyclohexylcarbodiimide (DCCD) further supporting the role of the $\Delta\mu_{H^+}$ in driving ATP synthesis by the membrane-bound F_1F_0-ATP synthase. While proton translocation via the respiratory chain generates the $\Delta\mu_{H^+}$ during respiration with oxygen as the terminal electron acceptor, it is not clear how the $\Delta\mu_{H^+}$ is established in the absence of oxygen under anaerobic growth conditions. Anaerobic bacteria are able to generate a significant $\Delta\mu_{H^+}$ (−100 mV) using their membrane-bound F_1F_0-ATP synthase in the ATP hydrolysis direction (Dimroth and Cook, 2004). The ATPase activity (proton pumping) of the enzyme is fuelled by ATP produced by substrate level phosphorylation. The F_1F_0-ATP synthase of *M. tuberculosis* appears to have latent ATPase activity when purified, suggesting that the enzyme may not function in the ATP hydrolysis direction (Higashi *et al.*, 1975). Whether the enzyme is also latent in actively growing cells is not known and therefore the potential exists for this enzyme to function as a primary proton pump in the absence of oxygen and a functional respiratory chain to generate the $\Delta\mu_{H^+}$. To our knowledge, the effect of DCCD on anaerobic cultures of mycobacteria has not been determined to address this hypothesis. Rao *et al.* (2008) have reported that hypoxic, non-replicating *M. tuberculosis* generate a total proton-motive force of −113, −73 mV of electrical component ($\Delta\psi$) and −41 mV of ZΔpH. The addition of thioridazine, a compound that targets NDH-2, results in dissipation of the electrical potential and significant cell death suggesting that NADH is an important electron donor for the generation of the $\Delta\psi$ under hypoxic conditions. The addition of R207910 (TMC207), a specific inhibitor of the F_1F_0-ATP synthase was bactericidal against hypoxic, non-replicating *M. tuberculosis*, but had no effect on the $\Delta\psi$ (Rao *et al.*, 2008), an observation consistent with the latent ATPase activity of this enzyme (see above).

A potential solution to the generation of a $\Delta\mu_{H^+}$ under hypoxia is the use of a respiratory nitrate reductase (Fig. 7). Breakdown of nitric oxide in mammalian tissue would provide a source of nitrate that could be used as

an alternative electron acceptor. A transport system for the exchange of nitrate and nitrite into and out of the cell are present in the genome of *M. tuberculosis* (e.g., NarK2) (Cole *et al.*, 1998). *M. tuberculosis* contains genes (*narGHJI*) that encode for a putative membrane-bound molybdenum-containing nitrate reductase complex similar to the corresponding *narGHJI* operon of *E. coli*. Moreover, the *narGHJI* operon of *M. tuberculosis* is able to functionally complement a *nar* mutant of *E. coli* to grow on glycerol and reduce nitrate anaerobically (Sohaskey and Wayne, 2003). Importantly, however, the expression of *narGHJI* operon in *M. tuberculosis* is not up-regulated in response to either hypoxia or stationary phase (Sohaskey and Wayne, 2003). Furthermore, Sohaskey and Wayne (2003) demonstrate that overexpression of recombinant *M. tuberculosis* nitrate reductase in either *M. tuberculosis* or *M. smegmatis* (low nitrate reductase) does not confer the ability of these cells to grow anaerobically. No growth of either species is observed with nitrate anaerobically even though the nitrate reductase activity of whole cells increases (Sohaskey and Wayne, 2003). The genome of *M. tuberculosis* also lacks orthologs of FNR which, in combination with NarL, are responsible for the transcriptional activation of the *narGHJI* operon anaerobically in *E. coli* (Unden and Bongaerts, 1997). A putative NarL (*Rv0884c*) has been identified in *M. tuberculosis* but the promoter of the *narGHJI* lacks consensus-like binding sites for this regulatory protein. Based on these observations, it is apparent that this enzyme does not support anaerobic growth of mycobacteria and therefore the role of this enzyme in the physiology of mycobacteria is unclear. Given the proposed membrane-bound location of the enzyme and the proton-pumping activity of the *E. coli* enzyme, perhaps the primary role of the mycobacterial enzyme is to generate a $\Delta\mu_{H^+}$ when the concentration of oxygen is low, and hence its activity increases but not its expression. An alternative role for nitrate reductase may be maintaining the redox balance of the cell during conditions of hypoxia. Sohaskey (2008) has reported that exogenously supplied nitrate has no effect on long-term persistence during gradual oxygen depletion, but played an important role during rapid adaptation to hypoxia (<18 hours). This effect required a functional nitrate reductase, suggesting that nitrate reduction may play a role in protecting cells during sudden changes in oxygen concentration leading to disruption of aerobic respiration. Sohaskey (2005) proposes a role for NarK2 in sensing the redox state of the cell such that nitrate is transported into the cell under reducing, but not oxidizing, conditions.

The role of endogenous electron acceptors may also fuel the generation of a membrane potential ($\Delta\psi$) in the absence of exogenous acceptors.

Mycobacteria utilize menaquinone/menaquinol as a conduit between electron-donating and -accepting reactions. Menaquinone has a lower midpoint redox potential ($E_m = -74\,mV$) compared to ubiquinone ($E_m = +113\,mV$) and is ideally poised to donate electrons to fumarate during anaerobic conditions (Cecchini *et al.*, 2002). Fumarate reductase (encoded by *frdABCD*) is present in *M. tuberculosis* and has been shown to be up-regulated during carbon starvation and oxygen depletion (Betts *et al.*, 2002) and in macrophages (Schnappinger *et al.*, 2003), suggesting a role for this enzyme in persistence (Fig. 7). A recent study suggests that fumarate may be an important endogenous electron acceptor for energy production and maintenance of redox balance (oxidation of NADH to NAD^+) in hypoxic non-replicating mycobacteria, but this remains to be experimentally validated (Rao *et al.*, 2008). Interestingly, the use of fumarate as an electron acceptor in *E. coli* requires complex I, and expression of the *nuo* operon is stimulated by the presence of fumarate (Unden and Bongaerts, 1997).

11.6. ATP Synthesis by Mycobacteria

In *M. tuberculosis*, ATP is synthesized via substrate level phosphorylation and oxidative phosphorylation using the membrane-bound F_1F_0-ATP synthases (encoded by the *atpBEFHAGDC* operon). No *atpI* gene is present in the operon, but an ORF (i.e., Rv1303) potentially may play this role being located immediately upstream of the *atp* locus. The F_1F_0-ATP synthase catalyzes ATP synthesis by utilizing the electrochemical gradient of protons to generate ATP from ADP and inorganic phosphate (P_i) and operates under conditions of a high $\Delta\mu_{H^+}$ and low intracellular ATP. The enzyme is also capable of working as an ATPase, hydrolyzing ATP to pump protons from the cytoplasm to the outside of the cell and operates under conditions of high intracellular ATP and an overall low $\Delta\mu_{H^+}$. In *M. smegmatis*, the *atp* operon has been shown to be constitutively expressed and an increase in expression is noted upon growth with non-fermentable substrates that are strictly coupled to oxidative phosphorylation (e.g., succinate) compared to fermentable carbon sources like glucose (Tran and Cook, 2005). The F_1F_0-ATP synthase in *M. tuberculosis* and *M. smegmatis* has been shown to be essential for growth (Sassetti *et al.*, 2003; Tran and Cook, 2005). In other bacteria, the F_1F_0-ATP synthase has been shown to be dispensable for growth on fermentable carbon sources (Friedl *et al.*, 1983; Santana *et al.*, 1994), where increased glycolytic flux can compensate for the loss of oxidative phosphorylation. This strategy does

not appear to be exploited by *M. smegmatis*: the F_1F_0-ATP synthase is essential for growth even on fermentable substrates, suggesting that ATP production from substrate level phosphorylation alone, despite increased glycolytic flux, may be insufficient to sustain growth of these bacteria. This would be reflected by an extraordinarily high value for the amount of ATP required to synthesize a mycobacterial cell, a possibility that requires further investigation. Alternatively, or in conjunction with a high ATP demand for growth, the ATP synthase may be an obligatory requirement for the oxidation of NADH by providing a sink for translocated protons during NADH oxidation coupled to oxygen reduction. Such strict coupling would imply that mycobacteria do not support uncoupled respiration; either they lack a conduit for proton re-entry in the absence of the F_1F_0-ATP synthase or they are unable to adjust the proton permeability of the cytoplasmic membrane to allow a futile cycle of protons to operate. In this context, the cytoplasmic membrane of *M. smegmatis* is extremely impermeable to protons (Tran *et al.*, 2005).

Several new anti-tubercular drugs have recently been reported (Andries *et al.*, 2005; Weinstein *et al.*, 2005). It is particularly noteworthy that both classes of drugs target oxidative phosphorylation in mycobacteria. The first class, diarylquinolines, have been shown to target the F_1F_0-ATP synthase to inhibit ATP synthesis by the enzyme (Andries *et al.*, 2005; Koul *et al.*, 2007). Given the essential role of this enzyme for mycobacterial growth (Tran and Cook, 2005), even on fermentable carbon sources, the ATP synthase would appear to represent a valid target. Genome sequencing of both *M. tuberculosis* and *M. smegmatis* mutants that are resistant to diarylquinolines (i.e., R207910) revealed that the target of these compounds is the oligomeric *c* ring (encoded by *atpE*) of the enzyme. Purified *c* ring from *M. smegmatis* binds R207910 with a K_d of 500 nm, and modeling studies suggest that R207910 blocks proton transfer by the enzyme (de Jonge *et al.*, 2007). The second class, phenothiazine analogs, target the NADH:menaquinone oxidoreductase (Weinstein *et al.*, 2005) suggesting that NADH oxidation via aerobic respiration coupled to oxidative phosphorylation is essential for the growth of mycobacteria.

11.7. Other Mechanisms to Recycle NADH During Hypoxia

Hydrogenases are found in a wide variety of microorganisms and catalyze the reversible conversion of H_2 to $2H^+$ and $2e^-$ thus enabling the bacterium to utilize H_2 as a source of reducing equivalents. Conversely, some bacteria use the enzyme to reduce protons to H_2 thereby releasing

the reducing equivalents. Hydrogen evolution is a mechanism commonly employed by anaerobic bacteria to recycle reducing equivalents obtained from anaerobic degradation of organic substrates. However, even strictly aerobic bacteria have been shown to produce H_2 under anaerobic conditions (Kuhn *et al.*, 1984). It has been shown that *M. smegmatis*, among other mycobacterial species, can oxidize molecular hydrogen in the presence of carbon monoxide, implying that *M. smegmatis* expresses a functional hydrogenase (King, 2003). No studies have reported the ability of mycobacteria to produce hydrogen, but analysis of the *M. tuberculosis* genome reveals the presence of a gene cluster (viz. Rv0082 to Rv0087) encoding components of a putative hydrogenase and formate hydrogen lyase that is up-regulated during infection of human macrophage-like THP-1 cells (Fontan *et al.*, 2008). Moreover, the transcription of the early genes of the *hycP*/*hycQ* containing operon was shown to be up-regulated during anaerobic adaptation in several studies (Sherman *et al.*, 2001; Park *et al.*, 2003; Voskuil *et al.*, 2003, 2004; Bacon *et al.*, 2004). The putative hydrogenase gene cluster of *M. tuberculosis* shows homology to components of hydrogenase 4 and 3 complex of *E. coli*, the latter of which has been shown to catalyze hydrogen evolution at acidic pH (Mnatsakanyan *et al.*, 2004). It is tempting to propose that the acidic, hypoxic, and lipid-rich environment in the macrophages might require the expression of a hydrogenase complex in *M. tuberculosis* to help with the recycling of reducing equivalents under these conditions. The requirement for a low potential redox carrier in hydrogen production could be satisfied by reactions that are coupled to ferredoxins and not NADH. For example, *M. tuberculosis* contains a putative pyruvate:ferredoxin oxidoreductase (*Rv2454c*/*Rv2455c*) that would catalyze the oxidation of pyruvate to acetyl-CoA with reduced ferredoxins acting as the electron donor for hydrogen production. The role of hydrogen in the cycling of reducing equivalents in mycobacteria during hypoxia warrants investigation.

11.8. Control of Energy Generation in Mycobacteria

In bacteria there is often a trade off between obtaining the maximum energy yield from a substrate and the maximum flux (rate of ATP production) or growth rate (Pfeiffer *et al.*, 2001). A cell that uses a pathway with a high yield and low rate (e.g., respirers) can produce more ATP from a given amount of substrate compared to a bacterium that produces ATP at a higher rate but a lower yield (e.g., fermenters). The growth yield of bacterial cultures has been used to estimate the efficiency of energy

generation during respiration or fermentation. Mycobacteria would appear to adopt the first strategy where the cells grow slowly using oxidative phosphorylation to generate large amounts of ATP at a slow rate. Furthermore, this strategy appears to be employed by both fast- and slow-growing mycobacteria; the growth yield on glycerol is comparable between *M. smegmatis* and *M. bovis* BCG at similar growth rates (see above).

In order for mycobacteria to utilize oxygen efficiently and obtain the maximum growth yield on a particular carbon and energy source, there must be co-ordinated regulation of terminal respiratory oxidase expression. For example, *E. coli* cytochrome *bo* (K_m for oxygen in the micromolar range) and cytochrome *bd* (K_m for oxygen in the nanomolar range) (D'Mello *et al.*, 1995, 1996) are coordinately regulated by the ArcBA system and FNR (Cotter *et al.*, 1997). Cytochrome *bo* is synthesized at high oxygen tension (optimal between 15 and 100% air saturation) and repressed as the oxygen concentrations decreases (Tseng *et al.*, 1994). This coincides with the induction of cytochrome *bd* at 7% air saturation, which is turned off (FNR-mediated repression) once the cells enter anaerobiosis (Cotter *et al.*, 1997; Tseng *et al.*, 1994). *E. coli* also uses non-coupling dehydrogenases (NDH-2) during aerobic growth that allow a fast metabolic flux (fast growth rate) and switches to coupling dehydrogenases (NDH-1) during anaerobic growth with fumarate (Unden and Bongaerts, 1997).

Mycobacteria also adopt regulation of oxidase expression to match oxygen supply. Under conditions of low oxygen tension (ca. 1% air saturation), cytochrome *bd* is induced in *M. smegmatis* as the transition to anaerobiosis is approached (Kana *et al.*, 2001), a value that is 10-fold lower than that observed in *E. coli* (ca. 10% air saturation). In *M. tuberculosis*, cytochrome *bd* is up-regulated in the early stages of NRP-1 (i.e., decreasing oxygen) (Voskuil *et al.*, 2004). A strategy that appears to be invoked by mycobacteria is one of down-regulation or a slowing of metabolism as cells enter NRP-1 and NRP-2. Transcriptional analysis of *M. tuberculosis* in the macrophage (phagosomal environment) has revealed that NDH-1, menaquinol–cytochrome *c* oxidoreductase and the ATP synthase are all down-regulated when compared to cells growing exponentially. Suggesting the reduced need for energy generation during bacteriostasis, that is, the growth state of intraphagosomal *M. tuberculosis* (Schnappinger *et al.*, 2003). Consistent with these observations is the repression of these operons during starvation. In contrast, fumarate reductase, nitrate reductase, and NDH-2 are all up-regulated under these conditions (Schnappinger *et al.*, 2003). Whilst these proteins do not appear to contribute to increased energy production, it has been suggested that they may play a pivotal

role in the recycling of NAD^+ as a result of β-oxidation of fatty acids (Schnappinger *et al.*, 2003). Furthermore, the induction of *ald*, encoding L-alanine dehydrogenase, may also contribute to the recycling of NAD^+ through the reductive amination of pyruvate to alanine and subsequent oxidation of NADH.

While various components of the oxidative phosphorylation machinery are down-regulated (e.g., ATP synthase) during persistence or dormancy, this does not mean they are entirely absent. Koul *et al.* (2008) demonstrate that R207910 kills dormant *M. tuberculosis* as effectively as aerobically grown *M. tuberculosis* with the same target specificity. Moreover, the authors show that dormant *M. tuberculosis* do indeed have residual ATP synthase activity.

12. SYSTEMS BIOLOGY AND THE MYCOBACTERIAL CELL

Molecular biology, biochemistry, and genetics have proved powerful disciplines in uncovering the individual functions of biomolecules and their interactions. However, whilst these traditional methods provide insight into the understanding of what constitutes a biological system, there is an increasing need to elucidate the overall working of a biological system (Kitano, 2001, 2002). In this regard, mathematical models and computational analyses are required to attain such system level understanding (Kitano, 2002; Stelling, 2004). Mathematical models quantitatively and dynamically describe molecular interactions in biological networks and enable simulations of complex biological behavior. Through such simulations, the ultimate goal of systems biology-understanding how global biological behavior (e.g., a phenotype) emerges from the local interactions of molecular components (Kitano, 2002) can be obtained.

A systems biology approach encompasses three main steps:

(i) Large-scale-omics measurement techniques (such as transcriptomics, proteomics, metabolomics, etc.) to provide quantitative experimental data on component concentrations.
(ii) In order to extract biological insight from these data, due to their size and the underlying complexity, computational tools need to be utilized. This step of computational analysis of large-scale data is typically referred to as ''top-down'' systems biology.

(iii) The knowledge about molecular components and interactions between those components does not allow for a system understanding *per se*. The ''bottom-up'' approach of systems biology is concerned with integrating the knowledge about the molecular interactions in a quantitative manner by means of mathematical models. Computational analysis of these models (i.e., through simulations) then leads to the envisioned system understanding.

In contrast to the field of *E. coli* or *S. cerevisiae*, little work along these lines has been performed with species of mycobacteria. In the following section, we will review the systems biology work that has been done to date in the mycobacterial field.

12.1. Large-Scale Omics Analyses and Computational Analyses of Such Data

12.1.1. Transcriptomics

Transcriptomics using microarray and quantitative PCR (qPCR) is the experimental analysis tool most commonly applied for studies with *M. tuberculosis*. Three recent reviews comprehensively summarize the transcriptome studies published until 2007 (Kendall *et al.*, 2004; Bacon and Marsh, 2007; Waddell and Butcher, 2007). The majority of microarray data have been derived from batch cultures with low oxygen tension or nutrient starvation or from *ex vivo* macrophage mouse models. Only a few studies are available where mycobacteria were grown under more defined conditions like continuous culture. In macrophage or mouse model studies, the amount of bacterial mRNA is the limiting factor due to the low bacterial numbers in such systems. However, in a recent study, mycobacterial RNA was reproducibly amplified from as little as 5 ng total RNA (equivalent to 2×10^5 bacilli) (Waddell *et al.*, 2008). This opens up new possibilities for *in vivo* large-scale transcriptomics studies.

The analysis of microarray data is usually limited to extracting the significantly up- or down-regulated genes and gene clusters, classification into functional categories and, less often, superimposition onto metabolic pathways (Schnappinger *et al.*, 2003). The latter method is most promising for the understanding of the metabolic state of *M. tuberculosis in vivo* (Bacon and Marsh, 2007). Some studies also provide a meta-analysis, revealing regulons and gene clusters, which are similarly regulated under different conditions (Beste *et al.*, 2007b; Kendall *et al.*, 2004; Voskuil *et al.*,

2004). Beste et al. (2007a, b) have used microarray data to validate an *in silico* genome-scale network model (see below).

A prerequisite for the use of transcriptional data in a systems biology approach is an exact knowledge of the growth parameters. However, in macrophage and *in vivo* studies, the composition and concentration of nutrients and the availability of terminal electron acceptors like oxygen or nitrate are difficult to assess. Furthermore, in the majority of gene expression studies, the control culture is growing at a different growth rate than the test culture. This difference alone can account for a different pattern of gene expression being observed (Ishii *et al.*, 2007; Beste *et al.*, 2007b). More defined *in vitro* approaches like continuous cultivation (chemostat), where bacteria can be grown on minimal medium in a carefully controlled environment, can help to dissect the complex *in vivo* environment (Bacon *et al.*, 2004, 2007; Beste *et al.*, 2007b). It will be important to use gene expression data from experiments with well-defined growth and environmental parameters (from *in vitro* approaches) to feed into the *in silico* models. *In silico* predictions can then be validated using *in vivo* data.

12.1.2. Proteomics

In order to gain a greater insight into mycobacterial physiology in response to the environment, the measurement of protein expression is an essential prerequisite. Many proteins are regulated post-transcriptionally and, therefore, transcriptome studies cannot account for these changes. Shotgun proteomics and 2D-gel analysis are tools that can complement and extend our knowledge gained from DNA microarray analysis. Several studies with mycobacteria have been performed (Jungblut *et al.*, 1999; Cho *et al.*, 2006; Radosevich *et al.*, 2007). Recently, a shotgun protein expression analysis of *Mycobacterium smegmatis* challenged with three different anti-tubercular antibiotics revealed drug-specific changes in metabolic pathways as well as putative drug targets (Wang and Marcotte, 2008). Proteomics though, is still limited to *in vitro* studies because *in vivo* experiments do not yield enough material for a global protein analysis. Nevertheless, as mentioned above, well-defined *in vitro* studies are valuable for the construction and iterative improvement of *in silico* models.

12.1.3. Other Omics

A global lipid profile (i.e., lipidomics) of *M. tuberculosis* has been performed by Jain *et al.* (2007). However, other omic techniques that are

widely used with unicellular model organisms, such as metabolomics (Dunn *et al.*, 2005; van der Werf *et al.*, 2007) or fluxomics (Sauer, 2006), have not been applied to mycobacteria. While metabolomics aims at measuring the concentration of as many as possible intracellular metabolites in a quantitative manner, fluxomics (or metabolic flux analysis) represents an approach that aims at determining the rates of the intracellular metabolic reactions (or, in other words, the metabolic fluxes).

12.2. Toward Obtaining a System Understanding Through Mathematical Modeling

Through systems biology omics analyses, as well as the traditional approaches to biology, molecular components, and the interactions between these components can be identified. In order to obtain an understanding about how these interactions actually result in system behavior, that is, how a certain environment makes a cell express a certain phenotype, the mere knowledge of biological components and their interactions has to be quantitatively integrated into mathematical models and analyzed by computational means (Kitano, 2002; Stelling, 2004). This approach is typically referred to as the bottom-up branch of systems biology (Bruggeman and Westerhoff, 2007). For *M. tuberculosis*, two different modeling approaches have been applied to study bacterial metabolism and physiology.

12.2.1. Constraint-Based Models

The first modeling approach is called constraint-based modeling, a valuable tool for studying genome-wide metabolism (Price *et al.*, 2003, 2004; Feist and Palsson, 2008). In this approach, a mathematical version of an organism-specific metabolic network chart is computationally interrogated and analyzed. With this type of analysis, questions can be answered as to how carbon flow is redirected or how redox and energy cofactors are balanced upon knocking out a specific metabolic gene (Price *et al.*, 2003). In principle, this is what experienced physiologists do when they ''manually'' inspect metabolic network charts. By means of computation, constraint-based modeling allows metabolic flux balancing on a genome-wide scale. With simple model organisms such as *E. coli* or *S. cerevisiae*, and also to a limited extend with mycobacteria, such analyses have proved to be useful in hypothesis generation and correction of errors in genome annotation and

Table 7 Overview of reconstructed stoichiometric network models of mycobacteria.

Intra-system reactions/proteins	Metabolites	Genes	Reference(s)
219/28	197	25	Raman *et al.* (2005)
849	739	726	Beste *et al.* (2007a)
939/543	828	661	Jamshidi and Palsson (2007)

to some extent in predicting phenotypic behavior (Duarte *et al.*, 2004; Feist *et al.*, 2007).

The most important input for this type of analysis is a mathematical model of the stoichiometry of an organism's metabolic network. For *M. tuberculosis*, three such models have been developed (Table 7). Raman *et al.* (2005) generated a stoichiometric model for the biosynthesis pathways of mycolic acid including 219 metabolic reactions. The two stoichiometric models reconstructed by Beste *et al.* (2007a) and Jamshidi and Palsson (2007) have genome-wide scope and provide a comprehensive view on the mycobacterial metabolic network. The network reconstructed by Beste *et al.* (2007a) was calibrated against experimentally determined mycobacterial physiology and other experimental data and thus can be considered a validated model.

As a first step in the development of such a stoichiometric network model, a comprehensive list of the organism's metabolic reactions needs to be assembled. In this context, the genome annotation is usually a rich source of information, but also information from biochemistry and physiology is typically used to generate such a list (Joyce and Palsson, 2007). In a next step, the reaction list is used to formulate mass balance equations for each metabolite present in the metabolic network. Applying the steady-state assumption to the balance equations, the set of differential equations translates into a set of linear equations that mathematically describes the metabolic reaction network at steady-state. Solely based on the genome annotation, typically the networks assembled are not complete, and gaps in essential biosynthesis pathways exist. In this context, information from the biochemical literature is exploited to close these gaps in the network. Additional further manual work and curation is necessary in order to define an equation that specifies the stoichiometry of a typical composition of the organism's biomass (as, e.g., done by Beste *et al.* (2005) for mycobacteria) and also typical directionalities of the metabolic reactions need to be defined (Kümmel *et al.*, 2006b). In addition to the reaction network described in such stoichiometric network reconstructions,

information is typically also provided about the proteins and genes that are linked to metabolic reactions. Such, metabolic reconstructions represent a comprehensive and manually curated resource for an organism's metabolism.

The metabolic network model can also be interrogated using a great variety of computational methods. One of the most important tools for this is flux balance analysis (Kauffman *et al.*, 2003). Flux balance analysis uses mathematical optimization of a certain biological objective (i.e., typically maximization of biomass production per molecule of substrate) to determine values for each of the metabolic fluxes (i.e., reaction rates) in the network, which are within the constraints as defined by the stoichiometry of the metabolic network and the specified reaction directions. For this, Beste *et al.* (2007a) have developed an internet-based tool to perform such calculations in a way that special mathematical expertise is not required besides a basic understanding of the concept behind this approach.

By applying the concept of flux balance analysis, *in silico* gene deletion studies were performed with all three presented model reconstructions for mycobacteria. With such computational analyses, the effect of gene deletion (i.e., removal of the corresponding metabolic reaction from the network) on an organism's viability under a given condition can be tested. Compared with the experimental global mutagenesis dataset on gene essentiality (Sassetti *et al.*, 2003), the *M. bovis* BCG model from Beste *et al.* (2007a) showed the best accuracy (78%) in correctly predicting growth phenotype.

A disagreement found between the experimental and computational analysis points to relevant science. For example, an incorrect *in silico* lethality prediction points to incomplete knowledge about the metabolic network. As suggested from the analysis of Jamshidi and Palsson (2007) on *M. tuberculosis* (*in silico*), alternative pathways or enzymes have to exist for amino acid metabolism. The same seems to hold true for both fatty acid metabolism and membrane and peptidoglycan biosynthesis (Jamshidi and Palsson, 2007). These findings might also be due to unknown, and thus not considered, changes in the biomass composition, which is a required input for the *in silico* analysis.

The capability for efficient computational testing of gene deletion or inactivation effects (single or multiple) can be used as a fast and simple screen for putative targets for anti-tubercular drugs in metabolism. For example, the computational analysis of Beste *et al.* (2007a) revealed a potential role for the enzyme isocitrate lyase during slow growth of mycobacteria, and experimentally it was indeed found that this enzyme

shows higher activities in slow growing cells. Isocitrate lyase has been shown to be essential for *M. tuberculosis* persistence in macrophages and mice (McKinney *et al.*, 2000).

Beyond the illustrated type of analyses mentioned, reconstructed stoichiometric network models can also be used as an input for ^{13}C metabolic flux analysis, a technique that allows for the experimental determination of the magnitude of metabolic fluxes (Sauer, 2006). Furthermore, stoichiometric networks can be used for analyzing transcriptome or metabolome data. The metabolic network can link measured entities (i.e., transcripts or metabolites) and provide a context for the measured quantities, allowing for an improved statistical or mechanistic-based analysis (Patil and Nielsen, 2005; Cakir *et al.*, 2006; Kümmel *et al.*, 2006a; Zamboni *et al.*, 2008). Overall, genome-scale stoichiometric network models have been shown to be powerful tools for studying an organism's metabolism and physiology as they allow for the interrogation of the highly interconnected network of many metabolic reactions in a systematic and unambiguous fashion, which is not possible with a mere inspection of the reaction map alone.

12.2.2. Detailed Mechanistic Models

A second modeling approach has recently been applied to *M. tuberculosis* where molecular interactions are described in detail with ordinary differential equations (Singh and Ghosh, 2006). This approach allows for dynamic and quantitative simulations of biological processes. Singh and Ghosh (2006) report some initial efforts to mathematically describe the kinetics of the tricarboxylic acid cycle and glyoxylate bypass in *M. tuberculosis*. Based on the developed mathematical model, these authors suggested isocitrate lyase as a potential drug target, which agrees well with experimental data (Munoz-Elias and McKinney, 2005).

12.3. Outlook and Challenges for Systems Biology with *M. tuberculosis*

Systems biology as a new biological discipline not only provides novel large-scale measurement techniques, but ultimately aims to achieve quantitative understanding of biological systems. Due to the complexity of biological systems, such understanding can most likely only be achieved by using mathematical models that can quantitatively and dynamically

describe molecular interactions. In the mycobacteria field, first efforts along these lines have been made only recently.

It should be noted that mathematical models, by means of computational simulations, can generate novel hypotheses about the modeled biological system. These hypotheses can then be experimentally tested. Unfortunately, in the papers outlined above, very little experimental follow-up has been performed to test the derived hypotheses. In order to realize the full potential of systems biology for mycobacterial research, two communities, that is, those with the computational expertise and mycobacterial biologists, will need to work together to harvest the potential inherent to the systems biology modeling and computational analyses.

13. CONCLUSIONS

M. tuberculosis was discovered in 1882 by Robert Koch and it is estimated that over two billion people are infected with this bacterium. This is a remarkable feat when one considers that there is no significant animal or environmental reservoir and the limited genetic diversity exhibited by strains of *M. tuberculosis*. The bacterium makes up for these apparent weaknesses through its ability to evade the host immune defenses and an extraordinary capacity to adapt to and survive under adverse conditions, including nutrient deprivation, hypoxia, and various exogenous stress conditions. *M. tuberculosis* is also able to adapt to very different intracellular environments including: phagosomes in macrophages and dendritic cells (Russell, 2003), granulomas (Ulrichs and Kaufmann, 2006), and even fat cells (Neyrolles *et al.*, 2006). A key requirement for this adaptation is sensory perception and *M. tuberculosis* contains an array of signal transduction systems involving two-component regulatory systems, STPKs and ECF sigma factors. The ability of *M. tuberculosis* to efficiently accumulate nutrients in these different environments is essential for its survival, but many of the proteins involved in this process have not been determined. Uptake of these nutrients is tightly coupled to metabolism and energy generation and metabolism in the absence of cell replication is a hallmark of mycobacterial persistence. The electron donors and acceptors utilized under these conditions is an important question for mycobacterial energetics during persistence.

The search for new drug targets to combat the threat of *M. tuberculosis* is an area of renewed interest, especially drugs that will allow a shorter therapy. The contents of this review reinforce the idea that bacterial

metabolism and energetics represents a virtually untapped source of new targets for anti-TB drugs. The hot prospects are those compounds that target the F_1F_0-ATP synthase (Andries *et al.*, 2005), electron transport chain (Weinstein *et al.*, 2005; Rao *et al.*, 2008) and outer membrane proteins (Niederweis, 2008b). Outer membrane proteins offer the advantage as drug targets because potential inhibitors may not need to cross the outer membrane, which is an extremely efficient permeability barrier in mycobacteria. In addition, they are likely to represent novel drug targets because they do not appear to show any similarity to other proteins (Faller *et al.*, 2004).

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APPENDIX

When cells are growing ideally (all cells have the same generation time) the age distribution is given by Equation (A.1) where a is a cell's age ($a = t/t_D$; $a = 0$ for newborn cells and $a = 1$ for cells about to divide), and $t \leq t_D$ is the time (hours) since the birth of a cell; n_c, is the number of cells per milliliter of culture; $n_{c(a)}$, is the number of cells aged a per milliliter of culture; $n_{c(a=0)}$, is the number of newborn cells per milliliter of culture.

$$n_{c(a)} = n_{c(a=0)} + e^{-a \bullet \ln 2} \tag{A.1}$$

The total number of cells, n_c, is given by the following equation.

$$n_{c(a)} = n_{c(a=0)} \int_{a=0}^{a=1} e^{-a \bullet \ln 2} da \tag{A.2}$$

Evaluation of Equation (A.2) leads to the following equation.

$$n_c = 0.72 n_{c(a=0)} \tag{A.3}$$

The age $a_{r(ORF_{(i)})}$ at which $ORF_{(i)}$ is replicated is given by Equation (A.4), where t_{G_1} is the period between cell division and the onset of DNA replication, τ_g (hours) is the period of DNA replication, $\gamma_{ORF_{(i)}}$ is the replication coefficient for $ORF_{(i)}$, and t_D is the doubling or generation time.

$$a_{r(ORF_{(i)})} = \frac{(t_{G_1} + \gamma_{ORF_{(i)}} \bullet \tau_g)}{t_D} \tag{A.4}$$

Compared with the time taken to replicate the genome the time taken to replicate $ORF_{(i)}$ is very small and can be neglected. Suppose that there is a single copy of $ORF_{(i)}$, per genome ($n_{ORF_{(i)}/g=1}$). The number of copies of $ORF_{(i)}$ per cell will depend on the cell's age. Before $ORF_{(i)}$ is replicated (cells younger than $a_{r(ORF_{(i)})}$) the number of copies of $ORF_{(i)}$ per cell is equal to $n_{ORF_{(i)}/g}$ or a single copy per cell. For cells aged $a_{r(ORF)_{(i)}}$ and older the number of copies of $ORF_{(i)}$ per cell will be equal to $2n_{ORF_{(i)}/g}$. The number $n_{ORF_{(i)}/c}$ of copies of $ORF_{(i)}$ per population-average cell is equal to the total number of copies of $ORF_{(i)}$ divided by the total number of cells. Thus, $n_{ORF_{(i)}/c}$ is defined in Equation (A.5).

$$n_{ORF_{(i)}/c} = \left(\frac{n_{ORF_{(i)}/g}}{n_c}\right)\left\{n_{c(a=0)} \int_0^{ar(ORF_{(i)})} e^{-a \bullet \ln 2} \bullet da + 2n_{c(a=0)} \times \int_{ar(ORF_{(i)})}^{a=1} e^{-a \bullet \ln 2} \bullet da\right\} \tag{A.5}$$

The equation can be simplified by substituting for $n_c = 0.72\, n_{c(a=0)}$. The integrated form of Equation (A.5) is presented in Equation (A.6)

$$n_{ORF_{(i)}/c} = \left(\frac{n_{ORF_{(i)}/g}}{(0.72 \ln 2)}\right)\left\{\left[e^{-a \bullet \ln 2}\right]_a^{ar(ORF_{(i)})} + 2\left[e^{-a \bullet \ln 2}\right]_{ar(ORF_{(i)})}^{1}\right\} \tag{A.6}$$

An approximate equation related to Equation (A.6) was derived for *E. coli* before genomic sequences were available (Chandler and Pritchard, 1975; Bremer and Churchward, 1977).

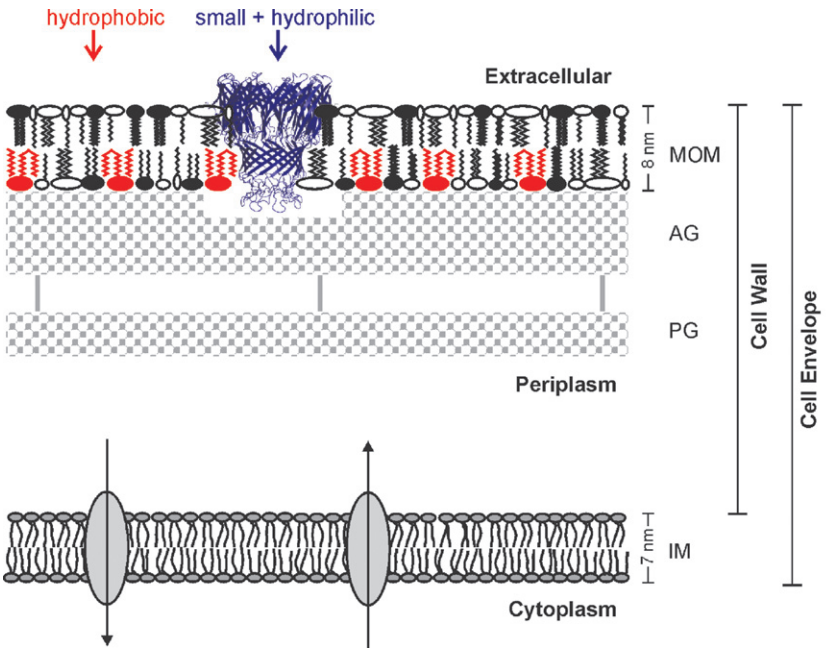

Plate 3 Transport processes across the mycobacterial cell envelope. Schematic representation of the mycobacterial cell envelope consisting of the inner membrane (IM) and the cell wall. This representation is based on cryo-electron micrographs (Hoffmann *et al.*, 2007, 2008). Mycolic acids are covalently linked to the arabinogalactan (AG)–peptidoglycan (PG) co-polymer and are an essential component of the inner leaflet of the outer membrane (OM). Extractable lipids are represented in black. The porin MspA mediates the uptake of small and hydrophilic nutrients such as sugars (Stephan *et al.*, 2005) and phosphates (Wolschendorf *et al.*, 2007) across the OM of *M. smegmatis*. The MspA channel is 9.6 nm long (Faller *et al.*, 2004). Approximately, 7 nm of the MspA surface are inaccessible to hydrophilic reagents (Mahfoud *et al.*, 2006). Hydrophobic compounds are assumed to diffuse directly across the OM. The dimensions are approximately to scale. (For b/w version, see page 107 in the volume.)

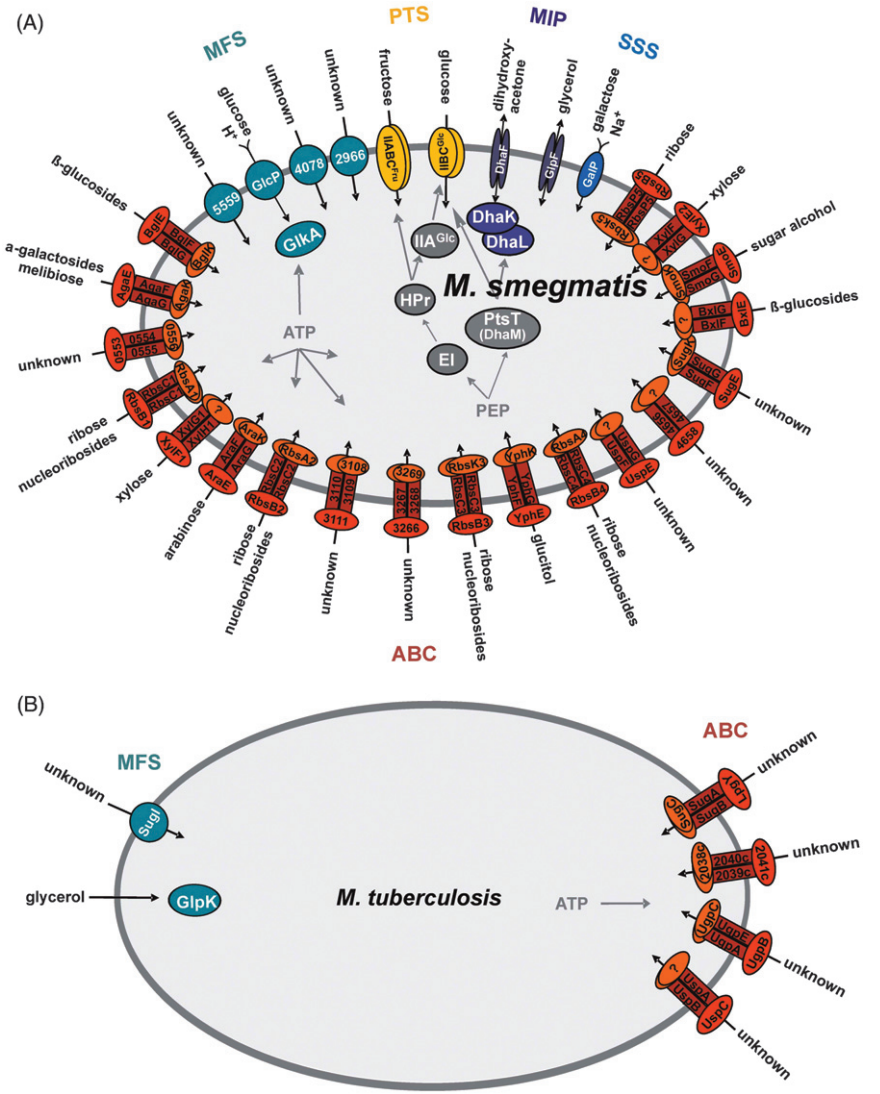

Plate 4 Transporters for uptake of carbohydrates across the inner membrane of *M. smegmatis* and *M. tuberculosis.* Shown are the transport proteins of the ATP-binding cassette (ABC, red), phosphotransferase system (PTS, yellow), major facilitator super family (MFS, green), major intrinsic protein family (MIP, dark blue), and the sodium solute super family (SSS, blue). The derived putative substrates are inferred from *in silico* analyses in combination with experimental data (Titgemeyer *et al.*, 2007). Systems are denoted by the protein name instead of their locus tags (MSMEG_XXXX) if the substrates were experimentally verified or were predicted with a high likelihood. Note that the OMs of *M. smegmatis* and *M. tuberculosis* were omitted for clarity reasons. The figure was taken from Niederweis (2008b) with permission. (For b/w version, see page 111 in the volume.)

Biology and Genomic Analysis of *Clostridium botulinum*

Michael W. Peck

Institute of Food Research, Norwich Research Park, Colney, Norwich, UK

ABSTRACT

The ability to form botulinum neurotoxin is restricted to six phylogenetically and physiologically distinct bacteria (*Clostridium botulinum* Groups I–IV and some strains of *C. baratii* and *C. butyricum*). The botulinum neurotoxin is the most potent toxin known, with as little as 30–100 ng potentially fatal, and is responsible for botulism, a severe neuroparalytic disease that affects humans, animals, and birds. In order to minimize the hazards presented by the botulinum neurotoxin-forming clostridia, it is necessary to extend understanding of the biology of these bacteria. Analyses of recently available genome sequences in conjunction with studies of bacterial physiology are beginning to reveal new and exciting information on the biology of these dangerous bacteria. At the whole organism level, substantial differences between the six botulinum neurotoxin-forming clostridia have been reported. For example, the genomes of proteolytic *C. botulinum* (*C. botulinum* Group I) and non-proteolytic *C. botulinum* (*C. botulinum* Group II) are highly diverged and show neither synteny nor homology. It has also emerged that the botulinum neurotoxin-forming clostridia are not overtly pathogenic (unlike *C. difficile*), but saprophytic bacteria that use the neurotoxin to kill a host and create a source of nutrients.

One important feature that has contributed to the success of botulinum neurotoxin-forming clostridia is their ability to form highly resistant endospores. The spores, however, also present an opportunity to control these bacteria if escape from lag phase (and hence growth) can be prevented. This is dependent on extending understanding of the biology of

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these processes. Differences in the genetics and physiology of spore germination in proteolytic *C. botulinum* and non-proteolytic *C. botulinum* have been identified. The biological variability in lag phase and its stages has been described for individual spores, and it has been shown that various adverse treatments extend different stages of lag phase. For example, heat treatment primarily extended germination, while incubation at a chilled temperature primarily extended outgrowth. The neurotoxin gene is present within a cluster of associated genes, and can be located on the chromosome, a plasmid or a bacteriophage. Two basic types of neurotoxin cluster have been identified. Evolution of the neurotoxin gene and cluster has occurred independently of the organism, and involved a series of recombination events but is still poorly understood. Factors affecting the regulation of neurotoxin formation also remain poorly understood, and will be the focus of much future research.

ABBREVIATIONS

HA	hemagglutinins
MLST	multi-locus sequence typing
NTNH	non-toxic-non-hemagglutinin

1. INTRODUCTION

Clostridium botulinum is a heterogeneous species defined by the ability to form botulinum neurotoxin. The name of *C. botulinum* is retained to emphasize the importance of neurotoxin formation, and this species comprises four phylogenetically and physiologically distinct bacteria, known as *C. botulinum* Groups I–IV (Hatheway, 1993). Some strains of *C. baratii* and *C. butyricum* also possess the capacity to form botulinum neurotoxin. Together these organisms are responsible for botulism, a severe and often deadly disease that affects humans, animals, and birds. Three types of botulism are most frequently encountered. Foodborne botulism is an intoxication caused by inadvertent consumption of preformed botulinum neurotoxin, infant/intestinal (adult) botulism is an infection of the gastrointestinal tract, and wound botulism is an infection of an abscess or lesion.

Two aspects of the biology of botulinum neurotoxin-forming clostridia are primarily responsible for the considerable hazards presented. The first is their ability to form endospores. The endospores are highly resistant, and enable these clostridia to survive adverse treatments such as heating. After such adverse treatment, botulinum neurotoxin-forming clostridia may find themselves in a pure culture, able to grow and form neurotoxin without competition. To better control the botulinum neurotoxin-forming clostridia, it is important to understand their mechanisms of spore heat resistance, and the physiological processes involved in germination and lag phase. While these are less well understood in clostridia than in *Bacillus* species, important progress is being made. The second important aspect of the biology of these clostridia is their ability to form a highly potent neurotoxin. The botulinum neurotoxin is the most toxic poison known, with as little as 30–100 ng potentially fatal. Knowledge of some aspects of the neurotoxin is well advanced, for example, the crystallographic structure has been solved, while many other aspects such as the mechanisms regulating neurotoxin formation are still poorly understood.

There have been a number of important recent developments in clostridial genomics, including the sequencing of the genome of several botulinum neurotoxin-forming clostridia and the building of DNA microarrays enabling whole genome analyses (Sebaihia *et al.*, 2007; Carter *et al.*, 2009). These developments allied with more traditional physiological approaches have created exciting new opportunities to extend understanding of key aspects of clostridial physiology, and provide a unique opportunity to dissect out and extend understanding of spore germination,

lag phase, and neurotoxin formation and its regulation in botulinum neurotoxin-forming clostridia. These physiological and genomic studies are already yielding interesting new insights in to the biology of botulinum neurotoxin-forming clostridia.

2. CHARACTERISTICS OF *C. BOTULINUM*

2.1. Taxonomy of the Six Bacteria that form the Botulinum Neurotoxin

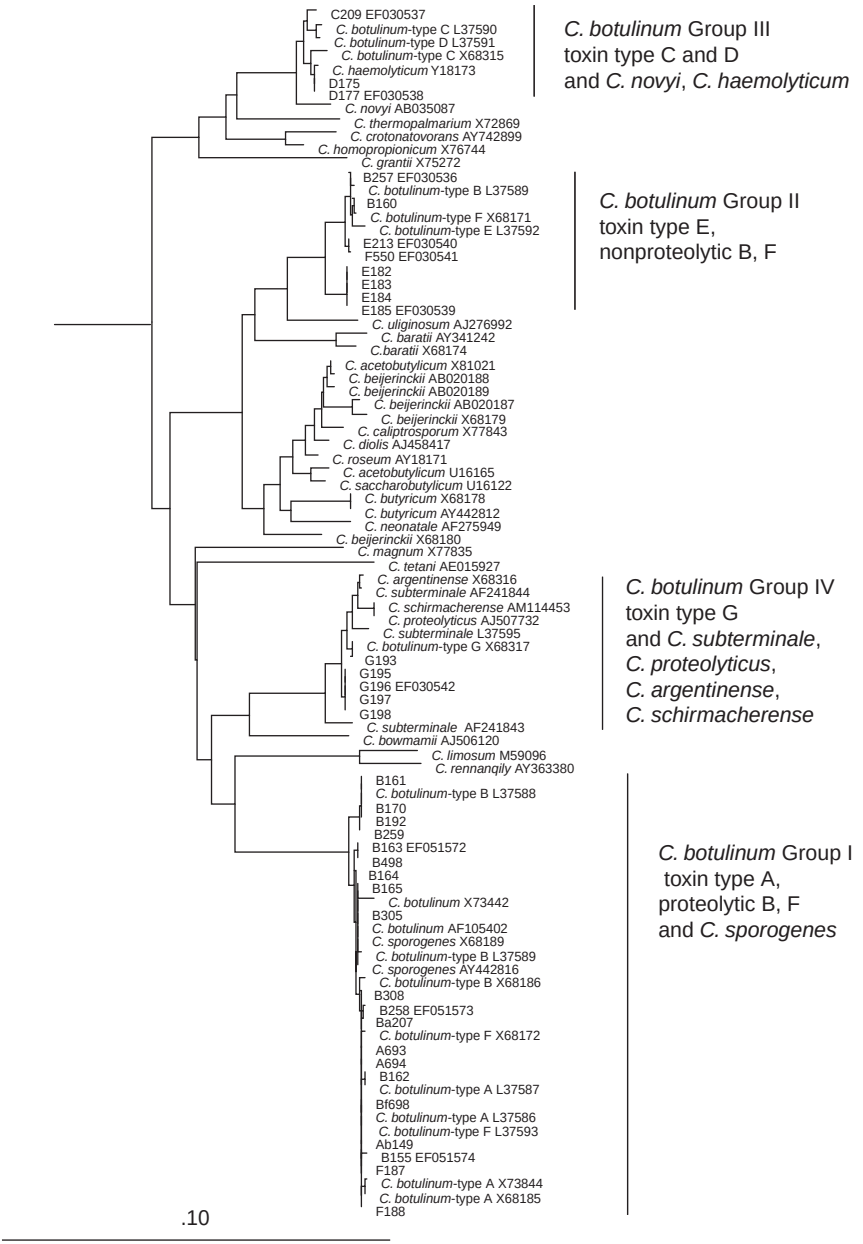
The genus *Clostridium* comprises more than 100 species. It is a phylogenetically disjointed genus that is traditionally defined as comprising Gram-positive rod-shaped endospore-forming anaerobic bacteria (Stackebrandt and Rainey, 1997). Some clostridia are important from a biotechnological perspective, such as *C. acetobutylicum* (acetone/butanol fermentation) (Durre, 2007). Other clostridia are pathogenic, with at least 35 pathogenic species described (Stackebrandt and Rainey, 1997). These include *C. botulinum* and *C. tetani* (the causative agents of botulism and tetanus, respectively), *C. perfringens* (responsible for gas gangrene and food poisoning), and *C. difficile* (associated with pseudomembraneous colitis). The wide genetic variability between members of the genus *Clostridium* is illustrated by comparison of sequenced genomes and 16S *rrn* genes. For example, 1,571 of the coding sequences (CDSs) (approximately 43%) in a representative *C. botulinum* Group I genome were absent from four other sequenced clostridia (*C. acetobutylicum, C. difficile*, *C. perfringens, C. tetani*), and only 568 (approximately 16%) of the CDSs in this *C. botulinum* Group I genome were shared by the genomes of all five clostridia (Sebaihia *et al.*, 2007). The genome of *C. botulinum* Group I did not show synteny with the genomes of *C. acetobutylicum, C. difficile, C. perfringens,* or *C. tetani*.

A recent comparison of clostridial 16S *rrn* sequences by Hill *et al.* (2007) extended the earlier work of Collins and others (Collins *et al.*, 1994; Stackebrandt and Rainey, 1997), and confirmed that *C. botulinum* is a heterogeneous species separated into four distinct phylogenetic groups (Fig. 1). *C. botulinum* is defined not on the basis of close phylogenetic or physiological characteristics, but by the ability to form botulinum neurotoxin. Indeed, the distinction between *C. botulinum* Groups I to IV is strong enough to merit the creation of four different species. The name of

C. botulinum is retained to emphasize the importance of neurotoxin formation. For each of the four Groups, a phylogenetically equivalent, but non-neurotoxigenic, organism has been described (Table 1). Strains of *C. botulinum* Group I (proteolytic *C. botulinum*) are very closely related to *C. sporogenes*, and only distantly related to strains of *C. botulinum* Group II, Group III, or Group IV. Non-neurotoxigenic strains of *C. botulinum* Group II (non-proteolytic *C. botulinum*) have been described in the literature but not given a species name (Peck and Stringer, 2005). *C. botulinum* Group III is closely related to *C. novyi*, and *C. botulinum* Group IV (also known as *C. argentinense*) is closely related to *C. subterminale* (Fig. 1). Strains of *C. baratii* and *C. butyricum* that form botulinum neurotoxin have also been described, and except for this ability are indistinguishable from other more typical strains (Lund and Peck, 2000). The wide genetic distance between the six clostridia that form the botulinum neurotoxin (*C. botulinum* Groups I–IV, *C. baratii* and *C. butyricum*) is also demonstrated by studies of DNA hybridization (Hatheway, 1993), comparative genomic indexing (Sebaihia *et al.*, 2007; Carter *et al.*, 2009), and comparison of sequenced genomes. While the genomes of two *C. botulinum* Group I (proteolytic *C. botulinum*) strains (type A strain ATCC 3502 and type F strain Langeland) were highly similar and showed substantial synteny, *C. botulinum* Group I (proteolytic *C. botulinum*) type A strain ATCC 3502 and *C. botulinum* Group II (non-proteolytic *C. botulinum*) type B strain Eklund 17B did not show homology or synteny (Fig. 2).

2.2. Genetic and Physiological Properties

The different physiological properties of the six neurotoxigenic clostridia reflect their different phylogenetic background. Proteolytic *C. botulinum* is a mesophile that forms spores of high heat resistance, and strains form either one or two neurotoxins of type A, B, or F (Table 1). Proteolytic *C. botulinum* type A is reported to be prevalent in soils in the western continental USA, South America, and China, and proteolytic *C. botulinum* type B appears more prevalent in soils in the eastern continental USA (Dodds, 1993). Many botulism cases in these locations are associated with proteolytic *C. botulinum*. Non-proteolytic *C. botulinum* is psychrotrophic, and forms spores of moderate heat resistance. Strains of this bacterium form a single neurotoxin of type B, E, or F (Table 1). Non-proteolytic *C. botulinum* type B appears to predominate in much of Europe, and non-proteolytic *C. botulinum* type E is primarily associated with marine and freshwater sediments in temperate regions of the world (e.g., Northern


C209 EF030537
C. botulinum-type C L37590
C. botulinum-type D L37591
C. botulinum-type C X68315
C. haemolyticum Y18173
D175
D177 EF030538
C. novyi AB035087
C. thermopalmarium X72869
C. crotonatovorans AY742899
C. homopropionicum X76744
C. grantii X75272
B257 EF030536
C. botulinum-type B L37589
B160
C. botulinum-type F X68171
C. botulinum-type E L37592
E213 EF030540
F550 EF030541
E182
E183
E184
E185 EF030539
C. uliginosum AJ276992
C. baratii AY341242
C.baratii X68174
C. acetobutylicum X81021
C. beijerinckii AB020188
C. beijerinckii AB020189
C. beijerinckii AB020187
C. beijerinckii X68179
C. caliptrosporum X77843
C. diolis AJ458417
C. roseum AY18171
C. acetobutylicum U16165
C. saccharobutylicum U16122
C. butyricum X68178
C. butyricum AY442812
C. neonatale AF275949
C. beijerinckii X68180
C. magnum X77835
C. tetani AE015927
C. argentinense X68316
C. subterminale AF241844
C. schirmacherense AM114453
C. proteolyticus AJ507732
C. subterminale L37595
C. botulinum-type G X68317
G193
G195
G196 EF030542
G197
G198
C. subterminale AF241843
C. bowmamii AJ506120
C. limosum M59096
C. rennanqily AY363380
B161
C. botulinum-type B L37588
B170
B192
B259
B163 EF051572
B498
B164
B165
C. botulinum X73442
B305
C. botulinum AF105402
C. sporogenes X68189
C. botulinum-type B L37589
C. sporogenes AY442816
C. botulinum-type B X68186
B308
B258 EF051573
Ba207
C. botulinum-type F X68172
A693
A694
B162
C. botulinum-type A L37587
Bf698
C. botulinum-type A L37586
C. botulinum-type F L37593
Ab149
B155 EF051574
F187
C. botulinum-type A X73844
C. botulinum-type A X68185
F188
.10
C. botulinum Group III toxin type C and D and C. novyi, C. haemolyticum
C. botulinum Group II toxin type E, nonproteolytic B, F
C. botulinum Group IV toxin type G and C. subterminale, C. proteolyticus, C. argentinense, C. schirmacherense
C. botulinum Group I toxin type A, proteolytic B, F and C. sporogenes

Europe, Japan, Canada, Alaska). Both are a frequent cause of foodborne botulism (Peck and Stringer, 2005). *C. botulinum* Group III is a mesophile that forms neurotoxins of types C and D. *C. botulinum* Group IV (*C. argentinense*) is a mesophilic organism that forms type G neurotoxin. Neurotoxigenic strains of *C. baratii* and *C. butyricum* form type F and type E neurotoxin, respectively. Both are mesophiles (Table 1). Proteolytic *C. botulinum* and non-proteolytic *C. botulinum* are responsible for the majority of cases of botulism in humans, and are the primary focus of this review.

2.2.1. Proteolytic C. botulinum (C. botulinum Group I)

Proteolytic *C. botulinum* strains form neurotoxin of types A, B, or F (Table 1). Strains with two toxin genes have also been described. Some of these form two active neurotoxins, with the major and minor neurotoxin types designated by an uppercase and lowercase letter, for example, Ab, Af, Ba, and Bf (Lund and Peck, 2000). Others form only one active neurotoxin, for example, A(B) strains possess a type A and type B neurotoxin gene, but only form active type A neurotoxin. Proteolytic *C. botulinum* is associated with foodborne, wound, and infant/intestinal botulism. This mesophilic bacterium has an optimum growth temperature of 37 °C, and the time to a 1,000-fold increase from a spore inoculum at this temperature can be as short as 12.6 hours (Peck, 2006). Growth and neurotoxin formation do not occur at 10 °C or below, and have been reported in 3–4 weeks at 12 °C from a large inoculum, although many strains grow poorly below 15 °C (Peck, 2006). Proteolytic *C. botulinum* is unable to multiply and form neurotoxin at pH $\leq$ 4.6, or at $\geq$10% NaCl (Table 1). The effects of various preservative factors and combinations of factors on growth and neurotoxin formation and on thermal death have been described, and predictive models developed (Lund and Peck, 2000). Both are freely available in software packages

Figure 1 Phylogenetic dendrogram of *Clostridium* species based on the sequence of 16S *rrn* (16S rRNA) genes [Hill *et al.* (2007), reproduced with permission from American Society for Microbiology]. A neighbor-joining tree of 90 representative sequences is shown, and illustrates the genetic diversity within the clostridia. *C. botulinum* Groups I, II, III, and IV are interspersed among 27 other clostridial species in this tree. The tree was constructed using an alignment of 16S *rrn* sequences, which was 1,329 bases in length after removal of columns containing more than 80% gap characters. The scale bar indicates the distance equivalent to 0.1 mutations per site (e.g., the 16S *rrn* gene of *C. botulinum* Group I strain F188 and *C. tetani* are 91.7% identical).

Table 1 Characteristics of the six physiologically and phylogenetically distinct clostridia that form the botulinum neurotoxin.

Neurotoxigenic clostridia	*C. botulinum* Group I (proteolytic *C. botulinum*)	*C. botulinum* Group II (non-proteolytic *C. botulinum*)	*C. botulinum* Group III	*C. botulinum* Group IV (*C. argentinense*)	*C. baratii*	*C. butyricum*
Neurotoxins formed	A, B, F[a]	B, E, F	C, D	G	F	E
Non-neurotoxigenic equivalent clostridia	*C. sporogenes*	No species name given	*C. novyi*	*C. subterminale*	All typical *C. baratii* strains	All typical *C. butyricum* strains
Ferment glucose	+[b]	+	+	−	+	+
Ferment fructose	+/−	+	+/−	−	+	+
Ferment maltose	+/−	+	+/−	−	+	+
Ferment sucrose	−	+	−	−	+	+
Optimum growth temperature	37 °C	25 °C	40 °C	37 °C	30–45 °C	30–37 °C
Minimum growth temperature	10–12 °C	2.5–3.0 °C	15 °C		10–15 °C	12 °C
Minimum pH for growth	4.6	5.0	5.1			4.8
NaCl concentration preventing growth	10%	5%		6.5%		
Minimum water activity for growth, humectant: NaCl/ glycerol	0.94/0.93	0.97/0.94				
Spore heat resistance[c]	$D_{121°C}$ = 0.21 minutes	$D_{82.2°C}$ = 2.4/ 231 minutes[d]	$D_{104°C}$ = 0.9 minutes	$D_{104°C}$ = 1.1 minutes		$D_{100°C}$< 0.1 minutes

Source: Data from Smith and Sugiyama (1988), Lund and Peck (2000), and Stringer and Peck (2008).
Where values are not given, they are not readily available in the literature.
[a]In dual-toxin strains, more than one toxin is formed.
[b]+, all strains positive; +/−, some strains are positive with others negative,−, all strains negative.
[c]Spore heat resistance determined in phosphate buffer, pH 7.0
[d]*D*-value without/with lysozyme during recovery.

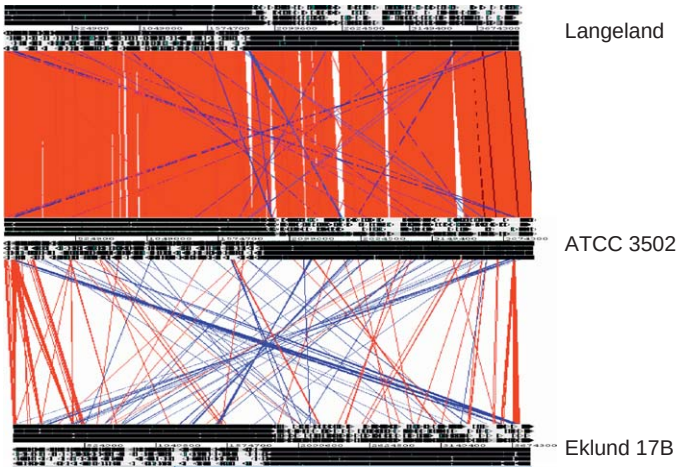


Figure 2 Artemis Comparison Tool (ACT) whole genome analysis of proteolytic *C. botulinum* type F strain Langeland and non-proteolytic *C. botulinum* type B strain Eklund 17B, each compared with proteolytic *C. botulinum* type A1 strain ATCC 3502 (Carter and Peck, unpublished data). Red lines connecting two genomes indicate both synteny and homology; blue lines indicate homology but breakdown in synteny. White gaps show lack of both parameters, that is, these regions are either diverged to the point where the BLAST analysis which forms the basis of this test gives a score which drops below a defined cut-off point, or the region is simply absent. The six possible reading frames (three forward, three reverse) of each genome are depicted as horizontal black bars, within which are shown potential coding sequences (CDSs) (open arrows, pointing in the direction of transcription). The two proteolytic *C. botulinum* strains (Langeland and ATCC 3502) share a high degree of both homology and synteny, whereas the non-proteolytic *C. botulinum* strain displays very little of either property. (See plate 5 in the color plate section.)

(www.combase.cc, www.combase.cc/predictor.html; www.ars.usda.gov/Services/docs.htm?docid = 6796).

Spores of proteolytic *C. botulinum* are of high heat resistance and are the principal concern for the safe production of low acid canned foods (Table 1). In response to a large number of botulism outbreaks involving home and commercially canned foods almost a century ago, the heat resistance of spores of proteolytic *C. botulinum* was extensively studied by Meyer, Esty, and co-workers. For example, Esty and Meyer (1922) measured the heat resistance of 109 strains, and established that heat resistance was dependent on the strain tested and the composition of the heating and recovery medium. These early studies formed the basis for the standard minimum heat treatment that has been adopted by the canning

industry as the ''botulinum cook'' for low acid canned foods for many years. The heat treatment is intended to reduce the number of spores of proteolytic *C. botulinum* spores by a factor of 10^{12} (12-D (12-decimal) process) (Stumbo *et al.*, 1975). Spores of the most heat resistant strains had a *D*-value at 121 °C of 0.21 min (Table 1), thus a 12-D process would be achieved by heating at 121 °C for 2.52 minutes. For commercial practice, this is rounded up to 3 minutes. The application of this heat treatment has ensured the safe production of low-acid canned foods for many decades, and botulism outbreaks have only occurred when the full heat treatment has not been appropriately delivered, or there has been post-process contamination.

The genomes of ten strains of proteolytic *C. botulinum* have been sequenced or are in progress (Table 2). Proteolytic *C. botulinum* type A

Table 2 Summary of completed/in progress genome sequences of strains of *C. botulinum*.

Toxin type	Strain	Genome size (Mb)	%G+C	Plasmid size (kb)
Proteolytic *C. botulinum*				
A1	ATCC 3502 (Hall 174)	3.89	28.2	16.3
A1	ATCC 19397 (NCTC 7272)	3.86	28.2	–
A1	Hall	3.76	28.2	–
A2	Kyoto-F	In progress		
A3	NCTC 2012 (Loch Maree)	4.26	28.1	266.8
A1(B)	NCTC 2916[a]	4.03	28.5	–
B	Okra	4.11	28.2	148.8
Bf	Bf (infant botulism)[a]	4.22	28.2	–
Ba4	CDC 657	In progress		
F	Langeland	4.01	28.3	17.5
Non-proteolytic *C. botulinum*				
B	Eklund 17B	3.80	27.5	47.6
E	Alaska E43	3.66	27.4	–
E	Beluga	In progress		
E	Iwanei	In progress		
C. botulinum Group III				
C	Eklund[a]	2.96	29.0	–
C. botulinum Group IV (*C. argentinense*)				
G	G	In progress		

Source: From Sebaihia *et al.* (2007); http://pathema.jcvi.org/cgi-bin/Clostridium/shared/Genomes.cgi; http://www.ncbi.nlm.nih.gov (accessed 17/11/08).
[a]Sequence being assembled.

strain ATCC 3502 (Hall 174) was the first of these strains to be sequenced, and this revealed interesting aspects of the biology and lifestyle of this dangerous pathogen (Sebaihia *et al.*, 2007). The genome comprised a chromosome (3.89 Mb, 3,650 CDSs (Coding Sequences)), and a single plasmid (16.3 kb, 19 CDSs) that encoded a bacteriocin. Both the chromosome and plasmid had a low %G+C content (28.2% for the chromosome and 28.6% for the plasmid). There was little evidence of recently acquired DNA, indicating a relatively stable genome. This is in strong contrast to the highly fluid genome of *C. difficile* (Sebaihia *et al.*, 2006). There was evidence that although proteolytic *C. botulinum* is a highly dangerous pathogen, it is well adapted to a saprophytic lifestyle. It appears to rely on the neurotoxin to kill a wide range of species, and then releases a variety of extracellular enzymes to obtain nutrients. The highly proteolytic nature of proteolytic *C. botulinum* type A strain ATCC 3502 is reflected by the presence of several CDSs encoding proteases in the genome (Sebaihia *et al.*, 2007). These proteases all appear to have an N-terminal signal sequence, indicating that they are likely to be secreted. The genome also contains 40 CDSs encoding transporters for amino acids and peptides. Proteolytic *C. botulinum*, like several other clostridia, is able to ferment amino acids in a coupled oxidation–reduction reaction known as the Stickland reaction (Stickland, 1935), where the oxidation of one amino acid is coupled to the reduction of another. A number of CDSs encoding enzymes capable of carrying out the Stickland reaction have been identified in the genome of strain ATCC 3502 (Sebaihia *et al.*, 2007). There are also CDSs encoding enzymes dedicated to carbohydrate catabolism, with proteolytic *C. botulinum* able to degrade complex carbohydrates such as starch and chitin. Chitin is an abundant polysaccharide comprising a linear homopolymer of *N*-acetyl-D-glucosamine, and is the major component of the skeleton of insects and crustaceans, and fungal cell walls. The genome of strain ATCC 3502 encodes five putative secreted chitinases, and these are widespread amongst strains of proteolytic *C. botulinum* and *C. sporogenes*, with 60/61 strains tested possessing all five putative secreted chitinases, and the other strain missing only one (Carter and Peck, unpublished data). This widespread distribution of all five putative secreted chitinases suggests that chitin is an important source of carbon and nitrogen for proteolytic *C. botulinum*, that it has carried this set of CDSs from a very early stage of its evolution, and that they have played an important part in this bacterium's lifestyle. Sugars are catabolized (Table 1), and the genome of ATCC 3502 contains 15 CDSs encoding putative phosphoenolpyruvate (PEP)-dependent phosphotransferase systems for sugar transport. Proteolytic *C. botulinum* strain ATCC 3502 has CDSs encoding a complete

glycolytic pathway, but an incomplete TCA cycle (Sebaihia *et al.*, 2007). The end products of catabolism are those typical of amino acid degradation (e.g., acetate, butyrate, iso-butyrate, iso-valerate, phenypropionate).

The genomes of the eight fully sequenced strains of proteolytic *C. botulinum* are of a similar size, 3.76–4.26 Mb, and similar low %G+C content (from 28.1 to 28.5%) (Table 2). Strains of *C. botulinum* are often referred to by the person who isolated the strain, for example, M.W. Eklund and I.C. Hall each isolated a great number of strains (see, e.g., Table 2). This may cause inadvertent confusion, I.C. Hall isolated many type A strains, and these are often called just "Hall A." Hill *et al.* (2007) examined 14 of Hall's strains that formed type A toxin, and found them to have slightly different properties. The genome of a strain known simply as "Hall" is marginally smaller than that of the first sequenced strain Hall 174 (ATCC 3502). The genomes of strains ATCC 19397, Hall, NCTC 2012, Okra and Langeland all showed synteny with the genome of strain ATCC 3502 (Carter and Peck, unpublished results). For example, Fig. 2 shows a comparison between ATCC 3502 and the type F strain Langeland. From whole genome analysis based on the genome of ATCC 3502 it is estimated that for 61 strains of proteolytic *C. botulinum* and *C. sporogenes*, the core gene set was 2155 of the CDSs (approximately 63%) of ATCC 3502 (Carter *et al.*, 2009). For 14 type A1 strains, the core set was 2775 (approximately 80%) of the CDSs, and for ten type A1 strains closely related to ATCC 3502, it was 3055 (approximately 89%) of the CDSs (Carter *et al.*, 2009). These observations provide further evidence for the close genetic relationship between strains of proteolytic *C. botulinum* and *C. sporogenes*, and for a relatively stable genome. Major areas of divergence include the plasmid, flagellar glycosylation island, and two prophages. Together these account for approximately 4.6% of the DNA (plasmid plus chromosome) of ATCC 3502. The core gene set is substantially larger than that of *C. difficile*, where only 20% of the CDSs present in the reference strain were also present in 75 test strains (Stabler *et al.*, 2006), suggesting that exchange of genetic information has occurred considerably more frequently in *C. difficile* than in proteolytic *C. botulinum*.

Plasmids are frequently present in strains of proteolytic *C. botulinum*, but most have no known associated phenotypic function (e.g., Strom *et al.*, 1984; Ferreira *et al.*, 1987; Eklund *et al.*, 1989; Table 2). In many strains of proteolytic *C. botulinum*, the neurotoxin gene(s) are located on the chromosome (e.g., ATCC 3502, 62A, CDC 5328 (all type A1 strains), Kyoto-F (type A2), NCTC 2916 (type A1(B)). However, it has been recently shown that in strains NCTC 2012 (Loch Maree) (type A3), Okra (type B) and CDC 657 (type Ba4), the neurotoxin genes are located on a large plasmid (Marshall *et al.*, 2007; Sebaihia *et al.*, 2007; Smith *et al.*, 2007;

Franciosa *et al.*, 2008). The plasmids in these three strains shared significant sequence identity and had a similar %G+C (25.4–25.6%), although the plasmid in strain Okra was smaller (148.8 kb) than the plasmids in the other two strains (266.8–270.3 kb). Smaller bacteriocin-encoding plasmids have been identified in strains of proteolytic *C. botulinum*, and characterized in the type A strain ATCC 3502 (plasmid of 16.3 kb) and the type B strain 213B (plasmid of 18.8 kb) (Dineen *et al.*, 2000; Sebaihia *et al.*, 2007; Carter *et al.*, 2009).

A number of complementary approaches have been used to assess the evolutionary and phylogenetic relationship between strains of proteolytic *C. botulinum*. Comparative genomic indexing using a DNA microarray based on the genome of the sequenced strain ATCC 3502 confirmed the high genetic relatedness between strains of proteolytic *C. botulinum* and *C. sporogenes*, and sub-divided 61 diverse strains into nine clades (Carter *et al.*, 2009). The 61 strains were of different toxin types, and originally isolated at different times over a period of more than 80 years, from the environment, or associated with various forms of botulism. Small numbers of closely related strains generally of the same toxin type grouped together within the clades, and there were a number of distinct groups for most toxin types often within different clades. Most clades contained strains of more than one toxin type (or sub-type), and most toxin types (or sub-types) were represented in more than one clade, suggesting that the evolution of the neurotoxin genes has not paralleled that of the remainder of the genetic complement. Furthermore, strains did not group together according to the type of botulism (foodborne, infant, or wound) with which they were associated, or location, environment, or time of isolation (Carter *et al.*, 2009). These findings are in accordance with those made using other typing methods (Nevas *et al.*, 2005a; Hill *et al.*, 2007; Macdonald *et al.*, 2008; Jacobson *et al.*, 2008b).

To date most emphasis has been placed on establishing the evolutionary and phylogenetic relationship between strains of proteolytic *C. botulinum* that form type A toxin (as this is the most potent of the neurotoxins). These studies have begun to identify outlying strains with unusual properties. By taking account of whole genome analysis, toxin gene type, and toxin cluster type, 31 strains that formed type A toxin were separated into 12 distinct groups (Table 3). Most type A1 strains were similar to the sequenced strain ATCC 3502, and present in clades 7 and 8 in the whole genome analysis. Strains 17A and 96A are worthy of further study, they appeared different to each other and all the other type A1 strains by whole genome analysis and also by analysis of the CDSs within the flagellar glycosylation island (Carter *et al.*, 2009). PFGE had previously showed that strain 96A was

Table 3 Grouping of 31 strains of proteolytic *C. botulinum* that form type A toxin based on toxin gene type, toxin cluster type, and clade type from whole genome analysis.

Group	Number of strains	Strains	Toxin gene type	Toxin cluster type	Whole genome clade
1	8	ATCC 3502, 16037, FE0101AJO, ATCC 25763, CDC 1690, ATCC 19397, NCTC 3805, VL1	A1	ha plus/orf-X minus	7
2	2	FE0205A1AK, F9801A	A1	ha plus/orf-X minus	8
3	1	17A	A1	ha plus/orf-X minus	1
4	1	96A	A1	ha plus/orf-X minus	3
5	2	F9604A, MUL0109ASA	A1	ha minus/orf-X plus	6
6	2	NCTC 9837, ZK3	A2	ha minus/orf-X plus	3
7	1	NCTC 2012 (Loch Maree)	A3	ha minus/orf-X plus	5
8	8	CDC 5001, CDC 13280, MDa10, NCTC 2916, FE0303A1YO, FE9909ACS, PE0101AJO, FE9504ACG	A1(B)	Both clusters[a]	4
9	3	NCTC 11199, FE0602ALS, FE0207AMB	A1(B)	Both clusters[a]	2
10	1	CDC 588	A1b	Both clusters[a]	2
11	1	CJ0611A	A1(B)	Both clusters[a]	6
12	1	CDC 657	Ba4	Both clusters[a]	6

Source: Derived from data in Carter *et al.* (2009).
[a]Both the ha plus/orf-X minus and the ha minus/orf-X plus clusters are present (see Fig. 6 for further details).

distinct from a number of other type A1 strains (Nevas *et al.*, 2005a). Strains F9604A and MUL0109ASA appeared similar to each other, but were in a different clade to the other type A1 strains (Table 3), and unusually have the type A1 toxin gene in a ha minus/orf-X plus cluster (see Section 4). Interestingly, these two strains were in the same clade as a type Ba4 strain (CDC 657). Strains F9604A and MUL0109ASA may be closely related to two other type A1 ha minus/orf-X plus strains (CDC 297 and CDC 5328) as they also grouped with strain CDC 657 when examined by AFLP, MLST, and MVLA (Hill *et al.*, 2007; Macdonald *et al.*, 2008; Raphael *et al.*, 2008; Jacobson *et al.*, 2008b). Two type A2 strains were in

clade 3; however the type A3 strain (NCTC 2012, Loch Maree) was the only member of clade 5 (Table 3). This strain was isolated following an outbreak of foodborne botulism at Loch Maree (Scotland) in 1922 (Leighton, 1923), and was responsible for the only food poisoning outbreak involving type A3 toxin. Other genetic indexing methods also showed NCTC 2012 (Loch Maree) to be unique and well separated from other strains of proteolytic *C. botulinum* (Hill *et al.*, 2007; Macdonald *et al.*, 2008; Jacobson *et al.*, 2008b). A majority of type A1(B) strains belonged to clade 4 and appeared to be closely related to each other, while four type A1(B) strains appeared to be divergent, with three strains belonging to clade 3, and strain CJ0611A to clade 6 (Table 3). Two strains formed both type A and type B toxin, albeit in different quantities, and these belonged to different clades. Strain CDC 588 forms a major amount of type A toxin and a minor amount of type B toxin, and is in the same clade as three type A1(B) strains, indicating that these strains are closely related to each other. Clade 6 included four strains that form type A toxin, two type A1 strains (F9604A, MUL0109ASA), a type A1(B) strain (CJ0611A), and a type Ba4 strain (CDC 657). It is likely that these strains are also closely related to each other. A multi-locus sequence typing (MLST) study, principally carried out with different strains of proteolytic *C. botulinum* that formed type A toxin, identified eight divergent strains (Jacobson *et al.*, 2008b). In this study, 24 distinct types were identified from the sequencing of seven housekeeping genes, and were classified into 4 major groups by bootstrap analysis (Table 4). Three of the divergent strains (NCTC 2012 (Loch Maree), CDC 657 and CDC 5328) have been identified as outliers using

Table 4 Grouping of strains of proteolytic *C. botulinum* that form type A toxin based on multi-locus sequence typing (MLST).

Group	Number of strains	Strains	MLST sequence type
1a	1	NCTC 2012 (Loch Maree) [type A3]	3
1b	3	CDC 657 [type Ba4], 14050 [type A(B)], CDC 5328 [type A1]	7, 14, 18
2	1	A207 [type A1]	17
3	3	14842 [type A(B)], 14843 [type A(B)], 14844 [type A(B)]	5
4	64	type A1 (41 strains), type A2 (2 strains), type A(B) (20 strains), *C. sporogenes* (1 strain)	Others

Source: Derived from data in Jacobson *et al.* (2008b).

other genetic indexing techniques, and each has an unusual property (as discussed above). Initial experiments also suggest that strains 14842, 14843, and 14844 may possess a unique neurotoxin cluster arrangement (Jacobson *et al.*, 2008b). It is expected that further type A strains with unusual properties will continue to be identified using whole genome analysis, MLST and other genetic indexing techniques. Further characterization of the outlier strains should contribute to our understanding of horizontal gene transfer within these strains, and to the phylogeny and evolution of proteolytic *C. botulinum*. The way forward is likely to include the sequencing of further strains of proteolytic *C. botulinum*. Suitable candidate outlier strains have been identified by whole genome analysis and MLST. Whole genome sequencing is rapidly becoming affordable for most laboratories, and is particularly attractive, as it will provide information on what has been inserted or lost and where on the genome this has taken place.

2.2.2. Non-Proteolytic C. botulinum (C. botulinum Group II)

In contrast to proteolytic *C. botulinum*, all strains of non-proteolytic *C. botulinum* possess a single neurotoxin gene of type B, E, or F (Table 1). Non-proteolytic *C. botulinum* is associated with foodborne botulism. It is a highly saccharolytic bacterium, and can ferment a number of sugars (Table 1). Typically, acetate and butyrate are the end products of catabolism. Non-proteolytic *C. botulinum* is a psychrotrophic bacterium, with an optimum growth temperature of 25 °C. It is estimated that when other environmental factors are optimal, a 1,000-fold increase from a spore inoculum is achieved at 25 °C in 10.4 hours (Peck, 2006). Growth and neurotoxin formation did not occur at 2.1–2.5 °C in 12 weeks, but has been reported at 3.0–3.3 °C in 5 to 7 weeks, although many strains grow poorly below 5 °C (Ohye and Scott, 1957; Schmidt *et al.*, 1961; Eklund *et al.*, 1967a, b; Graham *et al.*, 1997). Non-proteolytic *C. botulinum* is unable to multiply and form neurotoxin formation below pH 5.0 or at a NaCl concentration above 5% (Table 1). The effect of other preservative factors (alone and in combination) on growth and neurotoxin formation has been described, and predictive models have been developed (Graham *et al.*, 1996a; Peck, 2006; Stringer and Peck, 2008; www.combase.cc; www.ars.usda.gov/Services/docs.htm?docid = 6796).

Spores formed by strains of non-proteolytic *C. botulinum* are substantially less heat resistant than those formed by strains of proteolytic *C. botulinum* (Table 1). The highest reported D-value for spores heated in phosphate buffer at 82.2 °C, and recovered in the absence of lysozyme is

2.4 minutes (Table 1). Spore heat resistance is not related to the type of neurotoxin formed (Peck, 2009). It has been demonstrated that heating spores for a few minutes at 80–85 °C inactivates the spore germination system, and results in sub-lethal injury (Peck *et al.*, 1992a; Lund and Peck, 1994). These sub-lethally injured spores can be recovered if lysozyme is present in the plating medium or broth. Lysozyme is able to diffuse through the coat of a fraction (typically 0.1–1%) of these spores inducing germination by hydrolyzing peptidoglycan in the cortex, enabling growth and giving biphasic survival curves (Peck *et al.*, 1992a, b, 1993). Spores recovered in this way have a measured heat resistance approximately 100-times greater ($D_{82.2^{\circ}C} = 231$ minutes) than those not recovered by lysozyme (Table 1). They are still, however, more heat sensitive than spores of proteolytic *C. botulinum*. This effect of lysozyme may be significant for food safety, as this enzyme is present in many foods and is relatively heat-stable (Stringer *et al.*, 1999; Peck and Stringer, 2005). In experiments where 10^6 spores of non-proteolytic *C. botulinum* were added to a model food with no added lysozyme, heating at 85 °C for 36 minutes or 90 °C for 10 minutes was sufficient to prevent growth and neurotoxin formation at 25 °C in 60 days. When hen egg white lysozyme was added prior to heating at 85 °C for 84 minutes or 90 °C for 34 minutes, however, growth was observed at 25 °C after 14 days (Peck and Fernandez, 1995; Peck *et al.*, 1995b; Graham *et al.*, 1996b; Fernandez and Peck, 1997, 1999). Lysozyme is present in unheated crabmeat at approximately 200 μg/g (Lund and Peck, 1994), and may have facilitated the recovery of sub-lethally damaged spores, since a heat treatment at 90.6 °C for 65 minutes was needed to prevent growth and neurotoxin formation from 10^6 spores of non-proteolytic *C. botulinum* at 27 °C in 150 days (Peterson *et al.*, 1997).

The mean genome size of the two sequenced strains of non-proteolytic *C. botulinum* (3.73 Mb) is marginally smaller than that of the eight sequenced strains of proteolytic *C. botulinum* (4.02 Mb), and the %G+C is marginally lower (Table 2). DNA hybridization tests and 16S *rrn* sequencing have shown that the two genomes are very different (Hatheway, 1993; Collins and East, 1998; Hill *et al.*, 2007 (see Fig. 1)). Comparison of 16S *rrn* sequences revealed that in terms of genetic difference, the divergences between the four Groups of *C. botulinum* is greater than that found between *Bacillus subtilis* and *Staphylococcus aureus* (Collins and East, 1998). More recently the wide phylogenetic and evolutionary distance between proteolytic *C. botulinum* and non-proteolytic *C. botulinum* has been demonstrated by the finding that strains of non-proteolytic *C. botulinum* types B and E were too divergent to give a meaningful response on a DNA microarray based on the genome sequence of proteolytic *C. botulinum* strain

ATCC 3502 (Sebaihia *et al.*, 2007; Carter *et al.*, 2009). DNA from strains of non-proteolytic *C. botulinum* types B and E gave a weak hybridization response (below the cut-off level) to most of the microarray probes. Since the cut-off level for a positive response was approximately 85% sequence identity (Carter *et al.*, 2009), it is possible that many of the CDSs in the non-proteolytic *C. botulinum* genome resembled CDSs in the genome of strain ATCC 3502 but were highly divergent. A direct comparison of the genomes of proteolytic *C. botulinum* strain ATCC 3502 and non-proteolytic *C. botulinum* strain Eklund 17B reveals that they do not show homology or synteny (Fig. 2).

Plasmids are present in many strains of non-proteolytic *C. botulinum* types B, E, and F (e.g., Strom *et al.*, 1984; Ferreira *et al.*, 1987; Eklund *et al.*, 1989; Table 2). Until recently, it was widely thought that the neurotoxin genes of non-proteolytic *C. botulinum* were located on the chromosome, but as with proteolytic *C. botulinum* it has now been shown that in some strains the neurotoxin gene can be located on an extrachromosomal element (Franciosa *et al.*, 2008). The plasmid encoding the neurotoxin gene in non-proteolytic *C. botulinum* strain Eklund 17B is smaller than those characterized in proteolytic *C. botulinum* (Table 2). Bacteriocin-encoding plasmids have not yet been identified in strains of non-proteolytic *C. botulinum*.

There is evidence that the genetic diversity between strains of non-proteolytic *C. botulinum* is greater than that between strains of proteolytic *C. botulinum* (Lindström and Korkeala, 2006). According to PFGE, RAPD, and AFLP studies, strains of non-proteolytic *C. botulinum* could be separated into two major groups, one containing strains that formed type E toxin, and one containing strains that formed type B or type F toxin (Leclair *et al.*, 2006; Hill *et al.*, 2007). A number of sub-groups were also identified.

2.2.3. C. botulinum Group III

Strains of *C. botulinum* Group III form either type C or type D toxin. *C. botulinum* Group III is responsible for avian botulism (e.g., Eklund and Dowell, 1987; Friend, 2002; Chou *et al.*, 2008), and botulism associated with animals, for example, recent outbreaks have involved dairy cows in the Republic of Ireland (Sharpe *et al.*, 2008), and 52,000 farmed foxes and mink in Finland (Lindström *et al.*, 2004a). The properties of this bacterium have been extensively reviewed by Eklund and Dowell (1987). It is saccharolytic, with a number of sugars degraded (Table 1). Acetate and butyrate (and sometimes propionate) are the principal end products of catabolism.

Strains are either weakly proteolytic or lack proteolytic activity (excepting the activity of the neurotoxin), although amino acids are catabolized. *C. botulinum* Group III is a mesophilic bacterium, with optimal growth at 40 °C and a minimum growth temperature of approximately 15 °C (Table 1). Spores are of an intermediate heat resistance.

The genome sequence of *C. botulinum* Group III type C strain Eklund is presently being annotated (Table 2). The genome is smaller (2.96 Mb) than the genome of strains of proteolytic *C. botulinum* or non-proteolytic *C. botulinum* (3.66–4.22 Mb), and the %G+C is marginally higher (Table 2). Physiologically and genetically related organisms are *C. novyi* and *C. haemolyticum*, as shown by DNA hybridization and 16S *rrn* sequencing (Hatheway, 1993; Collins and East, 1998; Hill *et al.*, 2007 (see Fig. 1)). Twenty-five strains of *C. botulinum* Group III types C and D formed four major groups according to AFLP. Three of these groups were closely linked, two contained type C strains and one contained type C and type D strains. Unexpectedly, two type C strains were most closely grouped with strains of *C. botulinum* Group IV (Hill *et al.*, 2007). The neurotoxin genes of *C. botulinum* Group III are located on a bacteriophage. Organisms deliberately cured of these bacteriophages no longer form neurotoxin, and re-infection of a cured organism re-establishes neurotoxin formation. The bacteriophages are easily lost in laboratory cultivation, thus maintenance of strains forming type C or type D toxin can be demanding. A strain of *C. botulinum* Group III type C cured of its phage and infected with phage NA1 from *C. novyi* produced the alpha-toxin of *C. novyi* and could not be distinguished from *C. novyi* type A. Thus, an organism can be converted between *C. botulinum* Group III types C and type D and *C. novyi*, depending on which phage is inserted (Eklund *et al.*, 1989; Hatheway, 1993). The genome of a *C. botulinum* Group III type C bacteriophage has been sequenced, and found to be linear double-stranded DNA of 186 kb with a low %G+C content (26.2%) (Sakaguchi *et al.*, 2005). Plasmids are also present in the genome of *C. botulinum* Group III types C and type D, but do not yet have an ascribed phenotypic function (Strom *et al.*, 1984). The genome sequence of *C. novyi* strain NT, that lacks the bacteriophage responsible for production of the alpha-toxin, has been published (Bettegowda *et al.*, 2006).

2.2.4. C. argentinense (C. botulinum Group IV)

All strains of *C. botulinum* Group IV form type G neurotoxin, albeit generally in low quantities. This organism is also known as *C. argentinense*. It was originally isolated from soils in Argentina (Gimenez and Ciccarelli,

1970) and is the least studied of the six botulinum toxin forming organisms. It has not been associated with botulism cases, although the type G toxin is known to be toxic to a range of animals. *C. botulinum* Group IV is proteolytic and asaccharolytic. Products of catabolism include acetate, butyrate, iso-butyrate, iso-valerate, and phenylacetate. *C. botulinum* Group IV is a mesophilic bacterium with an optimum growth temperature at 37 °C, and spores are of an intermediate heat resistance and similar to those of *C. botulinum* Group III (Table 1).

DNA hybridization and 16S *rrn* sequencing have established that *C. botulinum* Group IV is closely related to other proteolytic asaccharolytic clostridia (Hatheway, 1993; Collins and East, 1998; Hill *et al.*, 2007; see Fig. 1). *C. subterminale* is considered to be a non-neurotoxigenic equivalent of *C. botulinum* Group IV (Table 1). The genome of a strain of *C. botulinum* Group IV is presently being sequenced (Table 2). Previous work has shown that this bacterium has a low %G+C (28–30%) typical of neurotoxin-forming clostridia. An AFLP study showed that seven strains that formed type G toxin (two from Argentina, and five from Switzerland) were closely related and grouped together in a single cluster (Hill *et al.*, 2007).

Six strains of *C. botulinum* Group IV (two from Argentina and four from Switzerland) each harbored an apparently identical single large plasmid of 123 kb (81 MDa) (Strom *et al.*, 1984). After a small number of sub-cultures, non-neurotoxigenic derivatives were isolated from two of these strains (an extended series of sub-cultures yielded non-neurotoxigenic derivatives of the other four strains). Subsequent sub-culturing of the non-neurotoxigenic isolates (at least 16 times over a period of 18 months) failed to produce revertants to the parent phenotype of type G neurotoxin production, indicating that the loss of the ability to form neurotoxin was permanent (Eklund *et al.*, 1989). All of the isolates that formed type G neurotoxin continued to harbor the 123 kb plasmid, while it was absent from the non-neurotoxigenic isolates, thereby providing strong evidence that the large plasmid is involved in neurotoxin formation (Eklund *et al.*, 1989). The non-neurotoxigenic isolates were indistinguishable from *C. subterminale*.

2.2.5. C. baratii type F

Neurotoxigenic strains of *C. baratii* form type F neurotoxin. DNA hybridization tests and 16S *rrn* sequencing have established that strains of *C. baratii* type F are closely related to typical strains that do not form neurotoxin, and distinct from other botulinum neurotoxin-forming

clostridia (Hatheway, 1993; Collins and East, 1998). Neurotoxigenic *C. baratii* type F is a mesophilic bacterium with an optimum growth temperature of 30–45 °C, and a minimum growth temperature of 10–15 °C (Table 1). Strains of *C. baratii* that do not form type F neurotoxin have a minimum growth pH 3.7, and growth is limited at 6.5% NaCl. Energy is derived from the degradation of sugars. Neurotoxigenic *C. baratii* type F has been associated with a limited number of human botulism cases, principally infant botulism and adult intestinal botulism (Hall *et al.*, 1985; McCroskey *et al.*, 1991; Paisley *et al.*, 1995; Trethon *et al.*, 1995; Koepke *et al.*, 2008), although a case of foodborne botulism was associated with consumption of spaghetti noodles and meat sauce (Harvey *et al.*, 2002).

2.2.6. C. butyricum type E

A majority of strains of *C. butyricum* are regarded as non-pathogenic. However, some strains of *C. butyricum* form type E neurotoxin. Neurotoxigenic strains of *C. butyricum* type E are closely related to typical non-pathogenic strains that do not form neurotoxin (as determined by DNA hybridization tests, 16S *rrn* sequencing, and AFLP analysis) and distinct from other clostridia that form botulinum neurotoxin (Hatheway, 1993; Collins and East, 1998; Hill *et al.*, 2007). *C. butyricum* type E is a mesophilic bacterium, with optimum and minimum growth temperatures of 30–37 °C and 12 °C, respectively (Table 1). While neurotoxigenic strains of *C. butyricum* type E have a $D_{100^\circ C} < 0.1$ minutes and a minimum pH for growth of 4.8, more typical non-neurotoxigenic strains of *C. butyricum* form more heat resistant spores ($D_{100^\circ C} = 4.7$ minutes) and are capable of growth at pH 4.4 (Stringer and Peck, 2008). *C. butyricum* is saccharolytic, and a number of sugars are catabolized (Table 1). Neurotoxigenic *C. butyricum* type E was first identified following two cases of infant botulism cases in Italy (Aureli *et al.*, 1986; McCroskey *et al.*, 1986), and has now been associated with a small number of further cases of infant botulism and adult intestinal botulism (Fenicia *et al.*, 1999; Aureli *et al.*, 2002; Fenicia *et al.*, 2002; Fenicia *et al.*, 2007; Koepke *et al.*, 2008), most recently in Japan (Abe *et al.*, 2008). Outbreaks of foodborne botulism involving neurotoxigenic *C. butyricum* type E have been reported in China, India, and Italy (Meng *et al.*, 1997; Chaudhry *et al.*, 1998; Anniballi *et al.*, 2002; Fu and Wang, 2008). *C. butyricum* type E has also been isolated from soil in China (Meng *et al.*, 1999; Fu and Wang, 2008), and strains isolated in Italy and China appear to be clonal (Wang *et al.*, 2000).

3. GERMINATION AND LAG PHASE IN *C. BOTULINUM*

Bacterial growth can be separated into a number of distinct phases: lag phase, exponential phase, and stationary phase. For a specified organism, the effects of environmental factors on exponential growth are reproducible and well described, for example, in predictive models. Lag phase is, however, relatively poorly understood, particularly for endospore-forming bacteria. Bacterial endospores are a dormant form that is highly resistant to a number of adverse treatments, such as heat, pressure, low pH, desiccation, and UV light, and their formation by neurotoxin-forming clostridia is a primary reason why these bacteria present a significant hazard. The lag period between a spore being exposed to conditions suitable for growth and the start of exponential growth includes a number of distinct stages, namely germination, emergence, elongation, and first cell division.

Spore germination results in the loss of spore resistant properties. It involves a cascade of membrane changes and the activities of enzymes that have been formed during sporulation, and does not require new macromolecular synthesis (Setlow, 2003; Moir, 2006). Germination is triggered in response to a number of nutrient, non-nutrient, enzymatic, and physical stimuli. The nutrient germinants penetrate the coats and cortex of the spore, and activate germination receptors located at the spore inner membrane committing the spore to undergo an irreversible reaction. This leads to the loss of potassium and sodium ions and protons, followed by calcium ions and dipicolinic acid from the spore core, leading to loss of resistance, and eventually hydrolysis of peptidoglycan in the spore cortex (Johnstone, 1994). Hydrolysis of the cortex leads to hydration and swelling of the spore core, creating conditions more typical of those in vegetative cells, allowing the resumption of enzyme activity, metabolism, and macromolecular synthesis. Germination-specific cortex lytic enzymes have been characterized in *B. subtilis* (CwlJ and SleB) and *C. perfringens* (SleC and SleM) (Shimamoto *et al.*, 2001; Setlow, 2003; Masayama *et al.*, 2006; Moir, 2006; Kumazawa *et al.*, 2007). Searching the genome sequences of proteolytic *C. botulinum* strains ATCC 3502, Okra, and Langeland, and non-proteolytic *C. botulinum* strains Eklund 17B and Alaska E43 revealed that putative homologues of these genes appeared to be present in several, but not all, strains (Carter and Peck, unpublished data). However, since these genes share homology with each other and with other cell wall hydrolases, further work is required to confirm the genetic complement of germination-specific cortex lytic enzymes in proteolytic *C. botulinum* and non-proteolytic *C. botulinum*.

Traditionally, the safety of foods with respect to the prevention of foodborne botulism has relied on severe treatments, such as the botulinum

cook given to canned foods. More sophisticated (milder) approaches are needed, however, to ensure the safety of the new processes required for high quality minimally heated chilled foods that are now demanded by consumers (Peck, 2006). The safety of these foods can be achieved by ensuring that growth (i.e., any escape from lag phase) of botulinum neurotoxin-forming clostridia is prevented, without reliance on a severe heat treatment. This approach may also be relevant to the control of infant botulism. A greater understanding of the duration and biology of lag phase and its stages may enable knowledge-led strategies to be devised to prevent botulinum toxin-forming clostridia from escaping lag phase and hence prevent growth and neurotoxin formation, or strategies to be devised to germinate spores and then destroy emergent vegetative cells. Of particular interest are the initial germination process, and the biovariability in germination and lag phase.

3.1. Nutrient Germinants and their Receptors

Spores of *Bacillus* and *Clostridium* species are triggered to germinate in response to a range of nutrient germinants. This process is more fully understood in *Bacillus* species than in clostridia, although full molecular details of the signal transduction process are not yet established. A number of nutrient germinant systems have been characterized in *Bacillus* species including, L-alanine, L-proline, inosine, and the AGFK mixture (L-asparagine, glucose, fructose, and potassium ions) (Corfe *et al.*, 1994; Johnstone, 1994; Moir *et al.*, 1994; Clements and Moir, 1998; Setlow, 2003; Moir, 2006). Spore germination in clostridia often involves a combination of nutrient germinants and generally proceeds more slowly than in *Bacillus* species. Studies with *Bacillus* species, especially the phenotypic characterization of mutants, provided the first insights into the nature of germinant receptors. The first two germinant receptor operons to be cloned and characterized were the *gerA* and *gerB* operons in *B. subtilis* (Zuberi *et al.*, 1987; Corfe *et al.*, 1994). Related germinant receptor operons are present in a majority of spore-forming bacteria (including proteolytic *C. botulinum* and non-proteolytic *C. botulinum*), and are probably derived from a common ancestor(s). The genes encoding the germinant receptor proteins are expressed during sporulation, but the product proteins are themselves active during germination. The majority of germinant receptor operons are tricistronic, and encode three proteins. In the case of *gerA*, these are GerAA, GerAB, and GerAC. GerAA is located in the spore inner membrane, and predicted to span the membrane at least five times.

GerAB is also membrane-bound, and is predicted to span the membrane ten times. GerAB is a single component membrane transporter and the only one of these proteins that has homologues outside the spore-forming bacteria (Moir, 2006). GerAC is predicted to be a lipoprotein. All three proteins are required for an operational germinant receptor, and the lack of functional interchange between genes in the various operons implies both that the genes within a specific operon have co-evolved, and that interaction between the resulting protein components is important for activity.

The presence of only one germinant receptor operon in the genome of *C. perfringens*, and none in the genome of *C. difficile* indicates the likely existence of unrecognized germinant receptor genes and potentially novel germination mechanisms (Sebaihia *et al.*, 2007). Spores of *C. difficile* are clearly able to sense their environment, as germination is triggered by cholate derivatives and glycine (Sorg and Sonenshein, 2008), and by potassium or inorganic phosphate ions (Paredes-Sabja *et al.*, 2008). Genes have not yet been associated with these phenotypes. An entirely new spore germination mechanism has been recently reported in *B. subtilis*, where germination was triggered by very low concentrations ($\leq$1 pg/ml) of muropeptides (Shah *et al.*, 2008). Muropeptides are peptidoglycan degradation products but, in order to germinate spores of *B. subtilis*, the amino acid *meso*-diaminopimelate needs to be at position three of the stem peptide linked to the muropeptide oligosaccharide backbone (*meso*-diaminopimelate is at this position in peptidoglycan in *B. subtilis* cells). The muropeptides triggered germination in the absence of all nutrient germinant (Ger) receptors, and signaled to PrkC, a serine/threonine protein kinase located in the spore inner membrane. Loss or mutation of PrkC prevented muropeptide-triggered germination (Shah *et al.*, 2008). A search of genome sequences reveals the presence of putative *prkC* homologues in a number of different clostridia, including strains of proteolytic *C. botulinum*, non-proteolytic *C. botulinum*, *C. sporogenes*, *C. perfringens*, and *C. difficile*. These putative homologues shared 34–37% amino acid identity with the encoded protein from the *prkC* gene in *B. subtilis* (Carter and Peck, unpublished data). It remains to be established whether the PrkC protein contributes to germination in proteolytic *C. botulinum*, non-proteolytic *C. botulinum* or other clostridia.

3.1.1. Spore Germination in Proteolytic C. botulinum

A majority of studies have shown that while spores of proteolytic *C. botulinum* and *C. sporogenes* germinate readily in the presence of

L-alanine, germination was generally more rapid and a greater proportion of spores germinated in the presence of L-alanine/L-lactate (Table 5; Barker and Wolf, 1971; Sarathchandra *et al.*, 1976; Broussolle *et al.*, 2002; Alberto *et al.*, 2003). Inconsistencies in observations made by different workers may reflect strain or methodological differences. For example, germination of spores of strain 213B was almost complete in response to L-alanine in 10 hours at 30 °C, but only 20% of spores of strains ATCC 3502 and NCIB 4301 germinated under these conditions (Alberto *et al.*, 2003). Germination of spores of ten strains of proteolytic *C. botulinum* in potassium phosphate buffer (pH 7.0) with $NaHCO_3$ at 30 °C was significantly higher in L-alanine and in L-alanine/L-lactate than in inosine or the AGFK mixture (Broussolle *et al.*, 2002; Alberto *et al.*, 2003). D-alanine failed to trigger spore germination for a number of strains of proteolytic *C. botulinum* and *C. sporogenes*, and inhibited L-alanine triggered germination, with the extent of the inhibition being strain dependent (Uehara and Frank 1965; Rowley and Feeherry 1970; Ando, 1973b; Montville *et al.*, 1985; Alberto *et al.*, 2003). L-lactate is not itself an effective trigger of germination, and high concentrations may inhibit germination of proteolytic *C. botulinum* (Ando, 1974; Foegeding and Busta, 1983; Houtsma *et al.*, 1994). Other amino acids (e.g., glycine, L-valine) also stimulated germination in the presence of L-lactate, but were less effective than L-alanine (Alberto *et al.*, 2003). L-alanine, glycine, and L-valine are hydrophobic amino acids with a short side chain. These findings are consistent with those made in *B. subtilis*, where L-alanine triggered germination is inhibited by various hydrophobic compounds (e.g., alcohols), suggesting that the L-alanine receptor may be hydrophobic (Yasuda-Yasaki *et al.*, 1978). Replacing $NaHCO_3$ with $KHCO_3$ had little effect on germination, but replacing $NaHCO_3$ with NaCl reduced germination for seven out of nine strains of proteolytic *C. botulinum* (Alberto *et al.*, 2003). The role of bicarbonate/CO_2 in promoting germination of spores of various clostridial species, but not those of *Bacillus cereus*, is well-recognized (Wynne and Foster, 1948; Rowley and Feeherry, 1970; Ando, 1974; Enfors and Molin, 1978; Smoot and Pierson, 1982; Montville *et al.*, 1985; Plowman and Peck, 2002).

Germinant receptor operons are well-conserved amongst five sequenced strains of proteolytic *C. botulinum*, and are found at a similar location on the chromosome of the different strains (Table 6). Sequencing the genome of the type A strain ATCC 3502 revealed the presence of three tricistronic germinant receptor operons, CBO0123-0125 (*gerXA1-XB1-XC1*), CBO1975-1977 (*gerXA2-XB2-XC2*), and CBO2797-2795 (*gerXA3-XB3-XC3*), and an orphan *gerXB* homologue (CBO2300) (Table 6). Unusually, CBO1975-1977 (*gerXA2-XB2-XC2*) was flanked by two additional *gerXB*

Table 5 Mixtures that trigger germination of spores of proteolytic *C. botulinum* and non-proteolytic *C. botulinum*.

Germinant mixture	Strains	Reference(s)
Proteolytic *C. botulinum*		
L-alanine and L-alanine/L-lactate (in potassium phosphate buffer (100 mM, pH 7.0) with $NaHCO_3$ (50 mM))	ATCC 3502, Eyemouth, 62A, 97A, NCTC 7272, B6, 213B, NCIB 4301, NCTC 7273, Langeland	Alberto *et al.* (2003)
L-alanine and L-alanine/L-lactate (in potassium phosphate buffer (200 mM, pH 7.0) with $NaHCO_3$ (100 mM))	NCTC 7273, NCIMB 701792[a]	Broussolle *et al.* (2002)
Three component system (L-alanine (or L-cysteine)/L-lactate (or sodium thioglycollate or alpha-hydroxy acids)/$NaHCO_3$) in neutral pH buffer	62A, 190, B-aphis, Ba410	Rowley and Feeherry (1970); Ando (1974); Smoot and Pierson (1982); Montville *et al.* (1985)
L-alanine/$NaHCO_3$ in Tris-hydrochloride buffer (pH 7.0)	62A, 12885A	Foegeding and Busta (1983)
L-alanine in citrate-phosphate buffer (pH 7.0)	62A, 12885A	Chaibi *et al.* (1996)
L-alanine/sodium pyrophosphate (pH 8.0)	3679h[a]	Uehara and Frank (1965)
Non-proteolytic *C. botulinum*		
L-alanine/L-lactate, L-serine/L-lactate, and L-cysteine/L-lactate (in potassium phosphate buffer (100 mM, pH 7.0) with $NaHCO_3$ (50 mM))	Eklund 17B, Beluga, Craig 610	Plowman and Peck (2002)
L-alanine (25 mM)/glucose (22 mM)/L-lactate (sodium salt, 9 mM)/$NaHCO_3$ (12 mM) in phosphate buffer (40 mM, pH 7.0)	Iwanai	Ando and Iida (1970)

(*Continued*)

Table 5 (*continued*)

Germinant mixture	Strains	Reference(s)
L-alanine/glucose/ L-lactate, L-alanine/ L-lactate/$NaHCO_3$, L-alanine/glucose/ $NaHCO_3$ in phosphate buffer (40 mM, pH 7.0)	Iwanai	Ando and Iida (1970)
Addition of glucose, galactose, maltose, glucose-6-phosphate, fructose-1,6-diphosphate, L-lactate, D-lactate, DL-lactate, guanosine, inosine or various alpha-hydroxy acids (2–20 mM) to L-alanine (25 mM)/ $NaHCO_3$ (60 mM) in phosphate buffer (40 mM, pH 6.8)	Iwanai	Ando (1971); Ando (1974)
Single amino acids (e.g., L-alanine, L-cysteine, L-serine, L-threonine) at pH 9.0 with $NaHCO_3$	Iwanai	Ando (1971)
Single amino acids (e.g., glycine, L-cystine) (2 mM, pH 7.0)	D8	Ward and Carroll (1966)
L-alanine/DL-lactate/90 mM glucose/12 mM $NaHCO_3$/2 mM sodium thioglycollate in phosphate buffer (pH 7.0)	NCIB 4270[a]	Peck *et al.* (1995a)

[a]Non-neurotoxigenic strain.

genes (CBO1974 and CBO1978), and is highly related to a germinant receptor gene cluster characterized in *C. sporogenes* NCIMB 701792 (82–84% amino acid identity of the encoded proteins) that was also flanked by two additional *gerXB* genes (Broussolle *et al.*, 2002), and a germinant receptor gene cluster present in the genomes of four other strains of proteolytic *C. botulinum* (ATCC 19397, NCTC 2012 (Loch Maree),

Table 6 Summary of germinant receptor CDSs present in the genomes of five strains of proteolytic *C. botulinum* (Carter and Peck, unpublished data).

CDS annotation	Approximate number of predicted amino acid residues	CDS annotation in designated proteolytic *C. botulinum* strain				
		ATCC 3502 (type A1)	ATCC 19397 (type A1)	NCTC 2012 (Loch Maree) (type A3)	Okra (type B)	Langeland (type F)
gerXA1	506[b]	CBO0123	CLB0159	CLK3298	CLD0663	CLI0178
gerXB1[a]	365	CBO0124	CLB0160	CLK3299	CLD0662	CLI0179
gerXC1	393	CBO0125	CLB0161	CLK3300	CLD0661	CLI0180
gerXB[a]	361	CBO1974	CLB1915	–	CLD2650	CLI2041
gerXA2	500	CBO1975	CLB1916	CLK1429	CLD2649	CLI2042
gerXB2[a]	363	CBO1976	CLB1917	CLK1430	CLD2648	CLI2043
gerXC2	370	CBO1977	CLB1918	CLK1431	CLD2647	CLI2044
gerXB[a]	369	CBO1978	CLB1919	CLK1432	CLD2646	CLI2045
gerXB[a]	368	CBO2300	CLB2245	CLK1751	CLD2262	CLI2363
gerXA3[e]	495	CBO2797	CLB2740	CLK2184	CLD1776	CLI2847
gerXB3[a,e]	377[c]	CBO2796	CLB2739	CLK2183	CLD1777	CLI2846
gerXC3[e]	381[d]	CBO2795	CLB2738	CLK2182	CLD1778	CLI2845
gerXC4[e]	364	–	–	–	CLD1669	CLI2929
gerXA4[e]	476	–	–	–	CLD1670	CLI2928
gerXB4[a,e]	361	–	–	–	CLD1671	CLI2927

Source: Data are from Sebaihia *et al.* (2007) and were analyzed using predicted amino acid sequences of each ATCC 3502 CDS to perform protein–protein BLAST (Basic Local Alignment Tool) searches of the non-redundant protein sequences database (National Center for Biotechnology Information (NCBI)).

[a]The identification of CDSs as *gerB* is tentative (described as a match with protein family HMM PFO3845 (i.e., a spore permease) in some genome annotations).

[b]486 residues in strains Okra and Langeland.

[c]373 residues in strain NCTC 2012.

[d]373 residues in ATCC 3502, and 377 residues in strain NCTC 2012.

[e]Encoded on the ''reverse'' strand.

Okra and Langeland), although NCTC 2012 (Loch Maree) lacked one of the additional flanking *gerXB* genes (Table 6). Interestingly, a CDS encoding alanine racemase immediately preceded this germinant receptor cluster in proteolytic *C. botulinum* strains ATCC 3502, ATCC 19397, NCTC 2012 (Loch Maree), Okra and Langeland (Carter and Peck, unpublished data). The germinant receptor gene cluster *gerXA3-XB3-XC3* (CBO2797-2795 in ATCC 3502) was also present in the genomes of the other sequenced strains of proteolytic *C. botulinum* (Table 6), and appears to be closely related to a germinant receptor gene cluster earlier characterized in the proteolytic *C. botulinum* type B strain NCTC 7273 (89–99% amino acid identity of the encoded proteins) (Broussolle *et al.*, 2002; Sebaihia *et al.*, 2007). The characterized GerXA and gerXB proteins in proteolytic *C. botulinum* NCTC 7273 and *C. sporogenes* NCIMB 701792 were predicted to possess six and ten transmembrane domains, respectively. Co-expression of *gerXA* and *gerXB* has been demonstrated in proteolytic *C. botulinum* NCTC 7273, and it was confirmed that *gerXA* was expressed during sporulation. Encoded proteins have been detected in the spore inner membrane, confirming that the genes are functional (Broussolle *et al.*, 2002; Alberto *et al.*, 2005). The complement of germinant receptor genes in strains Okra (type B) and Langeland (type F) were virtually identical, and both contained an additional tricistronic germinant receptor operon (*gerXC4-XA4-XB4*) not present in the genome of the type A strains (Table 6). There are two curious features to this additional germinant receptor operon. Firstly, the order of the three CDSs is unexpected, with the *gerXA4* gene located in the center of the operon. Secondly, while the three CDSs in strains Okra and Langeland shared strong homology (99% amino acid identity) they are more closely related to germinant receptor operons present in other clostridia than to the other germinant receptor operons in six strains of proteolytic *C. botulinum* (five strains in Table 6, plus strain Hall). The three CDSs in strain Langeland shared homology with CDSs present in *C. sporogenes* ATCC 15579 (89–91% amino acid identity), *C. tetani* E88 (39–51%), *C. acetobutylicum* (35–50%), non-proteolytic *C. botulinum* strains Eklund 17B and Alaska E43 (29–48%), and several strains of *C. perfringens* (28–49%). Amino acid identity was highest for CLI2928 (*gerXA4*) (Carter and Peck, unpublished data). The presence of this additional operon may allow strains Okra and Langeland to respond to a wider range of germinants. It remains to be established whether the germination systems characterized in proteolytic *C. botulinum* and *C. sporogenes* are responding to L-alanine, or L-alanine/L-lactate or presently uncharacterized germinant(s). The recent development of the ClosTron system for generating mutants in proteolytic

C. botulinum and other clostridia (Heap *et al.*, 2007, 2009) allows us for the first time to begin to dissect the germinant receptor system in this pathogen, and to associate phenotype with genotype.

3.1.2. Spore Germination in Non-Proteolytic C. botulinum

Germination of spores of a number of strains of non-proteolytic *C. botulinum* at a neutral pH is triggered by an amino acid with a second component, typically L-lactate (Table 5). Pairs of germinants (L-alanine/L-lactate, L-serine/L-lactate, and L-cysteine/L-lactate, at 50 mM) in 100 mM potassium phosphate buffer (pH 7.0)/50 mM $NaHCO_3$ triggered germination of $\geq 90\%$ of spores of three strains (Eklund 17B (type B), Beluga (type E), and Craig 610 (type F)) within 6 hours at 20 °C (Plowman and Peck, 2002). Other germinants (individually or in combination, including amino acids, carboxylic acids, purine ribosides, sugar phosphates, and sugars) were ineffective, inducing germination of $<10\%$ of spores within this period. Well-characterized germination triggers of *Bacillus* spores, including L-alanine, AGFK mixture and inosine failed to germinate spores of non-proteolytic *C. botulinum* (Plowman and Peck, 2002). Earlier studies with non-proteolytic *C. botulinum* type E strain Iwanai also identified the need for combinations of germinants at a neutral pH. Ando and Iida (1970) reported on a four component system (L-alanine/glucose/L-lactate/$NaHCO_3$ in phosphate buffer), and various three component systems (Table 5). Germination in strain Iwanai was not triggered by L-alanine/$NaHCO_3$ in phosphate buffer at a neutral pH, but addition of various organic compounds (including L-lactate) induced germination (Table 5). While L-alanine (or other amino acid) and a second component (e.g., L-lactate) were essential for germination, non-essential components ($NaHCO_3$, sodium thioglycollate, and heat shock) increased the rate and extent of germination (Plowman and Peck, 2002). For this reason, bicarbonate/carbon dioxide is often added to clostridial growth media (Holdeman *et al.*, 1977). Growth from spores of non-proteolytic *C. botulinum* is stimulated by small concentrations of carbon dioxide, but inhibited by high concentrations (Gibson *et al.*, 2000; Fernandez *et al.*, 2001). Interestingly, the effect of $NaHCO_3$ and sodium thioglycollate was additive suggesting that they may play a different role and have a separate site of action. A similar proposal was made for non-essential components (L-lactate, L-phenylalanine, and L-arginine) that promote germination triggered by L-alanine/sodium ions in *C. bifermentans* (Bright and Johnstone, 1987). Activation by sub-lethal heat-shock increased the rate and extent of germination, and it has been proposed that heat-shock leads to a conformational change in the

germination receptor making it more responsive to germinants (Johnstone, 1994).

A second type of nutrient germination system was described by Ando (1971) in strain Iwanai. It appears to respond to single amino acids at high pH but not at a neutral pH (Table 5), and could not be detected in strains Eklund 17B, Beluga or Craig 610 (Ando, 1971; Plowman and Peck, 2002). Lysozyme brings about enzymatic germination of spores of non-proteolytic *C. botulinum* (Peck *et al.*, 1993). It is able to permeate the spore coat, and bring about direct enzymatic cleavage of peptidoglycan in the spore cortex, leading to swelling and hydration of the core.

Spore germination in *C. bifermentans*, but not proteolytic *C. botulinum*, non-proteolytic *C. botulinum* or *B. subtilis* was triggered by D-alanine. In *C. bifermentans*, D-alanine replaced L-alanine in the L-alanine/sodium ion germination response. Alanine racemase inhibitors did not prevent germination suggesting that the effectiveness of D-alanine was not due to the enzymic production of L-alanine (Bright and Johnstone, 1987). D-alanine inhibited L-alanine triggered germination in proteolytic *C. botulinum* and *B. subtilis*, and L-alanine/$NaHCO_3$ triggered germination at pH 9.0 in non-proteolytic *C. botulinum* (Uehara and Frank, 1965; Rowley and Feeherry, 1970; Ando, 1971, 1973b; Montville *et al.*, 1985; Johnstone, 1994; Alberto *et al.*, 2003). Intriguingly, D-alanine did not inhibit L-alanine/L-lactate/$NaHCO_3$ triggered germination at pH 6.8–7.0 in non-proteolytic *C. botulinum*, providing evidence of a difference in the germination of proteolytic *C. botulinum* and non-proteolytic *C. botulinum* (Ando, 1971; Plowman and Peck, 2002). The potentially more important role of alanine racemase in germination of spores of proteolytic *C. botulinum* than spores of non-proteolytic *C. botulinum* may also be indicated by the presence of five putative alanine racemase genes in the genome of proteolytic *C. botulinum* strains ATCC 3502, ATCC 19397, Okra and Langeland, but only one in the genome of non-proteolytic *C. botulinum* strains Eklund 17B and Alaska E43 (Carter and Peck, unpublished data).

Germination occurs outside the growth range for several *Bacillus* and *Clostridium* species, and spores may germinate in conditions where subsequent outgrowth does not follow. For example, the temperature and pH range for germination are both wider than the growth range for proteolytic *C. botulinum* (Rowley and Feeherry, 1970; Ando, 1973a), non-proteolytic *C. botulinum* (Strasdine, 1967; Ando, 1971; Grecz and Arvay 1982; Plowman and Peck, 2002), and *C. botulinum* Group IV (Takeshi *et al.*, 1988). Germination is also less susceptible to oxygen inhibition than is subsequent outgrowth. Spores of proteolytic *C. botulinum* and non-proteolytic *C. botulinum* germinate under aerobic and anaerobic conditions

regardless of redox potential (Ando and Iida, 1970; Ando, 1973a; Plowman and Peck, 2002).

Analysis of the genome sequence of non-proteolytic *C. botulinum* strains Eklund 17B (type B) and Alaska E43 (type E) for germinant receptor genes revealed a pattern very different to that found with strains of proteolytic *C. botulinum*. The three tricistronic germinant receptor operons present in all five strains of proteolytic *C. botulinum* (Table 6) were absent. Instead, strains Eklund 17B and Alaska E43 possessed a single tricistronic germinant receptor operon (*gerXC-XA-XB*) reminiscent of the operon present in proteolytic *C. botulinum* strains Okra and Langeland, but not found in the four type A strains (Table 6). Unusually, the *gerXA* CDS was present in the center of the operon (Carter and Peck, unpublished data). The CDSs present in strain Eklund 17B shared the most homology with CDSs present in non-proteolytic *C. botulinum* strain Alaska E43 (CLH2914-2916; 92–96% amino acid identity), and then several *C. perfringens* strains (36–63% identity), *C. tetani* E88 (CTC2447-2449; 30–51% identity), *C. acetobutylicum* ATCC824 (CAC0643-0645; 29–51%), *C. sporogenes* ATCC 15579 (32–48%), and proteolytic *C. botulinum* strains Okra (CLD1669-1671; 29–48% identity) and Langeland (CLI2927-2929; 29–48% identity). The CDSs for *gerXC*, *gerXA*, and *gerXB* are predicted to encode 373, 466–469, and 366 amino acids, respectively. It remains to be established whether an active germinant receptor is encoded by this tricistronic germinant receptor operon, and whether it is responding to amino acids (e.g., L-alanine, L-cysteine, L-serine) together with L-lactate. This repertoire would seem very small, and it may be that there are other germinants, and other types of germinant receptor genes. For example, the presence of a putative *prkC* gene in non-proteolytic *C. botulinum* raises the possibility that muropeptide triggered germination may be important.

3.1.3. Spore Germination in other Botulinum Neurotoxin-Forming Clostridia

Relatively little is presently known of spore germination in other botulinum neurotoxin-forming clostridia, but again combinations of germinants appear to be required. Germination of spores of *C. botulinum* Group IV type G was optimal in a defined three-component system of L-cysteine/L-lactate/$NaHCO_3$ in phosphate buffer (pH 7.0), with all components necessary for germination (Takeshi *et al.*, 1988). Germination of spores of a neurotoxigenic strain of *C. butyricum* type E was triggered in mixtures of L-alanine/L-lactate/glucose/$NaHCO_3$, L-alanine/glucose/$NaHCO_3$, and L-cysteine/glucose/L-lactate/$NaHCO_3$. These and other germinant

mixtures were also effective for a non-neurotoxigenic strain of *C. butyricum* (Takeshi *et al.*, 1991).

3.2. Biovariability in Germination and Lag Phase

Measurement of the kinetic responses of populations of *C. botulinum* to environmental conditions, and the subsequent creation of predictive models has made an important contribution to microbiological food safety (Baranyi and Roberts, 2000; Tamplin, 2005; Membre and Lambert, 2008). There are, however, some limitations to these population based approaches. For example, they may be of limited relevance when the initial spore concentration is low (as is likely to be the case with *C. botulinum* (Lund and Peck, 2000)) or when additional information beyond point values is required. Growth of *C. botulinum* in foods is likely to initiate from just a few spores, and the distribution of times to growth or neurotoxin formation will reflect the heterogeneity of lag time from individual spores. Quantifying the biological variability in lag time for individual spores of *C. botulinum* is, therefore, essential. Data need to be generated specifically for this purpose, as this information cannot be derived from population measurements as there is an intrinsic stochastic variability between individual spores within a population, and a complex relationship between population and individual lag time (Baranyi, 1998). Furthermore, a greater understanding of the duration and biology of the various stages of lag phase may enable knowledge-led strategies to be devised to prevent botulinum toxin-forming clostridia from escaping lag phase.

Tests with individual spores of non-proteolytic *C. botulinum* placed in wells of an automated turbidometric reader situated in an anaerobic cabinet illustrate the extent of the intrinsic biological variability between individual spores within a population (Fig. 3). These tests were conducted in a rich medium at 22 °C (conditions near to the optimum for growth) with unheated and heated (80 °C/20 s) spores of strain Eklund 17B. The variability was considerably less for unheated spores than for heated spores. Most wells containing an unheated spore became turbid in 20–30 hours, while for heated spores there was pronounced tailing in the time to turbidity (Fig. 3). The first well became turbid after about 30 hours, and further wells became turbid after more than 200 hours (Fig. 3). While such experiments begin to characterize the biovariability of lag phase, they do not provide information on the variability of the individual stages of lag phase (germination, emergence, elongation, and first cell division), or on any relationship between these individual stages. For example, questions to

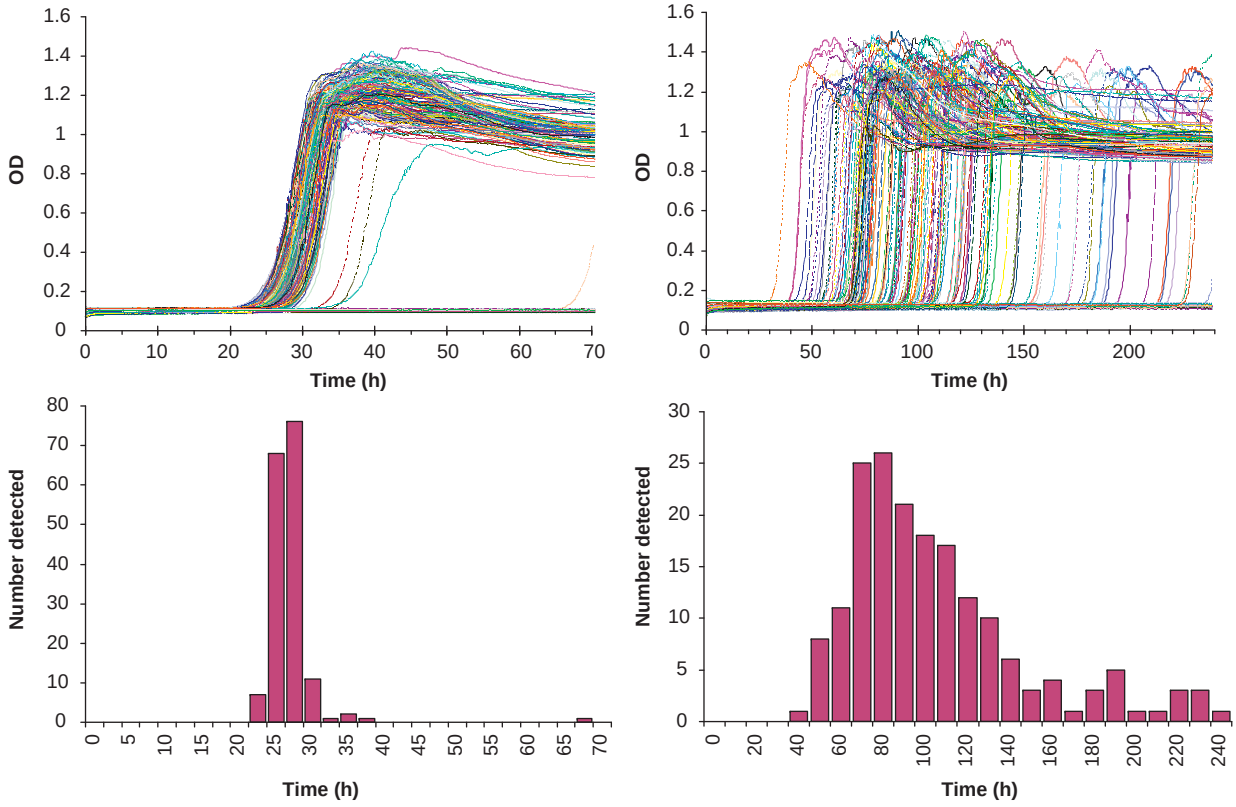


Figure 3 Effect of prior heat treatment on lag time of single spores of non-proteolytic *C. botulinum* type B strain Eklund 17B at 22 °C (Stringer and Peck, unpublished data). Left hand plots are for unheated spores, right hand plots are for spores heated at 80 °C/20 s. Upper plots are observations made with an automatic turbidometric reader for growth from single spores, and lower plots are the derived frequency distribution of lag times for single spores (as time to detection, $OD_{600} = 0.14$, approximately 10^5 cfu/ml).

arise from the data presented in Fig. 3 are which stage(s) of lag phase has been extended, and whether all stage(s) have been extended equally.

A majority of studies of spore germination have been population based (such as those in Table 5). A few studies, however, have quantified biovariability associated with germination (but not subsequent outgrowth) of individual spores of proteolytic *C. botulinum* (Billon *et al.*, 1997; Chea *et al.*, 2000), and *Bacillus* species (Vary and Halvorson, 1965; Coote *et al.*, 1995; Leuschner and Lillford, 1999; Chen *et al.*, 2006). Billon *et al.* (1997) used phase-contrast microscopy with image analysis to quantify the biovariability in germination of single spores of proteolytic *C. botulinum* strain 62A at five temperatures between 20 and 37 °C. At all temperatures, the distribution of germination times was skewed with considerable tailing. At the higher temperatures, germination started sooner and proceeded more quickly (mean germination time at 37 °C = 52 minutes, at 20 °C = 193 minutes), and the distribution had a lower standard deviation and showed less tailing (Billon *et al.*, 1997). Chea *et al.* (2000) used phase-contrast microscopy to measure the effect of temperature (15–30 °C), pH (5.5–6.5), and sodium chloride concentration (0.5–4.0%) on germination of individual spores of proteolytic *C. botulinum* strain 56A. These data were used to develop a mathematical model of the effect of environment on the fraction of germinated spores.

Smelt and colleagues quantified and then modeled the effect of heat treatment on the rate of germination and subsequent outgrowth of *B. subtilis* spores (Smelt and Brul, 2007; Smelt *et al.*, 2008). A semi-automated system that combined phase contrast microscopy and image analysis has been used to follow individual spores of non-proteolytic *C. botulinum* strain Eklund 17B in an anaerobic microscope slide from dormancy, through germination, emergence and elongation, to the first cell division (Stringer *et al.*, 2005; Webb *et al.*, 2007; Stringer *et al.*, 2009). Initial studies in good growth conditions (22 °C, rich microbiological medium) showed that the spore population demonstrated considerable heterogeneity at all stages of lag phase, with no significant relationship between the time spent in the various stages of lag phase for individual spores. This variability was so great, that the first spore to germinate was not the first to reach two cells, and it was not possible to predict the lag time of individual spores from their germination time (Stringer *et al.*, 2005). Furthermore, lag time variability resulted from variability in each stage of lag, rather than one very variable stage. The lack of correlation between the various stages of lag phase for individual spores was attributed to metabolic and non-metabolic differences between individual spores. Germination is a series of degradative reactions involving preformed enzymes and no new macromolecular synthesis, while

post-germination events involve macromolecular synthesis with different metabolic processes active at different times. An example of a non-metabolic factor is the strength of the spore coat affecting time to emergence (Stringer *et al.*, 2005; Webb *et al.*, 2007).

Adverse treatments extended lag phase and increased its biovariability (see, e.g., Fig. 3). An important issue that has been recently resolved is that of whether different stages of lag phase are affected by different adverse treatments. Addition of NaCl to either the sporulation (3% w/v) or subsequent growth medium (2% w/v) increased the mean times for, and variability of, lag time and each of its stages approximately equally. It was also found that spores formed in medium with added NaCl (3%) were sub-lethally damaged, and were not better adapted to subsequent growth at 2% NaCl than spores formed in medium with no added NaCl (Webb *et al.*, 2007). A moderate heat treatment (80 °C/20 s) significantly increased the duration and variability of lag phase of surviving spores (Fig. 3). However, all stages of lag were not affected equally. Fig. 4 shows time to germination and outgrowth (time for a germinated spore to reach one cell) for individual spores. The cluster of heated spores is located to the right of the unheated spores on this plot, that is, germination time was considerably increased, but time to outgrowth was only marginally increased (Fig. 4). The variability of individual spore germination times and median time to germination were increased, the latter from 0.93 to 15.20 hours (16-fold increase) with the result that it was now longer than the median time for outgrowth. There was a long tail of late germinating spores. Although the heat treatment mainly extended germination time, there was also evidence of damage to systems required for outgrowth (median time to outgrowth increased from 5.83 to 8.53 hours (46% increase)), but this damage was quickly repaired and was not evident by the time the cells started to double (doubling time (from one cell to two cells) 1.32 and 1.21 hours) (Fig. 5). It would seem that while significant damage to the spore germination apparatus prevented germination from heated spores of non-proteolytic *C. botulinum* (Peck *et al.*, 1992a, b), moderate heat damage brought about a substantial increase in germination time (Stringer *et al.*, 2009). For individual unheated spores incubated at 10 °C, germination was only marginally increased compared to that at 22 °C, but outgrowth was considerably extended and made a greater contribution to the duration and variability of the lag phase (Fig. 4). At 10 °C, median germination time was increased to 1.26 hours (35% increase compared to 22 °C), while median outgrowth time was increased to 17.39 hours (3.0-fold increase) and doubling time increased to 4.36 hours (3.3-fold increase). The proportion of

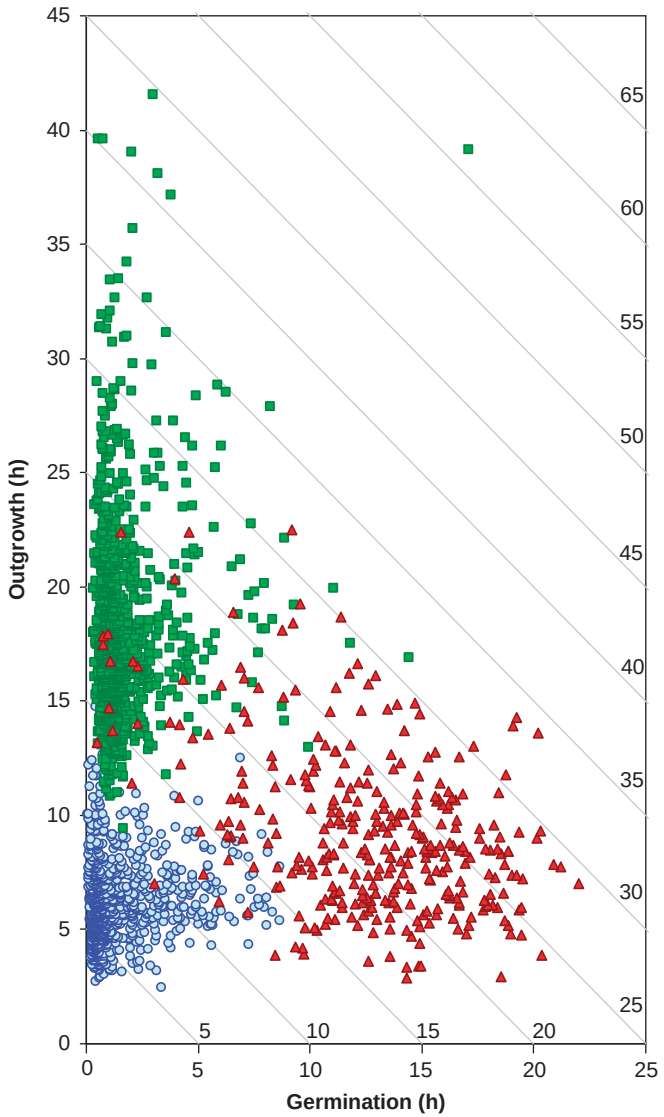


Figure 4 Effect of prior heat treatment and incubation at 10 °C on germination and outgrowth of single spores of non-proteolytic *C. botulinum* type B strain Eklund 17B (Stringer and Peck, unpublished data). Each point shows the germination time and outgrowth time for a single spore. Spores were: unheated and incubated at 22 °C, ○; heated at 80 °C/20 s prior to incubation at 22 °C, ▲; unheated and incubated at 10 °C, ■.

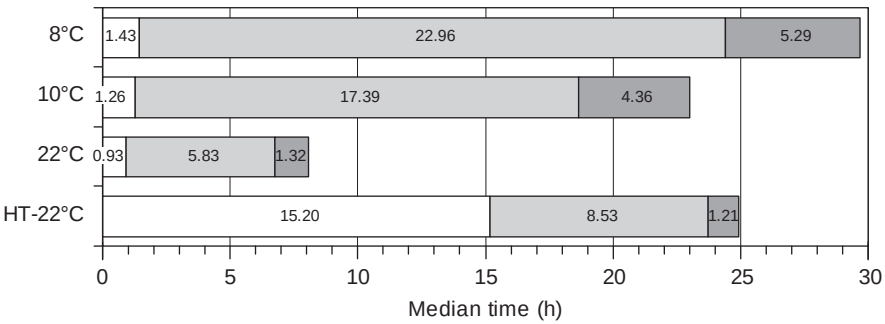


Figure 5 Effect of prior heat treatment and incubation temperature on median times for germination, outgrowth, and first doubling for spores of non-proteolytic *C. botulinum* type B strain Eklund 17B. [From data in Stringer *et al.* (2009)]. Spores were unheated prior to incubation at 8, 10, or 22 °C, or heated at 80 °C/20 s prior to incubation at 22 °C (HT-22 °C). Germination time ▭; Outgrowth time ▭; First doubling time ▭.

lag time associated with germination was less at lower incubation temperatures (Fig. 5) (Stringer *et al.*, 2009). Thus, the duration and variability of lag phase and its stages are dependent on the historical treatment (e.g., sporulation conditions, heat treatment) and the subsequent growth conditions (e.g., temperature, NaCl concentration).

4. BOTULINUM NEUROTOXINS

4.1. Genetics and Evolution of Neurotoxin Genes and Toxin Complex

There are seven major types of botulinum neurotoxin (types A–G), and a significant number of sub-types, including five sub-types of type A toxin (termed A1, A2, A3, A4, A5), five type B sub-types, and six type E sub-types (Arndt *et al.*, 2006; Chen *et al.*, 2007; Hill *et al.*, 2007; Smith *et al.*, 2007; Carter *et al.*, 2009). Variability within the various sub-types is between 2.6 and 32% at the amino acid level, compared to up to 70% between different types of botulinum neurotoxins (Smith *et al.*, 2005; Arndt *et al.*, 2006; Raffestin *et al.*, 2009). The type of neurotoxin formed is dependent on the organism (Table 1). In the case of proteolytic *C. botulinum* and non-proteolytic *C. botulinum*, the neurotoxin genes are

located either on the chromosome or a plasmid (Marshall *et al.*, 2007; Sebaihia *et al.*, 2007; Smith *et al.*, 2007; Franciosa *et al.*, 2008). In *C. botulinum* Group III and Group IV, the neurotoxin genes are located on a bacteriophage and plasmid, respectively (Eklund *et al.*, 1989; Hatheway, 1993).

The neurotoxins are present in complexes of various sizes up to 900 kDa (depending on the serotype) that also include neurotoxin associated proteins (Lund and Peck, 2000; Poulain *et al.*, 2008). The genes encoding these proteins are located together in the genome. Two major neurotoxin cluster types have been identified, and are called the ''ha plus/orf-X minus'' cluster and the ''ha minus/orf-X plus'' cluster (Fig. 6, Table 7). The designation of the genes within the neurotoxin cluster has been inconsistent. This review has followed the approach of Sebaihia *et al.* (2007) and used *cnt* (Clostridial NeuroToxin) as the suffix for all genes of known function located within the botulinum neurotoxin clusters. In this scheme, all botulinum neurotoxin genes are designated *cntA*, with the type A neurotoxin gene designated *cntA*/*A* and the type B neurotoxin gene *cntA*/*B*, etc. The ha plus/orf-X minus cluster is the most studied, and comprises two transcriptional units. The genes for the neurotoxin (*cntA*) and a non-toxic-non-hemagglutinin (NTNH) protein (*cntB*) are co-transcribed, while the hemagglutinins (HA) (*cntC, cntD, cntE*-previously known as *ha*34, *ha*17, and *ha*70, respectively) are in a second transcriptional unit and are

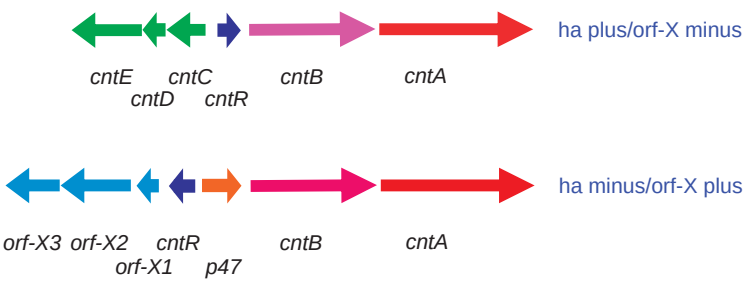


Figure 6 Two major neurotoxin cluster arrangements for botulinum neurotoxin-forming clostridia. This is based on full and incomplete sequences that are available at present, and further refinement may be necessary as further complete sequences become available. [From data in East *et al.* (1996, 1998), Bhandari *et al.* (1997), Collins and East (1998), Jovita *et al.* (1998), Santos-Buelga *et al.* (1998), Quinn and Minton (2001), Dineen *et al.* (2003), Zhang *et al.* (2003), Dineen *et al.* (2004), Franciosa *et al.* (2004, 2006), Chen *et al.* (2007), Sebaihia *et al.* (2007), Smith *et al.* (2007), Jacobson *et al.* (2008a), Carter *et al.* (2009), Lindström *et al.* (2009), and Raffestin *et al.* (2009).]

Table 7 Type of neurotoxin gene cluster present in botulinum neurotoxin-forming clostridia.

Organism	Type of neurotoxin present in designated cluster		Comments
	ha plus/orf-X minus cluster	ha minus/orf-X plus cluster	
Proteolytic *C. botulinum*	A1, A5, B	A1, A2, A3, A4, F	Most type A1 genes in single toxin strains in ha plus/orf-X minus cluster Few type A1 genes in single toxin strains, and all A1 genes in dual toxin gene strains in ha minus/orf-X plus cluster
Non-proteolytic *C. botulinum*	B	E, F	*cntR* is absent from type E strains
C. botulinum Group III	C, D	–	*cntR* is in same orientation as shown in Fig. 6, but downstream of *cntE*
C. botulinum Group IV	G	–	*cntC* is absent
C. baratii type F	–	F	Presence of *orf-X1*, *orf-X2* and *orf-X3* not confirmed
C. butyricum type E	–	E	*cntR* is absent

Note: See Fig. 6 for references used to compile this table.

transcribed in the opposite direction (Fig. 6). Located between these transcriptional units is gene encoding a sigma 70 factor (*cntR*) involved in the positive regulation of the neurotoxin gene (Dupuy and Matamouros, 2006). The ha minus/orf-X plus cluster is less studied. It also includes genes for the neurotoxin (*cntA*), NTNH (*cntB*), and the positive regulatory protein (*cntR*) (in this case historically also known as *p21*). In strains of proteolytic *C. botulinum*, the *cntB* and *cntR* genes carried by the ha minus/orf-X plus cluster are distinguishable from the *cntB* and *cntR* genes carried by the ha plus/orf-X minus cluster, as shown by sequencing and the use of specific probes (East *et al.*, 1996; Collins and East, 1998; Smith *et al.*, 2007; Jacobson *et al.*, 2008a; Carter *et al.*, 2009). The three genes encoding HA are absent from the ha minus/orf-X plus cluster, and the cluster additionally contains a group of three open reading frames (*orf-X1*, *orf-X2*, *orf-X3*) and

a single CDS (*p47*) of unknown function (Fig. 6). Transcriptional analysis of a proteolytic *C. botulinum* type A2 strain demonstrated that *cntA*, *cntB*, and *p47* were co-transcribed, as were *orf-X1*, *orf-X2*, and *orf-X3*, both from conserved neurotoxin cluster promoters (Dineen *et al.*, 2004). In non-proteolytic *C. botulinum* type E, the proteins encoded by *p47*, *orf-X1*, *orf-X2*, and *orf-X3* spontaneously associated with the neurotoxin (Kukreja and Singh, 2007). Different intragenic spacings have been reported in the ha minus/orf-X plus cluster. In clusters containing type A2, A3 and A4 neurotoxin genes, there is a 1.2 kb space between *cntR* and *orf-X1*, while in clusters containing a type A1 neurotoxin gene (single and dual strains) there is a 0.6 kb space between *orf-X2* and *orf-X3* (Raphael *et al.*, 2008; Jacobson *et al.*, 2008a). Although most strains contain a single neurotoxin gene and a single neurotoxin cluster, many strains of proteolytic *C. botulinum* are dual toxin strains and possess two neurotoxin genes and both types of neurotoxin cluster (Franciosa *et al.*, 1994; Cordoba *et al.*, 1995; Hutson *et al.*, 1996; Kirma *et al.*, 2004; Carter *et al.*, 2009).

Knowledge of the distribution of the two neurotoxin clusters has been substantially increased by sequencing and by whole genome analysis using a DNA microarray, although our information is presently far from complete. The ha plus/orf-X minus cluster appears to be associated with type A1, B, C, D, and G neurotoxin (Table 7, Fig. 6). In proteolytic *C. botulinum*, the ha plus/orf-X minus cluster is present in the genome of the sequenced type A1 strain ATCC 3502 (Sebaihia *et al.*, 2007) and is associated with type A1 toxin in most other single toxin strains, and type B toxin in single and dual toxin strains. The ha plus/orf-X minus cluster is also associated with type B toxin in strains of non-proteolytic *C. botulinum*, and with type C, D, and G neurotoxin in *C. botulinum* Group III and *C. botulinum* Group IV (Table 7). In the case of type C and D toxins, *cntR* is located downstream of *cntE*, while the type G neurotoxin gene appears to be associated with only two genes encoding hemagglutinins. The ha minus/orf-X plus cluster is associated with type A1, A2, A3, A4, E, and F neurotoxin (Table 7, Fig. 6). In proteolytic *C. botulinum*, the ha minus/orf-X plus cluster is associated with type A2, A3, A4, and F neurotoxin genes in single and dual toxin strains (where found), and the type A1 gene in dual toxin strains. A recent unexpected finding was that a small number of single toxin strains existed that possessed a type A1 neurotoxin gene in a ha minus/orf-X plus cluster (Raphael *et al.*, 2008; Carter *et al.*, 2009). There is presently no evidence for genetic crossover between the two clusters. Whole genome analysis using a DNA microarray demonstrated that 31 single neurotoxin strains of proteolytic *C. botulinum* either possessed all the genes present in the ha plus/orf-X minus cluster or all those present in

the ha minus/orf-X plus cluster. No strain possessed part of either cluster (Carter *et al.*, 2009). The ha minus/orf-X plus cluster is also associated with type E and F neurotoxin genes in non-proteolytic *C. botulinum*, *C. baratii* type F, and *C. butyricum* type E (Table 7, Fig. 6). No *cntR* homologue has been identified in strains containing a type E neurotoxin gene.

The original of the botulinum neurotoxins is unclear. It seems contradictory that the highly potent botulinum neurotoxin is formed by a collection of six bacteria that are not overtly pathogenic. It has been proposed that the neurotoxins are derived from viral proteases (DasGupta, 2006). An alternative proposal, based on an analysis of the structure of the neurotoxin and associated cluster in proteolytic *C. botulinum* type A is that the neurotoxin and adjacent genes originally arose from an ancestral collagenase-like gene cluster (Doxey *et al.*, 2008). The lack of correlation between the distribution of neurotoxin genes, neurotoxin cluster type, and the organism (as determined by whole genome analysis, 16S *rrn* sequencing, PFGE, AFLP, MLST) (Collins and East, 1998; Nevas *et al.*, 2005a; Hill *et al.*, 2007; Jacobson *et al.*, 2008a; Carter *et al.*, 2009) suggests that the neurotoxin genes and neurotoxin clusters have evolved independently of each other, and of the organism, and are likely to have arisen from a number of distinct recombination events. The center of the *cntB* gene (encoding NTNH) is reported to be a hot-spot for recombination events within the neurotoxin cluster (Collins and East, 1998; Smith *et al.*, 2007; Jacobson *et al.*, 2008a), and putative mobile genetic elements located close to the neurotoxin cluster may also have played a role in mobilization and gene transfer (Smith *et al.*, 2007). The transfer of plasmids and bacteriophages containing neurotoxin genes may also contribute to the horizontal movement of neurotoxin and associated genes. There is still much to learn about the transfer of neurotoxin and associated genes amongst these six clostridia.

4.2. Properties of the Neurotoxins

The seven botulinum neurotoxins (types A–G) were originally distinguished on the basis that polyclonal antibodies raised against purified neurotoxins neutralized the toxicity of neurotoxins of the same serotype, but not that of different serotypes. This classification is supported by comparison of neurotoxin gene sequences and studies of the site of action of the neurotoxins. Sub-types of each botulinum neurotoxin serotype may not be distinguished by polyclonal antibodies, but can be distinguished

by comparison of gene sequences and by the use of monoclonal antibodies (Smith *et al.*, 2005; Hill *et al.*, 2007). The crystallographic structure of a number of the botulinum neurotoxins has now been solved (e.g., Lacy *et al.*, 1998; Swaminathan and Eswaramoorthy, 2000).

The botulinum neurotoxins are formed initially as single-chain polypeptides with a molecular weight of about 150 kDa. The single-chain form (protoxin) is converted to a di-chain form, a process termed ''nicking'' (DasGupta, 1989), by an extracellular bacterial protease (or an added protease, such as trypsin) to form a molecule consisting of a heavy chain of about 100 kDa and a light chain of about 50 kDa linked by a disulfide bond. Nicking results in a substantial increase in toxicity. In foods and in cultures the neurotoxins may be present either as the single-chain, or in a di-chain form, or as a mixture of the two. Sebaihia *et al.* (2007) proposed that in proteolytic *C. botulinum* the orthologue of alpha-clostripain (CBO1920 in strain ATCC 3502) was the endogenous protease responsible for proteolytic nicking of the neurotoxin. Whole genome analysis revealed that this CDS was present in the genome of all 58 strains of proteolytic *C. botulinum* and three strains of *C. sporogenes* examined using a whole genome microarray (Carter and Peck, unpublished results). CBO1920 was constitutively expressed throughout lag, exponential and stationary phase of growth by strain ATCC 3502 (Carter and Peck, unpublished results) indicating that this protease is likely to contribute to the overall proteolytic activity of this organism, rather than it being solely concerned with post-translational modification of the neurotoxin. In contrast, neurotoxin formed by non-proteolytic *C. botulinum* often remains in a poorly active, single-chain form. It can be activated by proteolytic enzymes in the gut, and the neurotoxin must be activated by addition of trypsin prior to assessment of potential toxicity in the mouse test (Lund and Peck, 2000). The botulinum neurotoxins are relatively heat labile, and are rapidly inactivated at temperatures greater than 80 °C. Heating at 85 °C for 5 minutes reduced the concentration of active neurotoxin by a factor of 10,000, although some foods afforded protection (Siegel, 1993).

The botulinum neurotoxins are commonly associated with other proteins (e.g., hemagglutinin and non-toxic-non-hemagglutinin) that protect the neurotoxin and facilitate its absorption into the body. The heavy chain of the neurotoxin has two functional domains. The C-terminal domain facilitates binding of the neurotoxin to synaptic membrane vesicle proteins at the nerve terminal, while the N-terminal domain delivers the light chain into the cytosol of the nerve cell. The light chain contains a single zinc molecule, and possesses endopeptidase activity. Four steps have been

recognized in the mode of action of the neurotoxin (Turton *et al.*, 2002: Simpson, 2004; Jahn, 2006; Poulain *et al.*, 2008):

(i) The C-terminal binding domain of the heavy chains recognizes specific receptors on the surface of the presynaptic membrane of the motor neurone. A dual-receptor model is proposed where the neurotoxins bind to a ganglioside (GD_{1b}/GT_{1b}) and a glycoprotein (Rossetto and Montecucco, 2007; Brunger *et al.*, 2008). The protein receptors have been identified as the synaptic vesicle membrane proteins SV2 (iso-forms A, B, and C) for type A neurotoxin (Dong *et al.*, 2006; Mahrhold *et al.*, 2006; Poulain *et al.*, 2008), synaptotagmin I or II for type B and type G neurotoxin (Chai *et al.*, 2006; Jin *et al.*, 2006; Poulain *et al.*, 2008), and SV2A or SV2B for type E neurotoxin (Dong *et al.*, 2008).

(ii) The bound neurotoxin is then internalized into the vesicle by receptor-mediated endocytosis. Once internalized, the neurotoxins can no longer be neutralized by antisera (Turton *et al.*, 2002).

(iii) The lumen of the endocytic vesicle is then acidified by a proton-pumping ATPase, causing a conformational change in the neurotoxin exposing its hydrophobic domains at the surface. This allows the N-terminal translocation domain of the heavy chain to form an oligomeric (possibly tetramer) structure in the vesicle membrane that has permeable channels through which the now unfolded light chain can pass into the cytosol of the motor neurone. Once in the neutral pH of the cytosol, the light chain refolds (Raffestin *et al.*, 2009).

(iv) The interchain disulfide bond is reduced leading to the release of the light chain in the motor neurone cytosol. The zinc-endopeptidase activity of the neurotoxins light chain then brings about cleavage of protein components of the neurotransmitter (acetylcholine)-containing synaptic vesicle docking/fusion complex (SNARE proteins). The SNARE proteins are only cleaved when dissembled, not when in the complex form. Each light chain demonstrates stringent substrate specificity, and selectively cleaves SNARE proteins. The light chains recognize their substrate at specific binding sites, and then cut at a single specific cleavage site (e.g., Chen and Barbieri, 2006; Poulain *et al.*, 2008). Natural isoforms or recombinant proteins that lack the specific binding sites are not cleaved. The light chain of type A and E neurotoxins targets the synaptosome-associated protein of 25 kDa (SNAP-25), while the light chain of type B, D, F, and G neurotoxins cuts the vesicle-associated membrane protein (VAMP). The type C light chain cleaves two proteins in the synaptic vesicle docking/fusion

complex (SNAP-25 and syntaxin) (Simpson, 2004; Montecucco and Molgo, 2005; Rossetto *et al.*, 2006). Cleavage of any one of the SNARE proteins reduces the stability of the SNARE complex, preventing binding of acetylcholine-containing synaptic vesicles, thereby preventing neurotransmitter release. This leads to flaccid paralysis of the muscle. It is reported that type A neurotoxin is the most potent of the neurotoxins, and that botulism caused by type A neurotoxin is the most severe with the longest duration of action (e.g., Schantz and Johnson, 1992; Cherington, 1998). One explanation for these observations is that type A neurotoxin persists for longer in the neurons (Montecucco *et al.*, 2005; Poulain *et al.*, 2008).

Botulinum neurotoxins are the most potent substances known. Estimates of the lethality of the botulinum neurotoxins are based on experiments carried out with animals, and that of the amount of neurotoxin consumed in cases of foodborne botulism. The specific toxicities of botulinum toxins, when assayed by parenteral administration (intraperitoneal or intravenous injection) typically ranged from 3×10^7 to 10^8 MLD_{50} (mouse minimum lethal doses)/mg protein for the various complexes of different toxins types (Hatheway, 1988; Smith and Sugiyama, 1988; Hauschild, 1989; Hatheway, 1990; Schantz and Johnson, 1992; Potter *et al.*, 1993; Ekong *et al.*, 1995; Lund and Peck, 2000; Johnson, 2005). The botulinum neurotoxins are less toxic by the oral route than by the intraperitoneal route, and the neurotoxin complexes have a greater toxicity than purified toxin, because of their greater stability. Smith (1977) reported that the lethal oral dose of type A toxin for monkeys was 6.5×10^2 MLD_{50}/kg. Thus, by extrapolation, for a 50 kg human this would be 3.25×10^4 MLD_{50}. Extrapolating data from mice, rabbits and guinea pigs, the lethal dose for a 50 kg human by the oral route would be 5×10^3 MLD_{50} to 1.2×10^4 MLD_{50}. It is noted, however, that less potent estimates have been made (Herrero *et al.*, 1967; Ohishi *et al.*, 1977). For example, Herrero *et al.* (1967) found that 1.2×10^5 MLD_{50}/kg was required to elicit symptoms of botulism in a rhesus monkey, equivalent to 6.0×10^6 MLD_{50} for a 50 kg human. In one case of human botulism, it was estimated that the death of a 104 kg man resulted from the consumption of cheese containing about 3.5×10^3 MLD_{50} of type B toxin (Smith and Sugiyama, 1988). Schantz and Johnson (1992) estimated that consumption of a total of 3×10^3–3×10^4 MLD_{50} of toxin can lead to symptoms of botulism or death in humans following accidental exposure, and Lund and Peck (2000) estimated that toxicity via the oral route might be caused by consumption of as little as 3×10^3 MLD_{50} of toxin. From these various estimates, it would seem that foodborne botulism might be caused

by consuming as little as 3×10^3 MLD_{50} of neurotoxin. This is equivalent to approximately 30–100 ng of botulinum toxin depending on the size of the toxin complex. The botulinum neurotoxins are, therefore, more than a million-fold more potent than ricin or alkali cyanides, for which the fatal oral doses are estimated to be in the region of 50–1,000 mg and 50–100 mg, respectively (CDC, 1996; Audi *et al.*, 2005). It is reported that the concentration of botulinum neurotoxin in culture supernatants may reach $1–4 \times 10^6$ MLD_{50}/ml (Schantz and Johnson, 1992). Thus, if this concentration could be reached in food, consumption of as little as one microliter could be sufficient neurotoxin to lead to botulism. The neurotoxin concentration of foods associated with two recent cases of foodborne botulism has been measured. It can be estimated that consumption of just 10 mg of a temperature abused commercially produced chicken enchilada meal (that contained 3×10^5 MLD_{50}/g of neurotoxin (King, 2008)) would have been sufficient to cause botulinum, and that consumption of only 5 µl of temperature abused carrot juice (that contained 7×10^5 MLD_{50}/ml (Sheth *et al.*, 2008)) would have been sufficient to cause botulinum.

4.3. Regulation of Neurotoxin Formation

At the present time, it is not fully understood how the various botulinum neurotoxin-forming clostridia regulate neurotoxin formation. What is known is that the quantity of toxin formed is strain dependent, and influenced by culture conditions and the nutritional status of the medium employed (e.g., nitrogen sources). Sharma (1999) used an endopeptidase assay developed by Sesardic and co-workers (Ekong *et al.*, 1997) to quantify the effect of incubation temperature on neurotoxin formation by proteolytic *C. botulinum* type A1 strain ATCC 19397. When neurotoxin was first detected within the cells, it was primarily present in the single-chain form (initial ratio of single-chain:di-chain = 100:1). However, as growth proceeded at 25 °C, nicking of the single-chain led to a steady and substantial increase in the proportion of neurotoxin in the di-chain form (final ratio of single-chain:di-chain = 1:18). At 25 °C, as the bacteria entered late exponential phase and the log viable count increased from 6.9 to 8.7 cfu/ml, the activity of type A toxin in the culture supernatant increased by six-orders of magnitude to reach more than 10^5 MLD_{50}/ml (Fig. 7). A smaller increase was recorded at 37 °C. Other studies with various strains of proteolytic *C. botulinum* type A (Call *et al.*, 1995; Bradshaw *et al.*, 2004; Couesnon *et al.*, 2006; Shin *et al.*, 2006; Rao *et al.*, 2007), and non-proteolytic *C. botulinum* type E (Couesnon *et al.*, 2006;

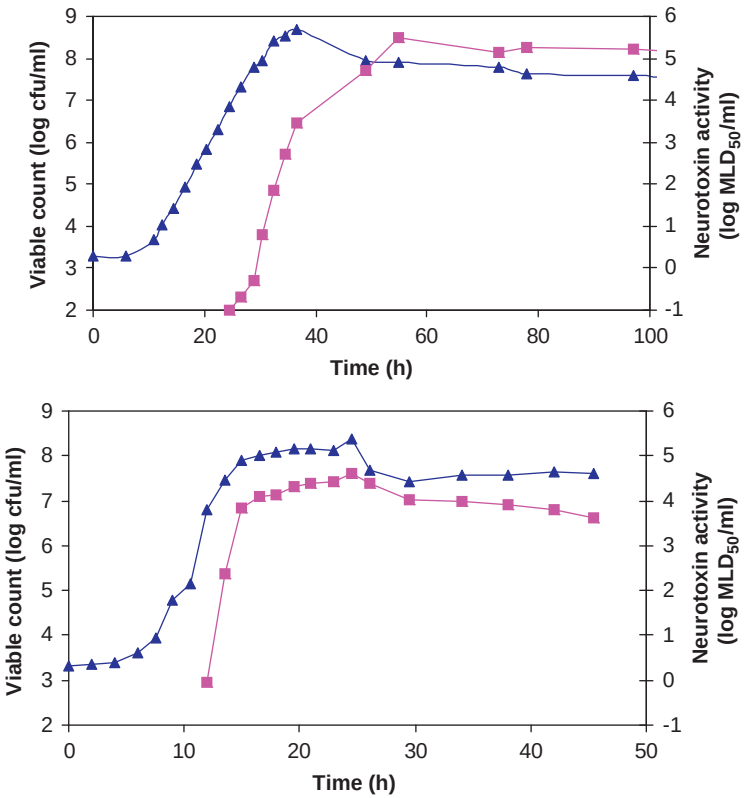


Figure 7 Effect of incubation temperature on growth and neurotoxin formation by proteolytic *C. botulinum* type A1 strain ATCC 19397. [Adapted from Sharma (1999)]. Data are mean of duplicate or triplicate bottles. Upper curves at 25 °C, lower curves at 37 °C. ▲, Viable count; ■, type A neurotoxin activity (as determined by endopeptidase activity).

Artin *et al.*, 2008) have also reported that neurotoxin formation is primarily associated with late exponential and early stationary phase. By measuring the neurotoxin concentration within the cells, it has been established that the dramatic increase in neurotoxin in the supernatant of strain ATCC 19397 was associated with *de novo* synthesis of neurotoxin, and not with the release of neurotoxin loosely attached to the cell, or with cell autolysis (Sharma, 1999). This raises the intriguing question as to how neurotoxin is released from the cell. An electron micrograph study by Call *et al.* (1995) with the type A1 strain 62A also concluded that neurotoxin released from the cell was not associated with autolysis, but cell wall exfoliation. Other

authors, however, have proposed a role for autolysis (e.g., Bonventre and Kempe, 1960), and it may be that the importance of autolysis is strain dependent and influenced by growth conditions. Rao *et al.* (2007) reported on a lack of any potential signal peptide at the N-terminus of each of the neurotoxin and associated proteins, and suggested that the neurotoxins may be secreted through a non-signal peptide mediated secretion system (e.g., flagella export apparatus, holins).

Interestingly, neurotoxin formation by several strains of proteolytic *C. botulinum* that form both type B and type F neurotoxin is temperature dependent. When incubated at 37 °C type B neurotoxin predominates, while at 30 °C type F toxin is the major form (Barash and Arnon, 2004). A fourfold greater concentration of neurotoxin was formed at 37 °C than 30 °C. Proteolytic *C. botulinum* strains NCTC 2012 (Loch Maree) and CDC 657 form low concentrations of neurotoxin, and this may be associated with the neurotoxin genes being located on plasmids (Marshall *et al.*, 2007). The ability to form neurotoxin can be rapidly lost, and it is not unusual for laboratory cultures to lose the ability to form neurotoxin. This is especially true of strains of *C. botulinum* Group III and *C. botulinum* Group IV that can lose the bacteriophage/plasmid that encode the neurotoxin genes (Eklund *et al.*, 1989; Hatheway, 1993).

The ha plus/orf-X minus and the ha minus/orf-X plus neurotoxin clusters both contain two transcriptional units, with a sigma 70 factor (*cntR*) involved in the positive regulation of the genes in the neurotoxin clusters generally located between them (Fig. 6, Table 7). Each cluster carries its own distinguishable *cntR* gene (Carter *et al.*, 2009), although *cntR* is not present in clusters that carry a type E neurotoxin gene (Table 7). Regulatory proteins highly similar to CntR are present in other toxigenic clostridia, *C. tetani* (TetR), *C. perfringens* (UviA), and *C. difficile* (TcdR), where they regulate expression of genes encoding toxins and bacteriocins (Raffestin *et al.*, 2004; Raffestin *et al.*, 2005; Dupuy and Matamouros, 2006). Indeed, these sigma factors were sufficiently closely related that they are able to substitute for one another (Dupuy *et al.*, 2006). The sequence to which CntR binds is highly conserved, and a survey of the genome of proteolytic *C. botulinum* strain ATCC 3502 failed to identify other possible binding sequences, suggesting that CntR specifically regulates expression of genes in the neurotoxin cluster (Sebaihia *et al.*, 2007). Expression of genes in the neurotoxin cluster has been extensively studied in type A1 strains of proteolytic *C. botulinum*, and all genes within the neurotoxin cluster follow the same profile reported for neurotoxin formation. There is a sharp peak of gene expression in late exponential and early stationary phase, and then a decrease during stationary phase (Bradshaw *et al.*, 2004;

Couesnon *et al.*, 2006; Shin *et al.*, 2006; Rao *et al.*, 2007). The rate of accumulation of *cntR* was lower than that of other genes in the neurotoxin cluster, as often found with a regulator (Couesnon *et al.*, 2006). Expression of genes in the neurotoxin cluster and associated neurotoxin formation by non-proteolytic *C. botulinum* type E follows a similar pattern to that reported for strains of proteolytic *C. botulinum* type A1 (Couesnon *et al.*, 2006; Artin *et al.*, 2008; Chen *et al.*, 2008). Thus, since *cntR* is absent from the neurotoxin cluster of these strains, it would appear that there is a presently unidentified positive regulator of the neurotoxin gene located elsewhere in the genome.

The nature of the signal that *cntR* is responding to remains to be identified. An effect of the nutritional status of the medium has been described (Johnson, 2005; Raffestin *et al.*, 2009). For example a raised carbon dioxide concentration is reported to increase neurotoxin formation in non-proteolytic *C. botulinum* types B and E (Lövenklev *et al.*, 2004; Artin *et al.*, 2008). A high growth temperature can reduce or prevent expression of sigma factors. For example, growth of *C. difficile* at 42 °C prevented expression of the sigma 70 factor *tcdR*, and the subsequent expression of *tcdA* and *tcdB* (Karlsson *et al.*, 2003). However, in proteolytic *C. botulinum* type A expression of *cntR* and other genes in the neurotoxin cluster were not temperature sensitive within the range 37–44 °C (Couesnon *et al.*, 2006). The type of neurotoxin formed by several strains of proteolytic *C. botulinum* type Bf was, however, temperature sensitive (Barash and Arnon, 2004). Neurotoxin formation by non-proteolytic *C. botulinum* type E was similar at 30 and 37 °C (Couesnon *et al.*, 2006). A possible role for a quorum sensing mechanism controlling expression of *cntR* in proteolytic *C. botulinum* strain ATCC 3502 has been proposed (Sebaihia *et al.*, 2007).

5. BOTULISM

There are three major types of botulinum in humans, foodborne botulism, infant/intestinal (adult) botulism, and wound botulism. The symptoms of botulism are primarily neurological and frequently commence with blurred vision. Other typical symptoms include dysphagia and dysphonia (difficulty swallowing and speaking), paralysis of face muscles, generalized weakness, descending bilateral flaccid paralysis, nausea/vomiting and dizziness/vertigo, and muscle weakness. In severe cases, flaccid paralysis of the respiratory or cardiac muscles may occur, and can result in death if not

treated. The rapid administration of antitoxin to adults suffering from foodborne or wound botulism, and supportive therapy has led to a reduction in the fatality rate to approximately 5–10% of cases. Severe cases may require an extended period of mechanical ventilation, and complete recovery can be very slow, in some cases taking months or even years. Arnon and co-workers have developed the use of human botulism immune globulin for treatment of infant botulism (Arnon *et al.*, 2006; Arnon, 2007). Prompt treatment led to a significant reduction in hospital stay, and duration of time spent in intensive care and on mechanical ventilation (Arnon *et al.*, 2006).

5.1. Foodborne Botulism

A disease reported in central Europe in the 18th and 19th century involving muscle paralysis, breathing difficulties, and a high fatality rate that was frequently associated with consumption of blood sausage came to be known as ''botulism,'' after the Latin word ''*botulus*'' meaning sausage (Novak *et al.*, 2005). Justinus Kerner investigated the ''sausage poison'' in the early part of the 19th century, and published monographs on the subject. He made suggestions for the prevention and treatment of botulism, and concluded that: (i) anaerobic conditions are necessary in the sausage for the development of the poison; (ii) the poison is a biological agent that is lethal even in small quantities; and (iii) the poison acts on the motor and autonomic nervous system (Erbguth, 2008). In December 1895, Emile van Ermengem investigated an outbreak of botulism involving home made raw salted ham in Ellezelles (Belgium) that affected 23 musicians (three fatally) who attended a funeral wake (van Ermengem, 1979; Pickett, 2008). He concluded, ''it is highly probable that the poison in the ham was produced by anaerobic growth of specific microorganisms during the salting process'' (Pickett, 2008). Importantly, van Ermengem established that foodborne botulism was an intoxication, not an infection, and that the toxin was produced by a spore-forming obligately anaerobic bacterium. He isolated an anaerobic sporulating bacillus from cultures of ham. The isolated organism was initially called ''*Bacillus botulinus*,'' and although these strains are now lost, their physiological properties are consistent with those of non-proteolytic *C. botulinum*. It was demonstrated that portions of macerated ham, filtered extracts and culture filtrates were toxic to various animal species.

A great number of botulism outbreaks occurred over the next few decades, particularly in Europe and North America, and were principally

associated with the wider use of canning and bottling processes to extend the shelf life of food. For example, between 1918 and 1924, there were 367 cases of foodborne botulism in the USA, of which 230 were fatal (Meyer and Eddie, 1965). An outbreak in Albany (OR, USA) involving inadequately processed home-canned string beans occurred in February 1924, and affected 12 people all fatally (Stricker and Geiger, 1924). The first recorded outbreak of foodborne botulism in the UK occurred at Loch Maree (Scotland) in August 1922, and was associated with consumption of a picnic lunch of sandwiches containing (under-processed) wild duck paste. This led to eight cases of botulism, all of which were fatal (Leighton, 1923). Extensive studies of the heat resistance of spores of proteolytic *C. botulinum* (e.g., Esty and Meyer, 1922) formed the basis for the standard minimum heat treatment (known as the botulinum cook) given to low-acid canned foods, and led to a substantial reduction in the number of botulism cases. Through the identification and implementation of a number of effective control measures, foodborne botulism is relatively uncommon, although outbreaks do occur when known control measures have not been implemented. These can involve home-prepared foods, and more rarely commercial products where they can be associated with significant medical and economic consequences (e.g., estimated cost per case of botulism in the USA is $30 million (Setlow and Johnson, 1997)).

The present issues are to ensure that known effective control measures are applied (e.g., botulinum cook) and that effective control measures are identified when new processing technologies (e.g., high hydrostatic pressure) are introduced or new types of foods such as minimally heated chilled foods are developed. Various guidelines have been produced with respect to the control of non-proteolytic *C. botulinum* in minimally heated chilled foods (e.g., Peck, 1997; ACMSF, 2007; CFA, 2006; FSA, 2008; Peck *et al.*, 2008; Stringer and Peck, 2008).

The true incidence of foodborne botulism is probably under-reported, since (i) it is not a reportable disease in all countries (Therre, 1999), and (ii) the efficiency of investigating potential outbreaks varies from country to country. Indeed, it is estimated that reporting efficiency is 50% in the USA (Mead *et al.*, 1999). Home-prepared foods, where known control measures have not been implemented, have been responsible for many recent foodborne botulism outbreaks. These can be associated with traditional practices (e.g., the home canning of vegetables is common in the USA), or difficult economic conditions placing an increased reliance on the preparation of food in the home. In 1999/2000, more than 2,500 cases of foodborne botulism were reported in Europe, with a high incidence reported in Armenia, Azerbaijan, Belarus, Georgia, Poland, Russia, Turkey, and

Uzbekistan (Peck, 2006). The highest reported incidence was in Russia (more than 800 cases), and were most frequently associated with consumption of smoked, salted and dried fish, and home-canned or bottled vegetables (Peck, 2006). The Republic of Georgia has one of the highest nationally reported rates of foodborne botulism, with most cases attributed to home-conserved vegetables (contaminated with type B toxin) or home-preserved fish (contaminated with type E toxin) (Varma *et al.*, 2004; Gottlieb *et al.*, 2007). One large outbreak at a wedding in 1994 affected 173 people and was associated with consumption of contaminated fish. Between 20 and 40 cases of foodborne botulism are presently recorded annually in France, Georgia, Germany, Italy, and the USA, with a higher incidence in China and Poland (Peck, 2006). Because of the potential severity of botulism, electronic alerting services (e.g., ProMED) post details of recalls linked to botulism concerns, for example, due to faulty processing, even if there are no associated human cases. A majority of outbreaks of foodborne botulism are associated with proteolytic *C. botulinum* or non-proteolytic *C. botulinum*, and since they are physiologically distinct and present different hazard scenarios, it is appropriate to consider the epidemiology of foodborne botulism for each separately.

Outbreaks of foodborne botulism have often been associated with proteolytic *C. botulinum* when either (i) canned or bottled foods have been inadequately processed, (ii) ingredients containing preformed neurotoxin have been added to a correctly refrigerated product, or (iii) products intended to be stored chilled have been temperature abused (Table 8). A very large outbreak in northern Thailand in March 2006 was associated with consumption of home-canned bamboo shoots. Spores of proteolytic *C. botulinum* type A survived the inadequate heat treatment, and led to growth and neurotoxin formation during subsequent ambient storage (CDC, 2006a). There were 209 cases of botulism, of which 134 were hospitalized, and 42 required mechanical ventilation (Ungchusak *et al.*, 2007; Wongtanate *et al.*, 2007). Inadequate thermal processing of cans of hot dog chili sauce at a commercial canning facility in Augusta (GA, USA) in June 2007 led to four cases of foodborne botulism (all required mechanical ventilation), and the recall of tens of millions of cans (CDC, 2007). Addition of ingredients containing preformed botulinum neurotoxin to a correctly refrigerated product has also been associated with foodborne botulism. The largest recorded botulism outbreak in the UK occurred in 1989 (27 cases, one fatal) and involved commercially produced hazelnut yogurt. Spores of proteolytic *C. botulinum* type B survived an inadequate heat treatment delivered to the hazelnut conserve, and led to neurotoxin formation. Hazelnut conserve containing type B neurotoxin was then

Table 8 Examples of incidents of foodborne botulism involving proteolytic *C. botulinum*.

Country (year)	Product	Toxin type	Cases (deaths)	Factors contributing to botulism outbreak	Reference(s)
Canada (1985)	Commercial garlic-in-oil	B	36	Bottled; no preservatives; temperature abuse	St. Louis *et al.* (1988)
Canada (1987)	Bottled mushrooms	A	11	Underprocessing and/or inadequate acidification	CDC (1987); McLean *et al.* (1987)
UK (1989)	Commercial hazelnut yoghurt	B	27(1)	Hazelnut conserve underprocessed	O'Mahony *et al.* (1990)
USA (1993)	Restaurant, commercial process cheese sauce	A	8 (1)	Contaminated after opening, then temperature abused	Townes *et al.* (1996)
Italy (1993)	Commercial canned roasted aubergine in oil	B	7	Insufficient heat treatment; improper acidification	CDC (1995)
USA (1994)	Restaurant; potato dip (''skordalia'') and aubergine dip (''meligianoslata'')	A	30	Baked potatoes held at room temperature	Angulo *et al.* (1998)
USA (1994)	Commercial clam chowder	A	2	No secondary barrier; temperature abuse	Anon (1995)
USA (1994)	Commercial black bean dip	A	1	No secondary barrier; temperature abuse	Anon (1995)
Italy (1996)	Commercial mascarpone cheese	A	8(1)	No competitive microflora; pH >6, temperature abuse	Franciosa *et al.* (1999); Aureli *et al.* (2000)
Italy (1997)	Home-made pesto/oil	B	3	pH 5.8, a_w 0.97	Chiorboli *et al.* (1997)
Germany (1997)	Home-prepared beans	A	1	Poor preparation	Gotz *et al.* (2002)

(*Continued*)

Table 8 (*continued*)

Country (year)	Product	Toxin type	Cases (deaths)	Factors contributing to botulism outbreak	Reference(s)
Iran (1997)	Traditional cheese preserved in oil	A	27(1)	Unsafe process	Pourshafie *et al.* (1998)
Thailand (1998)	Home canned bamboo shoots	A	13(2)	Inadequate processing (?)	CDC (1999)
Argentina (1998)	Meat roll (“matambre”)	A	9	Cooked and heat-shrink plastic wrap; temperature abuse	Villar *et al.* (1999)
UK (1998)	Home bottled mushrooms in oil (imported from Italy)	B	2(1)	Unsafe process	CDSC (1998); Roberts *et al.* (1998)
France (1999)	Commercial chilled fish soup	A	1	Temperature abuse at home	Carlier *et al.* (2001)
Japan (1999)	Commercial boil in bag curry	A	1	Temperature abuse	Kobayashi *et al.* (1999)
France (2000)	Home-made asparagus soup	B	9	Inadequate processing (?)	Abgueguen *et al.* (2003)
USA (2001)	Commercial frozen chili sauce	A	16	Temperature abuse at salvage store	Kalluri *et al.* (2003)
South Africa (2002)	Commercial tinned pilchards	A	2(2)	Corrosion of tin, permitted secondary contamination	Frean *et al.* (2004)
Canada (2002)	Restaurant, baked potato in aluminum foil	A	1	Baked potato held at room temperature (?)	Bhutani *et al.* (2005)

USA (2004/5)	Home-made pruno (2 outbreaks)	A	5	Dangerous process	Vugia *et al.* (2009)
UK (2005)	Travel from Georgia	A	1	Not known	McLauchlin *et al.* (2006)
Turkey (2005)	Homemade suzme (condensed) yogurt	A	10(2)	Dangerous process	Akdeniz *et al.* (2007)
USA (2006)	Home fermented tofu	A	2	Dangerous process	Meyers *et al.* (2007)
Thailand (2006)	Home canned bamboo shoots	A	209	Inadequate processing	CDC (2006a); Ungchusak *et al.* (2007); Wongtanate *et al.* (2007)
Canada/ USA (2006)	Commercial refrigerated carrot juice	A	6 (1)	Temperature abuse	Anon (2006a); CDC (2006b); Sheth *et al.* (2008)
USA (2007)	Commercial hot dog chili sauce	A	4	Inadequate processing	CDC (2007)
Australia (2007)	Commercial nacho meal	A	1	Inadequate processing (?)	Anon (2007)
France (2008)	Commercial chicken enchiladas	A	2	Temperature abuse	King (2008)

added to natural yogurt (O'Mahony *et al.*, 1990). An outbreak in the USA in 1994 (30 cases, 4 of which required mechanical ventilation) was associated with consumption of *skordalia* in a restaurant (Angulo *et al.*, 1998). Foil-wrapped potatoes were baked, and then stored at ambient temperature, enabling growth and neurotoxin formation by proteolytic *C. botulinum* type A. Potatoes contaminated with neurotoxin were then added to natural yogurt to give *skordalia* (Table 8). Outbreaks of foodborne botulism have also occurred when foods intended to be stored chilled have been temperature abused (Table 8). A recent severe outbreak involved commercial chilled carrot juice in August and September 2006 in Canada (Toronto) and the USA (Florida and Georgia). Six people were affected, and all are likely to have consumed extremely large amounts of toxin (it is estimated that as little as 5 μl may have constituted a lethal dose (see above)). All patients required mechanical ventilation, one of whom died, and two were still dependent on mechanical ventilation 1 year after the initial intoxication (Anon, 2006a; CDC, 2006b; Sheth *et al.*, 2008). An outbreak in France in August 2008 was associated with growth and neurotoxin production by proteolytic *C. botulinum* type A in temperature abused commercial chicken enchiladas. Two people were affected, and both required mechanical ventilation (Table 8).

While most reported outbreaks of foodborne botulism involving non-proteolytic *C. botulinum* are associated with strains producing type E toxin (Table 9), strains forming type B (and to a lesser extent type F toxin) are also important. A high proportion of botulism outbreaks in Europe have been associated with type B toxin, and it appears that many of these outbreaks are due to strains of non-proteolytic *C. botulinum* (Lucke, 1984; Hauschild, 1993). The original strain isolated by van Ermengem was probably non-proteolytic *C. botulinum* type B. Recorded outbreaks of botulism involving non-proteolytic *C. botulinum* have been most frequently associated with: (i) meat (e.g., sausage, home-cured ham), (ii) fish (e.g., salted, dried, vacuum, or smoked), and (iii) home-made foods prepared by the peoples of Alaska and north Canada (e.g., fermented seal flipper, fermented beaver tail and paw, fermented salmon roe, ''muktuk''). A large outbreak in Egypt in 1991 involved consumption of commercially produced uneviscerated salted fish (''faseikh''). The fish was stored in a cool room for 1 day to allow putrefaction prior to salting, enabling growth, and neurotoxin formation by non-proteolytic *C. botulinum* type E. More than 91 people were affected, of which 18 died (Weber *et al.*, 1993). Several individuals have contracted botulism more than once (Sobel *et al.*, 2007; Bilusic *et al.*, 2008). For example, a New Jersey resident contracted foodborne botulism in 1992 and then again in 2005 each time after consuming home-prepared unevicerated salted fish

Table 9 Examples of incidents of foodborne botulism involving non-proteolytic *C. botulinum*.

Country (year)	Product	Toxin type	Cases (deaths)	Factors contributing to botulism outbreak	Reference(s)
USA (1981)	Uneviscerated salted, air-dried fish (''kapchunka'')	B	1	Poorly controlled salting, lack of refrigeration	CSHD (1981)
Madagascar (1982)	Commercial pork sausage	E	60 (30)	Inadequate preservation	Viscens *et al.* (1985)
USA (1985)	Uneviscerated salted, air-dried fish (''kapchunka'')	E	2 (2)	Poorly controlled salting, lack of refrigeration	CDC (1985)
USA/Israel (1987)	Commercial uneviscerated salted, air-dried fish (''kapchunka'')	E	8(1)	Poorly controlled salting, lack of refrigeration	Slater *et al.* (1989)
Sweden (1991)	Vacuum-packed hot-smoked rainbow trout	E	?	?	Korkeala *et al.* (1998)
Egypt (1991)	Commercial uneviscerated salted fish (''faseikh'')	E	>91(18)	Putrefaction of fish before salting	Weber *et al.* (1993)
USA (1992)	Commercial uneviscerated salted fish (''moloha'')	E	8	Insufficient salt	CDC (1992)
Sweden (1994)	Vacuum-packed hot-smoked rainbow trout	E	?	?	Korkeala *et al.* (1998)
Canada (1995)	''Fermented'' seal or walrus (4 outbreaks)	E	9	Unsafe process	Proulx *et al.* (1997)
France (1997)	Fish	E	1	?	Boyer *et al.* (2001)
Germany (1997)	Commercial hot-smoked vacuum-packed fish (''Raucherfisch'')	E	2	Suspected temperature abuse	Jahkola and Korkeala (1997); Korkeala *et al.* (1998)
Argentina (1997)	Home cured ham	E	6	?	Rosetti *et al.* (1999)
Germany (1997)	Home smoked vacuum-packed fish (''Lachsforellen'')	E	4	Temperature abuse	Anon (1998)

(*Continued*)

Table 9 (*continued*)

Country (year)	Product	Toxin type	Cases (deaths)	Factors contributing to botulism outbreak	Reference(s)
Germany (1998)	Commercial smoked vacuum-packed fish	E	4	?	Therre (1999)
France (1998)	Commercial frozen vacuum packed scallops	E	1	Temperature abuse (?)	Boyer *et al.* (2001)
France (1998)	Commercial frozen vacuum packed prawns	E	1	Temperature abuse (?)	Boyer *et al.* (2001)
Finland (1999)	Whitefish eggs	E	1	Temperature abuse	Lindström *et al.* (2004b)
France (1999)	Salmon or fish soup	E	1	?	Boyer *et al.* (2001)
France (1999)	Grey mullet	E	1	Temperature abuse (?)	Boyer *et al.* (2001)
Australia (2001)	Reheated chicken	E	1	Poor temperature control	Mackle *et al.* (2001)
USA (2001)	Home-made fermented beaver tail and paw	E	3	Temperature abuse	CDC (2001)
Canada (2001)	Home-made fermented salmon roe (two outbreaks)	E	4	Unsafe process	Anon (2002)
USA (2002)	Home-made ''muktuk'' (from Beluga whale)	E	12	Unsafe process	McLaughlin *et al.* (2004)
Germany (2003)	Home salted air-dried fish	E	3	Temperature abuse (?)	Eriksen *et al.* (2004)
Germany (2004)	Commercial vacuum-packed smoked salmon	E	1	Consumed after ''use-by date''	Dressler (2005)
USA (2005)	Home-salted unevicerated fish	E	5	Insufficient salt	Sobel *et al.* (2007)
Iran (2006)	Traditional soup (''Ashmast'')	E	11	?	Vahdani *et al.* (2006)
Finland (2006)	Commercial vacuum-packed smoked whitefish	E	1	Temperature abuse (?)	Lindström *et al.* (2006)

contaminated with type E neurotoxin (Sobel *et al.*, 2007). Time and/or temperature abuse of commercial refrigerated foods has led to several outbreaks of foodborne botulism (Table 9).

5.2. Other Types of Human Botulism

While foodborne botulism is an intoxication, infant and intestinal (adult) botulism are infections. Spores of proteolytic *C. botulinum*, or more rarely *C. baratii* type F or *C. butyricum* type E, that have been consumed are able to colonize the large intestine and form neurotoxin *in vivo* (Mills and Arnon, 1987). Typical symptoms include extended constipation and flaccid paralysis. The infectious dose is estimated to be between 10 and 100 spores (Arnon, 2004). These clostridia are only able to multiply and form neurotoxin in the gastrointestinal tract in the absence of a fully mature gut flora. Infants less than 12 months of age (especially those between 2 weeks and 6 months) are most susceptible (Arnon, 2004), as are adults when the normal intestinal flora has been suppressed (e.g., by antibiotic treatment). Infant botulism is considerably more common than intestinal (adult) botulism (Fenicia *et al.*, 2007). The first clinical cases of infant botulism were described in 1976, although subsequent investigations revealed a number of earlier cases, including a misdiagnosed case from 1931 (Arnon *et al.*, 1979; Arnon, 2004). Infant botulism has been reported in many countries. Between 1976 and 2006, 2,419 cases were reported in the USA (where it is now the most frequently reported type of botulism), 366 cases in Argentina, and 158 cases in other countries. The fatality rate is lower than that for foodborne botulism, approximately 1% of cases (Koepke *et al.*, 2008). Infant botulism has been proposed as one possible cause of sudden infant death syndrome (Bohnel *et al.*, 2001; Fox *et al.*, 2005; Nevas *et al.*, 2005b). Two major sources of spores have been identified: general environmental contamination (e.g., soil, dust) and honey (Aureli *et al.*, 2002; Arnon, 2004). Honey may contain a high loading of *C. botulinum* spores (Lund and Peck, 2000), and a history of exposure to honey was identified for approximately half of the cases of infant botulism reported in Europe to 2002 (Aureli *et al.*, 2002), but a smaller fraction of cases reported in California (where most cases from the USA occur) (Koepke *et al.*, 2008). The association with infant botulism has led to recommendations in several countries that jars of honey should carry a warning label indicating that the product should not be consumed by infants less than 12 months of age.

Wound botulism is an infection, in which proteolytic *C. botulinum* grows and forms neurotoxin in a wound in the body. A majority of cases have

involved strains of proteolytic *C. botulinum* that form type A or type B neurotoxin, although a recent case in Sweden was associated with an organism that formed type E neurotoxin (Artin *et al.*, 2007). The first case of wound botulism was described in 1943 and, for about half a century, this disease was uncommon with most cases involving gross trauma (e.g., crush injury) to an extremity (Passaro *et al.*, 1998; Lund and Peck, 2000). Recently, however, there has been a significant increase in reports of wound botulism in many countries, and this has been associated with drug abuse (Passaro *et al.*, 1998; Werner *et al.*, 2000; Sandrock and Murin, 2001; Brett *et al.*, 2004; Akbulut *et al.*, 2005; Galldiks *et al.*, 2007). Wound botulism had not been reported in the UK prior to 2000, but between 2000 and 2007 a total of 141 suspected cases were reported, all involving heroin injection (Anon, 2008). Two of the 22 cases reported in the UK in 2006 were fatal (Hough, 2007). Spores of proteolytic *C. botulinum* are either present in heroin or on contaminated needles and enter the body via subcutaneous or intramuscular injection (skin-popping) of heroin. Germination, growth, and neurotoxin formation then occur *in vivo*, with a wound or abscess providing suitable anaerobic growth conditions. Inhalation of cocaine has also led to cases of wound botulism where *C. botulinum* has grown and formed neurotoxin in the sinus (Kudrow *et al.*, 1988; Roblot *et al.*, 2006).

Other types of human botulism are extremely rare. Iatrogenic botulism has been reported as a result of injection of botulinum neurotoxin intended for therapeutic purposes. A number of various botulinum neurotoxin preparations (e.g., Botox®, Dysport®) have been approved in various countries for therapeutic and cosmetic use. One case of iatrogenic botulism in a 3-year-old pediatric patient was associated with injection of an approved botulinum neurotoxin preparation (Crowner *et al.*, 2007). Four cases of iatrogenic botulism in the USA in 2004 were associated with injection of an unlicensed product. It is estimated that more than 2,500-times the human lethal dose may have been given (Chertow *et al.*, 2006). There is concern that counterfeit pharmaceutical products may present a health risk (Aoki, 2008). Three cases of inhalational botulism that occurred in Germany in 1962 were associated with inadvertent inhalation of aerosolized type A neurotoxin by veterinary workers disposing of contaminated animal fur (Arnon *et al.*, 2001). The deliberate release of aerosolized botulinum neurotoxin (and also deliberate neurotoxin release by other routes) by terrorists is a concern (Arnon *et al.*, 2001; Wein and Liu, 2005; Villar *et al.*, 2006). Aerosols of botulinum neurotoxin were dispersed by the Aum Shinrikyo cult in Japan on at least three occasions between 1990 and 1995, although these attacks apparently failed (Arnon *et al.*, 2001).

5.3. Botulism in Animals and Birds

C. botulinum Group III is responsible for a majority of cases of botulism involving animals and birds, although proteolytic *C. botulinum* has been associated with botulism in cattle and horses, and non-proteolytic *C. botulinum* has been associated with botulism involving cannibalism of dead fish by other fish (Lund and Peck, 2000). Most of these cases of botulism involve inadvertent consumption of pre-formed botulinum neurotoxin, although young mammals can also suffer from a disease analogous to infant botulism, and wound botulism can occur (Duprez, 2006; Liguori *et al.*, 2008). Recent animal-associated outbreaks of botulism have involved; farmed foxes and mink (Lindström *et al.*, 2004a), sheep (Anon, 2006b; van der Burgt *et al.*, 2007), horses (Wilkins and Palmer, 2003; Duprez, 2006; Whitlock and McAdams, 2006), and cattle (Yeruham *et al.*, 2003; Anon, 2004; Livesey *et al.*, 2004; Wenzel *et al.*, 2005; ACMSF, 2006; Anon, 2006c; Sharpe *et al.*, 2008). An increase in the reported incidence of botulinum in cattle in the UK since 2003 is at least partly associated with access to poultry litter containing carcasses of dead birds (Anon, 2003, 2004; Livesey *et al.*, 2004; ACMSF, 2006; Hogg *et al.*, 2008). The potential risks to human health associated with botulism in cattle have been evaluated (ACMSF, 2006). Outbreaks of avian botulism typically involve waterbirds or farmed birds (such as chickens), and can involve thousands or in extreme circumstances even millions of birds (e.g., Eklund and Dowell, 1987; Friend, 2002; Adams *et al.*, 2003; Trampel *et al.*, 2005; Ueng *et al.*, 2006; Neimanis *et al.*, 2007; Chou *et al.*, 2008).

6. CONCLUDING REMARKS

Whole genome sequences have recently become available for botulinum neurotoxin-forming clostridia, and have enabled DNA microarrays to be built and whole genome analyses to be carried out. The information derived from these studies and associated studies of bacterial physiology have already yielded a number of important findings on the biology of botulinum neurotoxin-forming clostridia. For example, an unsuspected result was that proteolytic *C. botulinum* is a highly chitinolytic bacterium, and that it is not an overt pathogen, but a saprophytic organism that uses its neurotoxin to create an energy source. Two basic types of neurotoxin gene clusters have been identified and characterized. It has also been demonstrated that differences in the response of spores of proteolytic

C. botulinum and non-proteolytic *C. botulinum* to nutrient germinants are reflected in the germinant receptor genes that they each possess. This is only the beginning. Genomes of several botulinum neurotoxin-forming clostridia have now been sequenced, and a detailed bioinformatic analysis of the similarities and differences will undoubtedly provide new and exciting insights into the biology of these bacteria. Whole genome transcriptomic studies are presently in progress in the author's laboratory and elsewhere and, in conjunction with studies of bacterial physiology, should make a considerable contribution to extending understanding of the biology of botulinum neurotoxin-forming clostridia. In particular the bioinformatic and transcriptomic analysis should generate further exciting testable hypotheses to enable dissection of key aspects of the biology (e.g., spore germination, lag phase, and neurotoxin formation and its regulation) of these highly dangerous bacteria.

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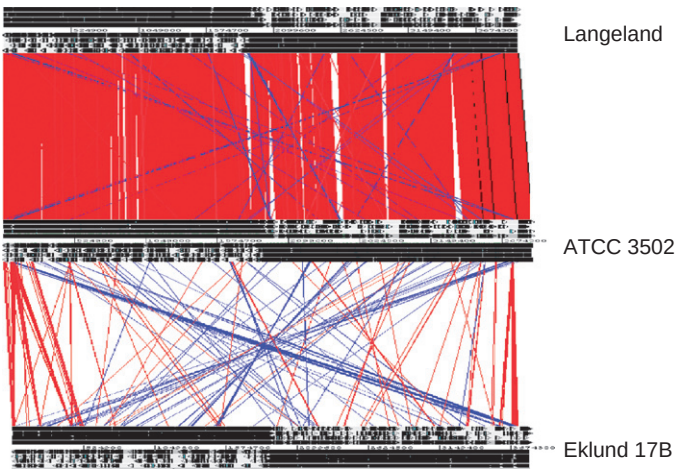

Plate 5 Artemis Comparison Tool (ACT) whole genome analysis of proteolytic *C. botulinum* type F strain Langeland and non-proteolytic *C. botulinum* type B strain Eklund 17B, each compared with proteolytic *C. botulinum* type A1 strain ATCC 3502 (Carter and Peck, unpublished data). Red lines connecting two genomes indicate both synteny and homology; blue lines indicate homology but breakdown in synteny. White gaps show lack of both parameters, that is, these regions are either diverged to the point where the BLAST analysis which forms the basis of this test gives a score which drops below a defined cut-off point, or the region is simply absent. The six possible reading frames (three forward, three reverse) of each genome are depicted as horizontal black bars, within which are shown potential coding sequences (CDSs) (open arrows, pointing in the direction of transcription). The two proteolytic *C. botulinum* strains (Langeland and ATCC 3502) share a high degree of both homology and synteny, whereas the non-proteolytic *C. botulinum* strain displays very little of either property. (For b/w version, see page 191 in the volume.)

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