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Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence

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Abstract

Two general strategies exist for the growth and survival of prokaryotes in environments of elevated osmolarity. The 'salt in cytoplasm' approach, which requires extensive structural modifications, is restricted mainly to members of the Halobacteriaceae. All other species have convergently evolved to cope with environments of elevated osmolarity by the accumulation of a restricted range of low molecular mass molecules, termed compatible solutes owing to their compatibility with cellular processes at high internal concentrations. Herein we review the molecular mechanisms governing the accumulation of these compounds, both in Gram-positive and Gram-negative bacteria, focusing specifically on the regulation of their transport/synthesis systems and the ability of these systems to sense and respond to changes in the osmolarity of the extracellular environment. Finally, we examine the current knowledge on the role of these osmostress responsive systems in contributing to the virulence potential of a number of pathogenic bacteria.

Keywords: Osmoadaptation; Osmosensing; Osmoregulation; Stress; Virulence

Contents

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1. Introduction

Bacterial species are perhaps the most versatile of all living organisms, inhabiting almost every environmental niche known to, and including, man. This successful occupancy of what are often hostile environments, uncongenial to other life forms, can be attributed at least in part to the development of complex stress management strategies, which have evolved to allow the bacterial cell to sense and respond to changes in its external environment. One such environmental parameter is the osmolarity of the extracellular medium. Bacterial cells are, in principle, required to maintain an intracellular osmotic pressure greater than that of the growth medium in order to generate cell turgor, generally considered to be the driving force for cell extension, growth and division [1,2]. The ability to adapt to changes in the osmolarity of the external environment is therefore of fundamental importance for growth and survival, and as such, prokaryotic cells have evolved a number of osmoadaptive strategies to cope with fluctuations in this important environmental parameter.

In light of the recent wealth of information concerning the physiological and genetic responses of bacteria to the osmolarity of the environment [3,4], the signals that regulate these responses [5,6] and the concepts underlying the perception of these osmoregulatory signals [7], we have undertaken to combine these specific and somewhat diverse aspects of the bacterial salt stress response into a single article. Our review begins with an outline of the principal strategies employed by prokaryotes to overcome salt stress, and continues with an in-depth analysis of the molecular mechanisms governing such responses. We then analyse the possible signals regulating these responses, and outline the current knowledge on bacterial osmotic signal transduction pathways. Finally we introduce a new aspect of the study of bacterial osmoadaptation, involving an analysis of the possible roles of some of the afore mentioned osmo stress responsive mechanisms in contributing to the virulence potential of a number of pathogenic bacteria.

2. Osmoadaptation

The term osmoadaptation describes both the physiological and genetic manifestations of adaptation to low and high water environments [8]. In principle, two strategies of osmoadaptation have evolved to cope with elevated osmolarity: (i) the salt in cytoplasm type and (ii) the organic osmolyte type [9]. Adaptation to hypo-osmotic shock, on the other hand, involves a combination of both specific (secondary transport) and non-specific (stretch-activated channel) solute efflux together with aquaporin-mediated water efflux.

2.1. Salt in cytoplasm; the halobacterial solution

This mechanism, which was discovered in and is typical of members of the Halobacteriaceae [9,10], achieves osmotic equilibrium by maintaining a cytoplasmic salt concentration (KCl) similar to that of the bathing solution. As a consequence, the cytoplasm is exposed to high ionic strength (up to 7 M KCl has been recorded in species of *Halobacterium* [11]) and as such requires extensive structural adaptations.

To achieve salt tolerance, halobacterial proteins have undergone extensive amino acid substitutions, involving enrichment in aspartyl, glutamyl and weakly hydrophobic residues [11]. The halophilic malate dehydrogenase (hMDH) from *Halobacterium marismortui*, for example, has an excess of 20 mol% acidic over basic residues as compared with only 6 mol% in its non-halophilic equivalent [12]. These modifications can be explained by the need to attract a hydration shell in a surrounding environment of low water activity. For example while native hMDH binds 0.8–1.0 g water and approximately 0.3 g salt g^{-1} protein the binding capacity of non-halophilic globular protein is much less (0.2–0.3 g water and approximately 0.01 g salt g^{-1} protein) [13,14]. Since the unusual hydration properties of the enzyme are absolutely dependent on its native structure, Zaccai et al. [14] proposed a model for the stabilisation of halophilic proteins in which the enzymes tertiary or quaternary structure is essential to coordinate hydrated salt at a local concentration higher than that in the solvent. The model proposed for hMDH (based on X-ray and neutron scattering studies) sees the protein with a core similar to that of its non-halophilic counterpart, but with loops (containing anionic amino acid residues) extending outwards, interacting with water and providing a large interface with the solvent [13]. The overall effect of salt in the cytoplasm therefore is structure stabilisation by means of tightening the folded conformation and strengthening hydrophobic interactions. Reducing the salt concentration (below 0.5 M NaCl) leads to a weakening of the enzyme conformation due to repulsive forces caused by the net negative charge on the enzymes surface, when the shielding cations (K^+) are removed (Fig. 1).

Organisms exhibiting the salt in cytoplasm mechanism

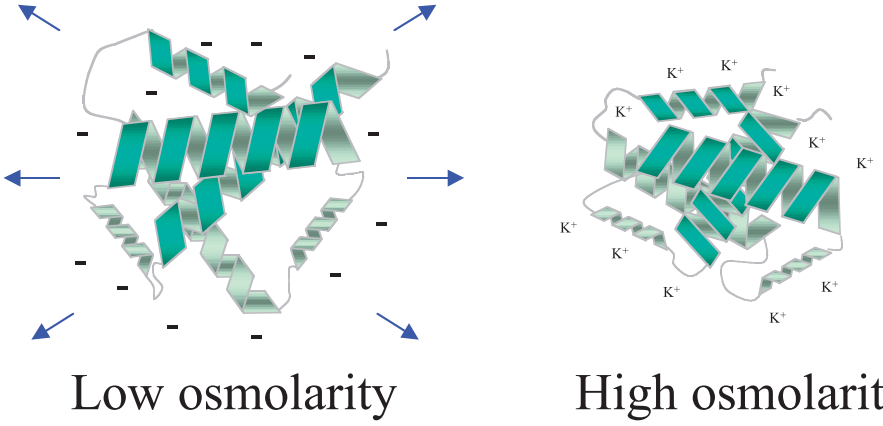

Fig. 1. Effects of low and high osmolarity on the structure and function of halophilic enzymes. Repulsive forces due to the net negative charge on the enzyme surface result in denaturation of the native protein structure at low osmolarity. At high osmolarity shielding cations (K^+) neutralise the negative charge, thus reducing the repulsive forces on the enzyme surface.

of osmoadaptation (the Halobacteriaceae as well as obligately halophilic eubacterial anaerobes such as *Halobacteroides acetoethylicus* [15]) are thus strictly confined to environments of elevated osmolarity. In contrast, all other organisms possess an adaptation strategy (involving organic osmolyte accumulation) that has as its hallmarks a minimal requirement for genetic change (so called 'genetic simplicity' [16]) and a high degree of flexibility in allowing organisms to adapt to significant fluctuations in external osmolarity.

2.2. Compatible solutes

Changes in the external osmolality trigger water fluxes along the osmotic gradient causing either swelling (and eventually cell lysis) in hypotonic environments or plasmolysis and dehydration under hypertonic environments. The compatible solute answer to the problem of elevated osmolarity involves a bi-phasic response in which increased levels of K^+ (and its counter-ion glutamate) have been observed as a primary response phenomenon [17], followed by a dramatic increase in the cytoplasmic concentration (either by synthesis and/or uptake) of osmoprotective compounds, representing the secondary response. Such compounds, given their compatibility with cellular functions at high internal concentrations, are often referred to as compatible solutes [18]. In general, compatible solutes are highly soluble molecules which carry no net charge at physiological pH [8] and do not interact with proteins; factors facilitating their accumulation to high intracellular concentrations (>1 mol kg^{-1} water [9]) without disrupting vital cellular processes such as DNA repair, DNA–protein interactions and the cellular metabolic machinery [19–22]. In addition to their role as osmotic balancers [18], compatible solutes function as effective stabilisers of enzyme function, providing protection against salinity, high temperature, freeze-thaw treatment and even drying [23,24].

A striking degree of convergent evolution of osmolyte systems has occurred in which each category of osmolyte is accumulated by species belonging to often only distinctly related phyla [16]. In general, the use of only a

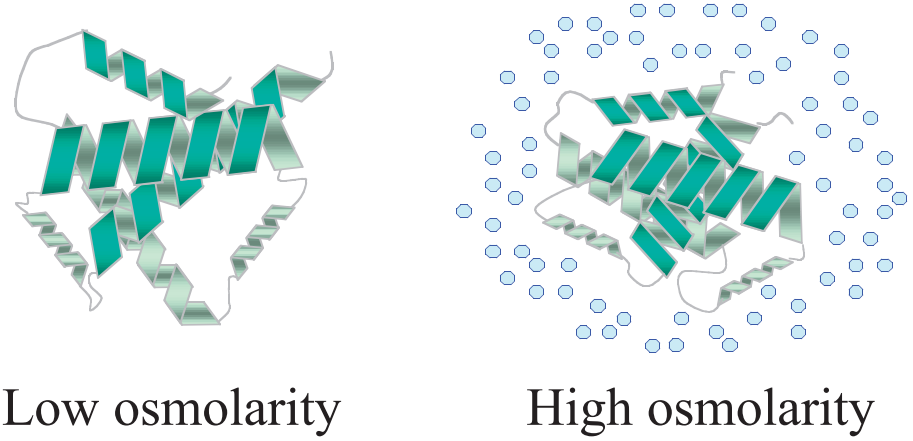

Fig. 2. Structure stabilisation of halotolerant enzymes at elevated osmolarity. Preferential exclusion of compatible solutes (blue circles) from the protein surface helps to maintain enzyme structure at elevated osmolarity, while also helping to increase cell volume.

small number of compounds as compatible solutes, not just in bacteria but also in higher forms of life, from amoeba to man [25], reflects fundamental constraints on the kinds of solutes that are compatible with macromolecular and cellular function [16]. Evolutionary pressures selecting for or against the accumulation of a specific compatible solute may depend not only on its osmotic function (as influenced by the degree of methylation [16] and hydrocarbon chain length [26]), but also secondary functions such as heat or cold tolerance [27].

2.2.1. Molecular principles of compatible solute function

Preferential exclusion from the immediate surface of proteins and other cytoplasmic macromolecules is the basis for the compatibility of nature's osmolytes [28] (Fig. 2). There are three possible explanations as to how the exclusion of these solutes from the protein–water interface occurs. A model proposed by Bull and Breese [29] suggests that compatible solutes may raise the surface tension of water, increasing the cohesive forces within the water structure thus making it energetically more difficult to disrupt water–water interactions in favour of protein–water complexes. Because solvation of the protein with the lower surface tension water is energetically more favourable, the bulk water will tend to hydrate the protein, expelling the high surface tension solute water from the protein surface. In addition to increased surface tension, steric incompatibility has been proposed to play an important role in osmolyte exclusion from macromolecular surfaces. In contrast to water, which (owing to its small size, polarity and hydrogen-bond potential) is capable of accommodating almost any protein surface geometry, most organic osmolytes are large, rigid molecules, which, although replete in hydrogen-bonding groups, are preferentially excluded from the protein surface in favour of the more accessible water molecules. The third and perhaps most trivial explanation for preferential exclusion centres on the existence of possible repulsive forces between solutes and certain protein surface groups [30]. Irrespective of the mechanism of solute exclusion, the thermodynamic consequence is the same: a general stabilising effect opposing the unfolding/denaturation of proteins and other labile macromolecular structures [31–33]. This stabilising effect extends not only to salt tolerance but also to a range of stress factors such as heating, freezing and drying [24].

In addition to the solute protection theory, Cayley et al. [34] have proposed that it is the free cytoplasmic volume (unbound water) which is the fundamental determinant of growth under hyper-osmotic stress, and that the secondary effect of volume increase by compatible solute accumulation (a consequence of preferential exclusion from cytoplasmic macromolecules and membrane components) is the key to their osmoprotective function. Thus, compatible solutes may serve a dual role in osmoregulating cells, restoring cell volume and stabilising protein structure.

3. The halotolerant response

3.1. Hyper-osmotic shock; solute accumulation

3.1.1. Initial phase of osmoadaptation; the primary response

The most rapid response to osmotic up-shock, both in Gram-positive and Gram-negative bacteria, is a stimulation of potassium (K^+) uptake [17,35]. However not all of the accumulated K^+ is osmotically active, a certain proportion being required to balance the net negative charge of the cytoplasmic macromolecules [36]. Only that fraction of the total K^+ concentration that is balanced by other small counter-ions contributes significantly to osmotic activity. The primary charge counterbalance for the rapid K^+ influx in *Escherichia coli* is endogenously synthesised glutamate [37], which accumulates at a much slower rate. On the other hand, the nature of the counter-ion in *Bacillus subtilis* is unclear since, in contrast to *E. coli*, glutamate levels increase only slightly after osmotic up-shock [4]. Under non-stressed conditions, Gram-positive bacteria already possess a large amino acid pool, of which a significant proportion is glutamate; similarly, the cellular concentrations of K^+ in non-stressed Gram-positive bacteria are usually much higher than their Gram-negative counterparts [36–41], a fact which is reflected in their higher turgor pressure (20 bar for Gram-positive bacteria as opposed to 3–10 bar for Gram-negative bacteria [42,43]). Given the observed differences in cytoplasmic osmolality and elevated electrolyte concentrations, it appears that under hyper-osmotic conditions, Gram-positive bacteria in particular benefit more from the accumulation of osmoprotective compounds such as glycine betaine, rather than the electrolyte pair K^+-glutamate. The primary function of K^+ accumulation in Gram-positive bacteria thus may be to signal induction of the secondary response [44]. This role of K^+ as a second messenger is inferred from the observed dependence of a number of osmotic responses on K^+ uptake (Section 5.2).

3.1.1.1. K^+ uptake. Molecular characterisation of K^+ uptake is most advanced for Gram-negative bacteria. *E. coli* possesses four constitutive low affinity K^+ transport systems: TrkG, TrkH, Kup (formerly TrkD) and TrkF, as well as an inducible high affinity system, Kdp.

3.1.1.1.1. Kdp. The Kdp system is highly specific for K^+, exhibiting a K_m of 2 μM and a V_{max} of 150 μmol min^{-1} g^{-1} cells [45,46]. A member of the P-type ATPases, the driving force for K^+ uptake via Kdp,comes from ATP hydrolysis [46]. The membrane-ssociated Kdp-ATPase (KdpFABC) is encoded by the *kdpFABCDE* operon, which includes the two-component regulatory system KdpDE. Located at the promoter-distal end of the operon the *kdpDE* genes (encoding the sensor kinase KdpD, and soluble transcriptional activator KdpE) are expressed as an operon from a promoter located within *kdpC*, however

read-through from the upstream *kdp* promoter has also been observed [47,48]. Kdp thus serves as an osmotically inducible system scavenging K^+ when the ion is present at low concentrations. Although instrumental in protecting the cell from the detrimental effects of elevated osmolarity, the primary function of the Kdp system is the maintenance of K^+ homeostasis particularly at limiting K^+ concentrations.

3.1.1.1.2. Trk. In media containing >1 mM K^+, the predominant uptake system is Trk. Encoded by constitutively expressed genes dispersed on the chromosome [49], K^+ uptake, previously attributed to TrkA, is now known to be mediated by two integral membrane-bound proteins, TrkG and TrkH [50]. While both membrane potential and ATP are required for K^+ uptake, ATP is thought to regulate, rather than drive, K^+ uptake via the Trk system. Existing as both cytoplasmic and membrane-associated forms [51], TrkA is believed to regulate TrkG/H, mediating activation by ATP, or by acting as a protein kinase [49,51]. In addition to TrkA, *trkE* represents a further regulatory domain which, when disrupted, eliminates and impairs K^+ transport via TrkH and TrkG, respectively. K_m and V_{max} values for K^+ uptake via TrkG and TrkH are 0.3–1 mM and 2.2–3.0 mM and >200 nmol min^{-1} (mg protein)$^{-1}$ and >300 nmol min^{-1} (mg protein)$^{-1}$, respectively [50,51]. As with Kdp, in addition to contributing to the salt stress response, Trk also plays a role in K^+ homeostasis.

3.1.1.1.3. Kup. The Kup system (also known as TrkD) represents a low affinity K^+ uptake system. Distinguished from the other systems by its ability to transport caesium [52] Kup exhibits a K_m of 0.3–0.4 mM and a V_{max} of 30 nmol min^{-1} (mg protein)$^{-1}$ for K^+ uptake [50,51]. The final and perhaps least studied K^+ transport system in *E. coli* is TrkF; as yet no gene has been linked to this system, which exhibits K_m and V_{max} values for K^+ uptake of 20–30 mM and <15 nmol min^{-1} (mg protein)$^{-1}$, respectively. It is unlikely that K^+ uptake via either Kup or TrkF plays any significant role in the osmoadaptation of *E. coli* [45,53].

While considerably less information is available concerning the mechanisms governing K^+ accumulation in Gram-positive bacteria, two transport systems, KtrI and KtrII, have been reported to exist in the Gram-positive lactic acid bacterium *Enterococcus hirae* [54]. KtrI, which is most likely constitutive, recognises K^+ as well as Rb^+ with an apparent K_m of 0.2 mM and requires both ATP (or an equivalent high-energy compound) and generation of proton potential. KtrII on the other hand appears to be independent of proton potential and selectively recognises K^+ with a K_m of 0.5 mM. Although largely uncharacterised at the molecular level recent evidence suggests that the *ntpJ* gene, a cistron located at the tail end of the vacuolar type Na^+-ATPase (*ntp*) operon of *E. hirae*, encodes a portion of the KtrII K^+ transport system [55]. NtpJ represents the membranous component of KtrII and resembles various K^+ transporters including KtrB of *Vibrio alginolyticus* [56], Trk1p and Trk2p in *Saccharomyces cerevisiae* and the TrkG and TrkH subunits of the *E. coli* Trk system [57]. K^+ uptake has been studied to a lesser extent in the acidophilic, moderate thermophile *Alicyclobacillus acidocaldarius* (formerly *Bacillus acidocaldarius*) [58–60]. Two transport systems have been identified in this strain; a high affinity system exhibiting immunological cross-reactivity with the KdpB subunit of *E. coli*, and a low affinity system displaying kinetic and substrate specificities similar to the *E. coli* TrkG/H systems [60].

In conclusion then, osmotically induced accumulation of K^+, representing the primary or initial phase of osmoadaptation, is mediated by rapid activation of low and high affinity systems in both Gram-positive and Gram-negative bacteria.

3.1.2. Secondary response; osmoprotectant accumulation

Given an upper limit of $\sim$400 mM for K^+-glutamate accumulation [61,62], the cut-off point for the primary response, at least in Gram-negative bacteria, appears set at $\sim$0.5 M NaCl [8] (though this value may vary considerably). Increases in the salt concentration above this level triggers the secondary response; i.e. accumulation of neutral osmoprotectants which, in contrast to the ionic osmolytes of the primary response, can be accumulated to high intracellular concentrations without adversely affecting cellular processes [16,18]. While the list of compatible solutes available to both prokaryotes and eukaryotes is extensive and varied [4], three compounds namely glycine betaine, carnitine and proline (listed in decreasing order of importance, in terms of the level of osmoprotection afforded to the utilising cells) have emerged as the principal compatible solutes in bacterial osmoadaptation [63]. Herein the molecular mechanisms governing the synthesis and transport of these compounds are reviewed, using *E. coli* and *B. subtilis* as models of Gram-negative and Gram-positive bacteria, respectively (Fig. 3).

3.1.2.1. Glycine betaine. The preferred compatible solute for the majority of prokaryotes and perhaps the most widely utilised osmolyte, spanning both the plant and animal kingdoms, is the trimethylammonium compound glycine betaine (*N*,*N*,*N*-trimethyl glycine). The earliest reports of bacterial osmoprotection by glycine betaine were for *Tetragenococcus halophilus* (formerly *Pedicoccus soyae*) by Sakaguchi in 1960 [64], however it was not until the early 1980s that the full potential of betaine as an effective and versatile compatible solute, both in prokaryotic and eukaryotic systems, was realised [9,65,66].

3.1.2.1.1. Glycine betaine synthesis. Despite confusion in the literature arising from the indiscriminate use of the term 'betaine synthesis' to describe situations in which precursor molecules such as choline or carnitine are enzymatically converted to betaine [67], de novo betaine synthesis is rare, being confined largely to oxygenic and an-

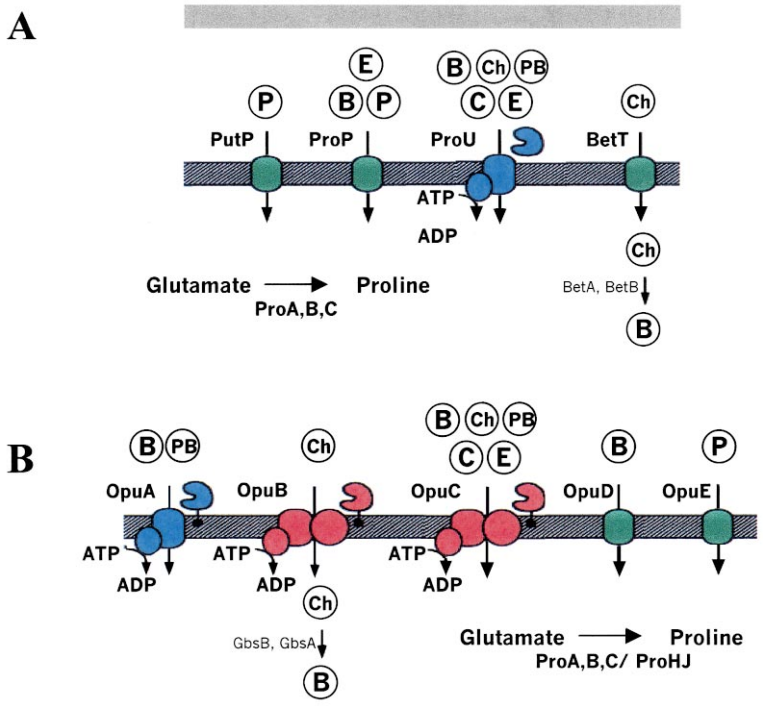

Fig. 3. Compatible solute transport/synthesis systems in (A) *E. coli* and (B) *B. subtilis*; models for Gram-negative and Gram-positive bacteria, respectively. B glycine betaine, C carnitine, Ch choline, E ectoine, P proline, PB proline betaine adapted from [148].

oxygenic phototrophic eubacteria, particularly those displaying salt tolerance [68–70].

Although incapable of de novo glycine betaine synthesis, *E. coli* can convert choline to betaine in a two-step enzymatic reaction. Choline, transported into the cell via the high and low affinity systems, BetT and ProU, respectively [71,72], is first oxidised to glycine betaine aldehyde by the enzyme choline dehydrogenase (BetA). A second oxidation step catalysed by glycine betaine aldehyde dehydrogenase (BetB) then converts glycine betaine aldehyde to glycine betaine [67]. The genes, *betA*, *betB* and *betI* (which encodes the choline-sensing repressor protein, BetI), are arranged in an operon (*betIBA*), located downstream of *betT* on the chromosome. Both gene systems are transcribed divergently under the control of separate though partially overlapping promoters [71]. Expression of *betA*, *betB* and *betT* is subject to osmotic induction. Addition of choline (in the absence of betaine) during osmotic stress results in a further induction of *betT* and *betA* by reducing BetI-mediated repression at the promoter region [73]. Under anaerobic conditions expression of both promoters is reduced by ArcA, the regulator protein of the ArcA–ArcB two-component regulatory system, controlling the activity of *E. coli* genes repressed under anaerobic conditions [74,75].

Genetic and physiological analysis of the osmoregulatory choline–glycine betaine pathway in *B. subtilis* reveals that, as with *E. coli*, glycine betaine production involves a two-step oxidation process with glycine betaine aldehyde as the intermediate [76,77]. Two enzymes act in concert for glycine betaine synthesis: a type III alcohol dehydrogenase (GbsB) that oxidises choline (transported into the cell by the OpuB and OpuC transporters; [78]) to glycine betaine aldehyde, and a glycine betaine aldehyde dehydrogenase (GbsA), which converts this intermediate to glycine betaine. The structural genes (*gbsAB*) for these enzymes are genetically organised in an operon, expression of which is enhanced by the presence of choline (but not salt) in the growth medium [76,79].

Although previously believed to be incapable of synthesising glycine betaine [27] recent studies demonstrating the existence of a choline transport system [80], coupled with the findings of Phan-Thanh and Mahouin [81] that *Listeria* harbours an alcohol dehydrogenase, exhibiting significant sequence homologies to GbsB in *B. subtilis*, prove that *Listeria*, at least in theory, has the necessary machinery to synthesise betaine from precursor molecules such as choline and/or glycine betaine aldehyde.

3.1.2.1.2. Glycine betaine transport. In addition to endogenous synthesis, bacteria have evolved sophisticated mechanisms for the uptake and accumulation of osmolytes released into the external environment either by primary microbial producers upon dilution stress, by decaying plant and animals, or by mammals in the form of excretion fluids (e.g. urine) [9,82]. Given that osmolyte uptake is often energetically more favourable than synthesis, accumulation of compatible solutes from exogenous sources generally inhibits endogenous synthesis, at least over a certain range of osmolarities [35,61]. In the presence of external glycine betaine, for example, both the *E. coli*

Bet and *B. subtilis* Gbs systems are inhibited [74,77], thus promoting glycine betaine uptake rather than synthesis. Two osmoregulated permeases, ProP and ProU, mediate uptake of most osmoprotectants in *E. coli* and *Salmonella typhimurium*. First recognised as proline transporters [83–87], the ProP and ProU systems were subsequently found to transport betaine and other osmoprotectants [88–93].

ProP. The ProP system transports betaine, proline and ectoine with similar affinities [87,92]. Possessing 12 transmembrane domains, a structural feature common in secondary transport systems [94], it is characterised additionally by the presence of an extended central hydrophilic loop and a carboxy-terminal extension predicted to form an α-helical coiled-coil [95]. Recently Culham et al. [96] demonstrated that this C-terminal extension plays an important role in the osmotic activation of ProP. A similar domain in the betaine transporter BetP of *Corynebacterium glutamicum* has also been linked to the osmosensing and osmoregulatory mechanisms of betaine uptake in this organism [97,98] (Section 5.2.3). Transcription of *proP* is directed from two promoters, P-1 and P-2, both of which are activated by osmotic up-shifts. While the cAMP–CRP complex normally represses *proP* P-1, the activity of *proP* P-2 appears dependent on both RpoS and the nucleoid-associated protein Fis [99,100]. Transport via ProP (which exhibits K_m and V_{max} values for betaine uptake of 44 μM and 37 nmol min^{-1} (mg protein)$^{-1}$, respectively) is enhanced by a combination of transcriptional induction (two to five-fold) and a five-fold stimulation of the activity of the ProP protein [85,89,101] in response to osmotic upshock.

ProU. The multi-component binding-protein-dependent transport system ProU belongs to a superfamily of prokaryotic and eukaryotic ATP-binding cassette transporters or traffic ATPases [102,103]. The components of the ProU system are encoded by an operon containing three cistrons: *proV*, *proW* and *proX*, encoding two cytoplasmic membrane-bound proteins, ProV and ProW, and the periplasmic binding protein ProX [88,104–107]. Two promoters upstream of *proU* have been identified in *E. coli*; an osmoregulated promoter recognised by the RpoD-RNA polymerase holoenzyme, situated downstream of a weak RpoS-dependent promoter [108,109]. In addition, evidence exists for the presence of a transcriptional activator site ~200 bp upstream of the RpoD-dependent promoter [110,111] together with a negative regulatory sequence within *proV* [108]. Both of these AT-rich regulatory regions are easily distorted, thus facilitating attachment of H-NS, a DNA-binding protein exhibiting a relatively high affinity for bent DNA [112]. As with ProP, maximal betaine uptake at elevated osmolarities is achieved by a combination of transcriptional induction (>100-fold) and stimulation of transporter activity [89,90,113]. However, unlike the ProP system, ProU transports glycine betaine with a much higher affinity than proline and exhibits K_m and V_{max} values for betaine of 1.3 μM and 12 nmol min^{-1} (mg protein)$^{-1}$, respectively.

As with their Gram-negative counterparts, both multicomponent ATP-dependent transporters and single-component ion-dependent secondary systems mediate glycine betaine uptake in Gram-positive bacteria. *B. subtilis* has three known betaine transporters [114] two of which, OpuA (osmoprotectant uptake) and OpuC (ProU), have been identified as members of the ATP-driven binding protein-dependent transporter family [115,116]. OpuA, closely related to BusA (the betaine uptake system of *Lactococcus lactis* [117–119]), comprises three components: OpuAA, an ATPase; OpuAB, an integral cytoplasmic membrane protein; and OpuAC, an extracellular substrate-binding protein [115]. Induced by high osmolarity growth conditions, transcription of *opuA*, like that of *proU*, is controlled by two separately regulated promoters, the osmoregulated *opuA* P-1, and *opuA* P-2, which does not respond to the osmotic stimulus. Both promoters show homology to the consensus sequence of σ^A-dependent promoters [120], and are thus likely transcribed by an RNA polymerase complex containing the main vegetative sigma factor (σ^A). With a K_m and V_{max} for betaine uptake of 2.4 μM and 282 nmol min^{-1} (mg protein)$^{-1}$, respectively, OpuA, like ProU in *E. coli*, represents the glycine betaine transporter of highest affinity in *B. subtilis* [115]. The OpuC system (exhibiting a K_m of 6 μM and a V_{max} of 65 nmol min^{-1} (mg protein)$^{-1}$ for betaine) is related to OpuA but contains an additional integral membrane component (OpuCD). The broad substrate specificity of OpuC (ectoine, crotonobetaine, γ-butyrobetaine, carnitine, choline-*O*-sulphate, choline, proline betaine and glycine betaine [121,122]) resembles that of EctP, the 'emergency system' accepting all known compatible solutes in *C. glutamicum* [97]. OpuD, the third betaine uptake system, is a single-component transporter exhibiting significant homologies to the betaine transporters BetP of *C. glutamicum* and BetL of *Listeria monocytogenes*, as well as the *E. coli* choline and carnitine transport systems, BetT and CaiT, respectively [71,123–125]. High osmolarity stimulates de novo synthesis of OpuD and activates pre-existing OpuD proteins to achieve maximal betaine uptake activity [114]. The K_m and V_{max} values for betaine uptake via OpuD were calculated as 13 μM and 61 nmol min^{-1} (mg protein)$^{-1}$, respectively.

While physiological investigations of osmolyte uptake in *Listeria* implicated a single highly specific, constitutive, energy-dependent, secondary transport system [126,127], genetic analysis has led to the identification of three independent betaine uptake systems. The first of these, BetL, homologous to OpuD in *B. subtilis*, is a highly specific secondary transporter with a K_m and V_{max} for glycine betaine uptake of 7.9 μM and 134 nmol min^{-1} (mg protein)$^{-1}$, respectively. As with OpuD, BetL is osmotically induced both at the level of transcription [128] and transporter activity [127]. The remaining systems, OpuC

(which also transports carnitine [80]) and GbuABC, are members of the traffic ATPases and as such resemble the multi-component transporters OpuA and OpuC in *B. subtilis*. An interesting feature of the listerial *betL* and *opuC* determinants is the presence of a consensus σ^B-dependent promoter-binding site upstream of the structural genes [80,125,128]. Given that σ^B-minus mutants of *Listeria* (in contrast to *Bacillus*) are significantly affected in their ability to accumulate glycine betaine and carnitine, both at elevated osmolarity and reduced temperatures [129,130], it is tempting to speculate that the observed phenotype is the consequence of reduced uptake via the σ^B-regulated BetL and OpuC transporters.

3.1.2.2. Carnitine. Playing a role in long chain fatty acid transport across the inner mitochondrial membrane of animal cells [131], the trimethyl amino acid carnitine (β-hydroxy-γ-*N*-trimethyl aminobutyrate) is widely distributed in nature, occurring predominantly in foods of animal origin (present in muscle tissue at concentrations of 0.05–0.2% on a fresh weight basis; [63]). For the majority of bacteria carnitine is transported from the external environment rather than being synthesised endogenously. The first reports of osmoprotection by carnitine were by Kets et al. [132], following nuclear magnetic resonance spectroscopy of cell extracts from *Lactobacillus plantarum* grown in medium containing added NaCl, and by Beumer et al. [63], who reported stimulation of *L. monocytogenes* by carnitine at elevated osmolarities. Later Verheul et al. [133] demonstrated that carnitine uptake in *E. coli* via ProP (K_m of 200–250 μM, V_{max} of 1.2 nmol min^{-1} (mg protein)$^{-1}$) and ProU (K_m of 200–250 μM and V_{max} of 1.9 nmol min^{-1} (mg protein)$^{-1}$) is osmotically significant while the CaiT system, implicated in anaerobic catabolism, has no known relationship to osmoadaptation [123,134]. While the OpuC system appears to function as the sole carnitine transporter in *B. subtilis* (K_m of 5.1 μM, V_{max} of 41 nmol min^{-1} (mg protein)$^{-1}$) [122] *opuC* mutants of *Listeria* are still capable of accumulating carnitine, albeit at a reduced rate. Thus, it would appear that unlike the situation in *B. subtilis*, carnitine uptake in *Listeria* might well be mediated by more than one system [135].

3.1.2.3. Proline. First reported as an osmoprotectant in *Salmonella oranienburg* by Christian in 1955 [136,137], proline has since been shown to accumulate to high intracellular concentrations in a variety of bacteria, following exposure to osmotic stress [40]. While many species of Gram-positive bacteria have been shown to increase their internal proline pool size by increased synthesis [34,43,62], Gram-negative bacteria, in general, achieve high intracellular concentrations of proline during osmotic stress as a consequence of enhanced transport [84,138–140].

3.1.2.3.1. Proline synthesis. For the majority of bacteria, proline is synthesised from glutamate via three enzymatic reactions catalysed by γ-glutamyl kinase (GK; *proB* product), γ-glutamyl phosphate reductase (*proA* product) and Δ^1-pyrroline-5-carboxylate reductase (*proC* product). In general, the *proB* and *proA* genes constitute an operon which is distant from *proC* on the chromosome. Regulated primarily through feedback inhibition of GK by proline [141], mutations in the *proB* gene have previously been linked to proline hyper-production (a consequence of reduced proline-mediated feedback inhibition of GK), leading to enhanced osmotic stress tolerance in *E. coli* and other bacteria [142–146]. In addition to *proBA*, sequence analysis of the *B. subtilis* chromosome [147] has recently uncovered an additional proline biosynthesis pathway: *proHJ*, which is apparently responsible for the high-level accumulation of proline under hyper-osmotic growth conditions [148].

Recent work in our laboratory has led to the identification and disruption of the listerial *proBA* homologue, which has been linked to the salt tolerance of *L. monocytogenes* [149]. Interestingly, while mutations in the listerial *proB* gene leading to proline overproduction had no obvious effects on listerial osmotolerance, heterologous expression of the mutated operon in an *E. coli proBA*$^-$ background resulted in a significant increase in the growth rate at elevated osmolarity [150]. In addition, the observed lack of growth of a listerial *proBA* mutant in proline deficient minimal medium (either at normal or elevated osmolarity) indicates that unlike *B. subtilis*, *Listeria* possesses only a single proline biosynthesis pathway.

3.1.2.3.2. Proline transport. The Gram-negative bacteria *E. coli* and *S. typhimurium* possess three proline transport systems: PutP, ProP and ProU [87]. The PutP system serves to transport proline solely for use as a carbon or nitrogen source [151], and as such plays little if any role in osmoadaptation [91,152]. The osmotically induced systems: ProP and ProU (described earlier in relation to glycine betaine uptake) are, on the other hand, highly responsive to osmotic up-shock. Measurement of growth in high osmolarity medium by mutants deficient in either ProP or ProU revealed that ProP is the major contributor to osmoprotection by proline [153].

Among the Gram-positive bacteria, osmoprotection by exogenous proline uptake has been most extensively studied in *Staphylococcus aureus* [154–157], *L. lactis* [117,158] and *B. subtilis* [159]. Proline uptake in *S. aureus* appears to be mediated by high and low affinity systems. The high affinity system, PutP, is highly specific for proline and, given its significant homologies with PutP in *E. coli*, appears to function independently of osmotic stimulation for the uptake of proline as a carbon, nitrogen or energy source. The low affinity system on the other hand is extremely responsive to osmotic up-shock and is capable of transporting both proline and glycine betaine. Proline uptake in both *L. lactis* and *L. plantarum* resembles that of *S. aureus*, in that the only osmotically significant proline transporter also functions as the major betaine uptake

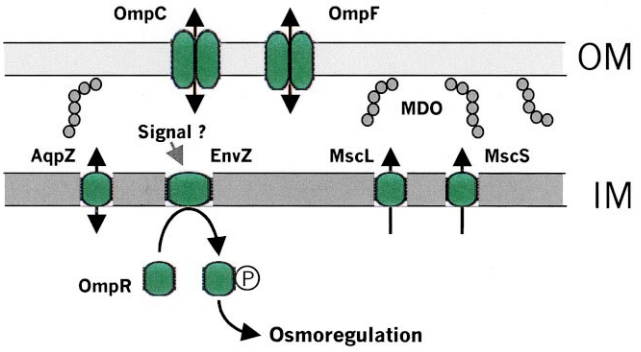

Fig. 4. Osmotic responses not involving compatible solute accumulation. Refer to text for details.

system in these strains (BusA (OpuA) in *L. lactis* [117] and QacT in *L. plantarum* [38]).

The situation in *B. subtilis* differs markedly from that in other Gram-positive bacteria studied to date, in that osmotically stimulated proline uptake in this strain is mediated by the high affinity, substrate-specific OpuE. While closely related to the proline-inducible PutP permeases, which have no apparent role in the osmostress response, expression of *opuE* is strongly induced by the osmolarity of the external environment, but not by proline [159,160]. Transcribed from two closely spaced, osmoregulated promoters: *opuE* P-1, which is recognised by the vegetative σ^A, and *opuE* P-2, which is dependent on the stress-induced σ^B [159,160], *opuE* was the first member of the σ^B regulon with a clearly defined physiological function in the *B. subtilis* osmostress response. However, σ^B is dispensable for the induction of the OpuE system under high osmolarity growth conditions, indicating that the activity of the σ^A-dependent *opuE* P-1 promoter is sufficient for overall osmotic control of *opuE*.

3.2. Osmotic responses not involving compatible solute accumulation (Fig. 4)

3.2.1. Outer membrane porins: OmpC and OmpF

The most extensively studied osmoregulated genes which do not directly contribute to compatible solute accumulation are *ompC* and *ompF*, encoding two structurally related Gram-negative outer membrane channel proteins OmpC and OmpF. Expression of these porins, which facilitate the non-specific diffusion of small ($\leq$500 Da) hydrophilic molecules across the outermost permeability barrier of the cell [161], responds in a reciprocal fashion to the external osmolarity (expression of *ompF* being depressed while that of *ompC* is enhanced at elevated osmolarity) [1]. Not restricted to salt stress, the levels of OmpC and OmpF appear to respond to a variety of environmental parameters including temperature, carbon source and oxygen availability as well as the pH of the medium [5].

3.2.2. Membrane adjustment

While the cytoplasmic interior of a bacterium employing compatible solutes may be protected from the damaging effects of the external salt, the outer surface of the cytoplasmic membrane (as well as the periplasmic space and outer membrane in Gram-negative bacteria) is permanently exposed and thus must undergo a number of adaptive changes. Perhaps the most obvious adaptation strategy involves an increase in the proportion of anionic over zwitterionic phospholipids. This structural modification adds additional surface charge to the membrane and as such parallels the mode of adaptation described for 'halophilic enzymes' (Section 2.1). Excess negative charge probably helps to maintain hydration of the interface, and has a pronounced effect on lipid phase behaviour [162,163].

3.2.3. Membrane-derived oligosaccharides (MDOs)

The periplasmic space of Gram-negative bacteria contains highly anionic polysaccharides, which in *E. coli* are referred to as MDOs [164]. Encoded by constitutively expressed genes, *mdoA* and *mdoB*, these anionic polymers (containing between six and 12 glucose units with an average charge of -5 [165]) generate a Donnan potential across the outer membrane, resulting in the accumulation of cations to a higher concentration in the periplasm than in the medium, consequently giving rise to hydrostatic pressure in the periplasmic space [165]. Unlike intracellular compatible solutes the levels of these oligosaccharides decrease with increasing osmolarity [166]. Interestingly, while MDOs appear to play an important role in periplasmic osmoregulation of Gram-negative bacteria, blocking MDO synthesis fails to inhibit growth of *E. coli* in media of high or low osmolarity [165,167].

3.2.4. Non-accumulated osmoprotectants

Identified by Gouffi and Blanco [168] as a new class of sinorhizobial osmoprotectants, these non-accumulated disaccharides include: sucrose, maltose, cellobiose, gentibiose, turanose and palatinose. Structurally, these disaccharidic osmoprotectants contain either two-glucosyl residues or a glucosyl residue linked to a fructosyl residue [169]. Unlike other bacterial osmoprotectants (e.g. betaine, carnitine or proline) these disaccharides do not accumulate as cytosolic osmolytes (or immediate osmolyte precursors) in salt-stressed *Sinorhizobium meliloti*. Instead they are catabolised during early exponential growth, contributing indirectly to enhance the levels of two endogenously synthesised osmolytes, glutamate (two-fold increase) and *N*-acetylglutaminylglutamine amide (six-fold), facilitating growth at elevated osmolarities.

3.3. Hypo-osmotic shock; solute and water efflux

Bacteria in their natural habitat are just as likely to encounter hypo-osmotic or dilution stress, as they are hyper-osmotic shock. Rapid increases in the water activity of the external environment (often a consequence of rainfall,

flooding etc.) leads to a massive influx of water into the cell, requiring the bacteria to react quickly to avoid cell lysis. As with salt stress, bacteria have evolved a number of mechanisms to counter the potentially detrimental effects of hypo-osmotic shock; essentially rapid increases in water activity are countered by both solute and water efflux.

3.3.1. Solute efflux

Ubiquitous amongst bacterial cells mechanosensitive or stretch-activated channels are the major routes for the release of cytoplasmic solutes to achieve a rapid reduction of turgor pressure during the transition from media of high to low osmolarity [170–174]. *E. coli* possesses between three and five stretch-activated channels, the best characterised of which are MscL and MscS [175,176]. In addition to stretch-activated channels, specific carrier-like systems (Section 5.4.1) appear to contribute to solute discharge, since the initial rapid efflux via stretch-activated channels is followed in some microbes by a slower process with different kinetic and metabolic parameters [21,38].

3.3.2. Water efflux

Recent evidence suggests that bacteria, like higher plants and animals, possess aquaporins (e.g. AqpZ; [177,178]), specific water channels that facilitate the rapid influx/efflux of water thus alleviating water stress without dissipating the transmembrane potential [179]. Expressed in diverse species [180,181], aquaporins have been shown to play essential roles in maintenance of turgor and transpiration in plants [182] as well as volume regulation and organismal fluid retention in animal cells [183].

4. Osmosensing

While much information is available concerning the genetic and physiological responses of bacteria to environmental osmolarity (as outlined in the previous section) considerably less is known about the signals regulating these responses. While regulation of most biological responses depends on the recognition of signal molecules by specific receptors, osmoregulation differs in that the information from the environment is not a specific molecule but a physiological parameter [184]. This section reviews the possible parameters, which (being subject to change in osmotically stressed cells) may be used as signals to trigger osmoregulatory responses (Fig. 5).

4.1. Possible osmosensing mechanisms

4.1.1. Internal hydrostatic pressure

Within the bulk liquid of the cell interior, changes in hydrostatic pressure are isotropic (uniform in all directions). While the observed pressure changes are low

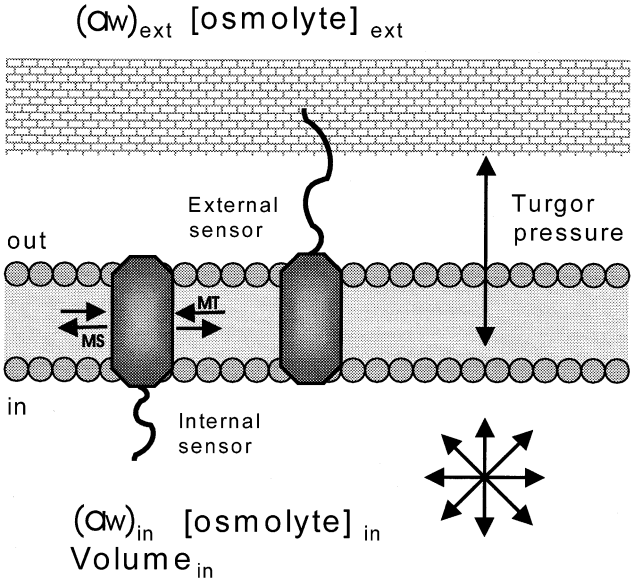

Fig. 5. Physiochemical parameters that may reflect the activity of osmoregulated transport systems. Two transport systems, one with an external and one with an internal osmosensing domain, are depicted schematically. The cell envelope represents that of a Gram-positive bacterium, i.e. the cytoplasmic membrane and peptidoglycan layer are shown. MS and MT refer to membrane stretch and tension while a_W (the water activity) refers to the mol fraction of water in solution. Adapted from [6].

(≤0.5 mPa [5]) they may bring about measurable changes in protein–protein and protein–ligand interactions [185].

4.1.2. Membrane pressure differential

Turgor pressure (the hydrostatic pressure difference which balances the osmotic pressure difference between the cell interior and exterior), acting normal to the wall, and membrane strain (MS/MT which occurs in response to the change in turgor pressure and affects the expansion/compression of the bilayer in the phase of the membrane) may be detected by pressure sensors located in the inner membrane.

4.1.3. Internal osmolarity

Once turgor is lost the cytoplasmic compartment behaves as an osmosensor [5]. In principle, either of three parameters: (i) cytoplasmic volume, (ii) accompanying changes in the concentration of one or more solutes (e.g. K^+ [17,44]), or (iii) the internal a_W, could serve as osmoregulatory signals.

4.1.4. External osmolarity or a_W

Possibly sensed by transmembrane proteins with outward-facing binding sites.

4.1.5. Cytoplasmic membrane area

As with the cytoplasmic volume, the cytoplasmic membrane area is responsive to changes in medium osmolarity. Such changes (around 7% in an elastic cell for a rise in

medium osmolarity of 100 mosmol kg^{-1} [5]) may be detected by a stretch-activated strain, e.g. MscL [7].

While not all acting in the same time scale [6], most of the above mentioned physiochemical parameters are not mutually exclusive but are instead interrelated. The observed flexibility of the cell wall peptidoglycan [186], for example, allows changes in turgor pressure to be accompanied by immediate changes in cytoplasmic volume, concentration of internal solutes and membrane area [5], thus allowing the cell to monitor three or more signals simultaneously.

While these signals are essential to trigger the activation of osmoregulated transport/synthesis systems, they will not solely determine the fluxes of compatible solutes across the membrane. The ultimate activity of an osmoregulated system, after initial activation, will depend on the state of the cell with respect to (i) the internal osmotic pressure or related parameter at the time of the shift (as described above); (ii) the internal concentration of the compatible solute, which may inhibit through 'feedback' or '*trans*'-inhibition; and/or (iii) physiological parameters such as the energy status and the internal pH of the cell [6]. Osmoregulatory mechanisms are thus inextricably linked to other cellular processes [5].

5. Osmoregulation

To date, extensive analysis of the signal transduction pathways originating from osmotic challenge and leading ultimately to immediate (activity) and long term (expression) modulation of the primary and secondary responses has been restricted to a handful of organisms; namely *E. coli* (Kdp, Trk, ProP, ProU and EnvZ/OmpR), *C. glutamicum* (BetP), *L. lactis* (BusA (OpuA)) and *L. monocytogenes* (BetL).

5.1. The primary response

5.1.1. Kdp

Osmotic regulation of the Kdp system occurs both at the level of transcription and enzyme activity [187]; however, more is known about the transcriptional regulation of the *kdp* genes. Induction of the *kdp* operon (mediated by a sensor kinase (KdpD)/response regulator (KdpE) system [47,48]) can be triggered by moderate osmotic pressure increases ($\geq$0.2 mPa [5]) elicited only by ionic and nonpolar solutes which are excluded from the membrane. While the latter observation rules out sensing by intra- or extracellular a_W, other parameters such as isotropic pressure, intracellular concentration of specific solutes, turgor pressure and membrane stretch remain as possibilities [5]. Of these the turgor model for Kdp regulation has received the most attention [188]. According to this model a drop in turgor pressure induces autophosphorylation of the KdpD kinase, which in turn transfers the phosphoryl group to an aspartate residue in the response regulator KdpE. The phosphorylated KdpE then activates expression of the *kdpFABC* operon. Transcription of the operon is only transiently induced following osmotic up-shifts, a regulatory pattern that is consistent with at least partial restoration of turgor by the accumulated K^+. In addition given that amphipathic compounds (which intercalate into the lipid bilayer altering the curvature stress of the membrane [189]) elicit a similar effect as osmotic up-shock, membrane stretch has also been proposed as a likely osmotic signal sensed by the transmembrane domain of the KdpD sensor kinase [190]. However, since K^+ uptake is still observed in the presence of glycine betaine (which restores turgor and consequently membrane stretch, to a normal level), stretch and/or turgor alone appear unlikely to function as sole regulatory signals for the *kdpABC* operon. Evidence that intracellular K^+ may function as a second signal regulating expression of the operon was originally put forward by Rhoads et al. [45] and later Gowrishankar [91]. Furthermore, Sugiura et al. [190] demonstrated that K^+ sensing can be separated mechanistically from medium osmolarity signals, as mutants which fail to perceive the K^+ signal respond normally to hyper-osmotic stress. Indeed, while the autophosphorylation of wild-type KdpD is negatively regulated by K^+, medium osmolarity has a positive effect. Recently Jung et al. [191] demonstrated that CsCl (which decreases the intracellular K^+ concentration) induces expression of the *kdpFABC* operon to a much higher level than does NaCl, thus stressing that in addition to osmoregulation the primary function of the Kdp system is K^+ homeostasis.

5.1.2. Trk

In contrast to Kdp, osmotic regulation of Trk is mainly at the level of transport activity. While the activity increases upon osmotic up-shift, the initial rate of influx appears dependent on the intracellular osmolarity as opposed to the external environment [192]. Since intracellular osmolarity and K^+ concentration are not well separated in the experimental setup, it has been proposed that the actual rate is determined by the intracellular K^+ concentration through feedback regulation [6].

Alkalisation of the cytoplasm, a consequence of K^+ uptake [104,193], has been suggested as a possible signal for increased glutamate (the K^+ counter-ion) synthesis following hyper-osmotic shock.

5.2. The secondary response

5.2.1. ProP

Effectively regulated by the external osmolarity, both at the level of expression and activity [85,89,101], it is the biochemical activation of the ProP protein that contributes most to the osmostress response. A number of possible signals have been proposed to modulate the activity of

ProP including turgor pressure [194], K^+ concentration [195] and intracellular pH [6]. Since 'activated' uptake occurs irrespective of whether turgor has been restored via uptake of K^+, it seems unlikely that ProP senses turgor pressure per se [6]. The requirement for K^+ to stimulate ProP activity, although well documented [195,196], remains ill-defined since K^+ is also required to support respiration [197] and energisation of ProP [198]. While stimulation of uptake via ProP can be observed following an increase in the intracellular pH upon K^+ uptake [41,199], the resulting activation is transient [195].

Perhaps the most likely mechanism governing sustained ProP activation at elevated osmolarity involves modulation of the α-helical coiled-coil formation of the ProP carboxy-terminus [96,198]. Stability of the coiled-coil structure (which often requires a second partner) may be modulated in response to either varying a_W or cytoplasmic solvent composition [200]. One cytoplasmic element exhibiting a significant influence on the osmotic activation of ProP is the 232-amino acid, basic, hydrophilic protein ProQ [201]. Mutating *proQ* reduces both the rate and extent of ProP activation by an osmotic up-shift [202]. Since neither transcription nor translation of *proP* appears to be altered by the mutation, it is proposed that ProQ may influence the osmotic activation of ProP at a post-translational level [96,202].

5.2.2. ProU

As with ProP, the ProU system is regulated both at the level of transcription and enzyme activity. However, unlike ProP, it is transcriptional activation of *proU* that is most important in terms of the osmostress response [89,90]. As with the *Kdp* operon, transcription of *proU* can be induced only by high concentrations of solutes that do not cross the membrane [5]. Also, since regulation by either turgor pressure or membrane stretch is unlikely given their transient nature, by elimination the most likely signal is the concentration of a specific solute or solutes. Intracellular K^+ concentration was originally proposed as a possible signal for *proU* expression by Sutherland et al. [203] and later by Ramirez et al. [204], who reported that expression of the operon in vitro was increased in proportion to the K^+-glutamate concentration in the assay buffer. Other workers have disputed this proposal and eliminated glutamate (but not K^+) as the inducing signal [205]. However, since multiple cellular processes are stimulated by K^+ [206], the dependence of *proU* transcription on K^+ may be a reflection of the general stimulatory effect of the ion on enzymatic reactions in general, rather than evidence for a specific osmoregulatory signal [42].

DNA supercoiling has also been suggested to function as a regulator of *proU* expression [207,208]. Mutations in *topA* (encoding topoisomerase I) were shown to increase *proU* expression [207], while disrupting *gyrA* and *gyrB* (genes specifying the two subunits of DNA gyrase) reduces expression of the operon (in *Salmonella* but not *E. coli*) at low osmolarity [207]. However, as with K^+, the effects of DNA supercoiling on the expression of *proU* may be the result of pleotrophic effects of supercoiling on transcription, rather than proof that supercoiling is a specific osmoregulatory signal [209].

In addition to *topA*, *gyrA* and *gyrB*, mutational alterations of a number of other DNA-binding proteins (which have no direct role in supercoiling) have been linked to modified *proU* expression. Mutations in *hns* (*osmZ*), encoding the DNA-binding protein H-NS, results in a moderately elevated expression of *proU* at all osmolarities. Under normal growth conditions H-NS binds to sites both up- [210] and downstream of the promoter [111] forming an extended nucleo–protein complex, which prevents binding of the RNA polymerase, thus blocking transcription. Dissociation of this complex occurs by an unknown mechanism at high osmolarity. Additionally, deletion of the negative regulatory sequence within *proV*, to which H-NS binds, prevents the formation of the nucleo–protein complex, consequently increasing expression of *proU* by up to 25-fold in low osmolarity medium [108]. Mutations in the gene for IHF (integration host factor), on the other hand, decrease the induced level of *proU* expression two-fold [111], while mutations in the gene for HU-B (histone-like protein) reduce both basal and induced levels of *proU* expression [109]. Since expression of *proU* remains osmotically controlled in strains mutated in H-NS, IHF or HU-B, it is apparent that these proteins function as modulators rather than regulators of *proU* expression [4].

Regulation of ProU at the level of enzyme activity has been linked to the periplasmic tail of the ProW protein, which is predicted to form an amphiphilic α-helix [211]. This protein domain has been implicated in osmosensing by monitoring alterations in membrane tension as well as changes in the intracellular osmolarity [6].

5.2.3. BetP

Although regulated both at the level of gene expression and enzyme activity, recent studies on betaine uptake in *C. glutamicum* have focused primarily on osmoregulation of the BetP protein [97,98]. Modulation of the activity of the protein by the amphipathic compound tetracaine indicates that at least part of the primary signal transferred to BetP comes directly from the membrane [97,98]. Additional evidence that a major factor modulating BetP activity originates via the membrane was obtained following heterologous expression against an *E. coli* background. A shift in the optimum of osmotic stimulation from 1.3 osmol kg^{-1} (in *C. glutamicum*) down to 0.5 osmol kg^{-1} (when expressed in *E. coli*), initially attributed to the difference in turgor pressure between *E. coli* and *C. glutamicum* [124], is now known to be linearly related to the increase in the content of phosphatidyl glycerol in the *E. coli* lipids [98]. Peter et al. [97] recently demonstrated that both the N- and C-terminal extensions of BetP function as putative

osmosensory domains. Deletions in the N-terminus (a 62-amino acid domain with an excess of negatively charged residues) shift the optimum of activation from 1.3 to 2.6 osmol kg^{-1}, while similar mutations in the C-terminus (a 55-amino acid extension with a large excess of positive residues) result in a complete loss of regulation.

5.2.4. BusA (OpuA)

As with BetP, a major factor modulating BusA (OpuA) activity originates via the surrounding membrane directly, as demonstrated both by the influence of tetracaine and the fatty acid composition of the membrane [118,212]. In addition to modulation of its translocation activity, *busA* is osmotically regulated at the level of gene expression [119].

5.2.5. BetL

While the secondary glycine betaine uptake system, BetL, has been shown to be effectively regulated at the level of gene expression [128], Verheul et al. [127] demonstrated that both betaine and carnitine uptake in *Listeria* are additionally regulated at the level of enzyme activity by a novel osmolyte sensing mechanism, in which regulation of uptake of both betaine and carnitine is subject to inhibition by pre-accumulated solute. Internal betaine inhibits not only transport of external betaine but also that of carnitine and vice versa. The observed *trans*-inhibition is alleviated upon osmotic up-shock, which suggests that alterations in membrane structure are transmitted to the allosteric binding sites for betaine and carnitine of both transporters at the inner surface of the membrane. The linkage of the *trans*-inhibitory effect to the osmotic strength of the environment is also observed in *L. plantarum* [6] and *S. aureus* [156] and thus may form a general strategy to tune the intracellular osmolarity and maintain the cell turgor within certain limits.

5.3. Outer membrane porins: OmpC and OmpF

The OmpC and OmpF porin levels are controlled predominantly at the level of gene expression, by the two-component regulatory system EnvZ/OmpR, but fine-tuning requires an additional level of control involving the antisense RNA MicF. Maximally expressed at 37°C [213], this 174-nucleotide RNA sequence, transcribed from a promoter upstream of the *ompC* gene, is highly complementary to the 5′ region spanning the translation initiation site of *ompF*. MicF thus acts as a negative regulator of *ompF* expression at the post-transcriptional stage [214].

While the signal transduction pathway for the transcriptional and post-transcriptional control of *ompC* and *ompF* has been well characterised with respect to the structures and interactions of its components, the signal or signals to which EnvZ responds remains to be determined. Given that activation occurs in response to both permeant and impermeant solutes [215] the most likely signals appear to be either the levels of specific solutes or the cytoplasmic, periplasmic or extracellular a_W [5]. Since the response to high osmolarity in minimal medium is markedly reduced in the presence of betaine [216], extracellular a_W is unlikely to function as the signal. This, together with the localisation and structure of the EnvZ sensor protein, which spans the cytoplasmic membrane, exhibiting both periplasmic and cytoplasmic domains [217–219], points to the sensing of a signal within either the periplasm or cytoplasm as opposed to the cell exterior. In this connection, sensing of the periplasmic derived MDOs has been proposed as a possible signal for EnvZ [167].

5.4. Solute efflux

5.4.1. Specific efflux systems

Osmoregulated efflux activity with specificity for compatible solutes has been described for a number of microbes [38,220,221]. In general, specific compatible solute efflux upon osmotic down-shock is characterised by two kinetic components; one with a $t_{1/2} < 1$ s and the other with $t_{1/2}$ of 4–5 min. The rapid ($t_{1/2} < 1$ s) efflux systems exhibit properties that mimic mechanosensitive channels (i.e. rapid stretch-activated efflux [222]) and are discriminated from the slower mechanisms (most probably mediated by bi-directional secondary transporters, e.g. BetP and BetL [223]) by a number of features including: a function independent of metabolic energy, and an observed insensitivity to substrate on the *trans* site of the membrane [6].

5.4.2. Mechanosensitive channels

Mechanosensitive or stretch-activated channels (of which MscL, MscM and MscS of *E. coli* are the best characterised members) are, as their name suggests, activated by membrane stretch (a fact demonstrated by their observed activation in the presence of amphipathic compounds which intercalate into the lipid bilayer [224]). Resolution of the crystal structure of the closed MscL homologue from *Mycobacterium tuberculosis* [225], TbMscL, has provided important insights into the structure and function of mechanosensitive channels in general. TbMscL was shown to comprise five identical subunits arranged around a central core. Each subunit consists of two transmembrane helices (TM1 and TM2) and a small intracellular domain at the C-terminus. All the transmembrane helices are slanted with respect to the plane of the membrane, and the five TM1 helices come together at their cytoplasmic ends, forming a gate held together primarily by hydrophobic interactions between neighbouring helices. In the closed state the minimum pore radius is less than 1 Å. It is believed that mechanical stretch pushes the cytoplasmic ends of the TM1 helices apart [225,226] forming a large (~30 Å), non-specific channel. Blount et al. [227] proposed that the mechanosensing domain of MscL might

be confined to the hydrophobic core (TM1 and 2) and the periplasmic loop in between the TMs. This proposal was later confirmed by the isolation of mutations in TM1, which result in an increased sensitivity of the channel to mechanical stress [228]. While MscL has been implicated in the release of both K^+ and small proteins such as thioredoxin during osmotic down-shock [228,229] it is not known whether it also mediates the efflux of other ions or non-ionic cosolvents [7]. While patch clamp analysis of *E. coli* revealed the existence of multiple mechanosensitive channel conductances, it seems likely that one or more of these activities correspond(s) to the observed efflux of compatible solutes upon hypo-osmotic shock (as described in Section 5.4.1).

6. Osmostress and virulence

Bacteria capable of causing foodborne illness must negotiate a long and tortuous passage from the environment to the site of infection of the susceptible host. As well as the stresses encountered during the production, preparation and storage of food, bacterial foodborne pathogens are additionally faced with the formidable defences of the host immune system. Following consumption they are exposed to the low pH of the stomach and subsequently the volatile fatty acids, bile salts, high osmolarity and low oxygen content of the small intestine. Bacteria surviving to this point are forced to compete with the established gut flora for niches and nutrients and encounter, among other insults, anti-microbial peptides produced by their competitors [230]. Organisms capable of invasion subsequently penetrate the gut epithelium (possibly via M cells located in Peyer's patches) and are rapidly engulfed by macrophages before being internalised by phagosomes, specialised organelles that prevent bacterial multiplication by means of acidic pH and the production of defensins (oxygen-independent mechanisms) as well as peroxide and superoxide radicals (oxygen-dependent mechanisms) [231].

In view of the variety of stresses encountered by pathogenic bacteria during the course of infection, it is becoming increasingly evident that in addition to 'true' virulence factors (those encoding toxins or invasins, for example), there also exists an additional class of proteins or contributory factors, involved in the complex stress management strategies which are essential for the pathogen to mount a successful infection. This section focuses on the link between osmostress and virulence, and reviews the role of various osmoregulatory systems in contributing to the virulence potential of certain pathogenic bacteria.

6.1. Osmoprotectant accumulation

In addition to their role in the salt tolerance response, there is increasing evidence to suggest that osmoprotective compounds, together with their transport/synthesis systems, may function as important virulence factors for certain pathogenic bacteria. Gowrishankar and Manna [113] first proposed that *proU* may function as a virulence gene in the pathogenic enterobacteria, while *E. coli*, a strain capable of causing urinary tract infections and pyelonephritis, has been shown to exhibit an abnormally high level of ProP activity. In addition, deletion of *proP* dramatically reduces the ability of the strain to colonise mouse bladders [232]. Similarly, inactivation of the *putP* homologue in *S. aureus* significantly reduces virulence in an experimental endocarditis model [233]. Recent evidence suggests that knockout of *opuC* in *L. monocytogenes* LO28 can reduce the virulence potential of this strain following intraperitoneal infection. Interestingly, this effect appears to be strain-specific and was not seen in a knockout mutant in *L. monocytogenes* ScottA. However, elimination of OpuC in both strains significantly reduced the ability to colonise the upper small intestine in mice following peroral administration [135].

Thus, while a number of osmolyte transport systems have clearly been linked to the virulence potential of certain pathogenic bacteria, the role of osmolyte synthesis in microbial pathogenesis has received considerably less attention. Recently we investigated the effects of osmolyte synthesis, specifically proline synthesis, in contributing to listerial pathogenesis [149,150]. While knockout of the *proBA* locus reduces salt tolerance in complex broth, it does not appear to affect virulence potential when administered to mice by the intraperitoneal or peroral routes [149]. This finding reflects that of an earlier study in which Marquis et al. [234], using an uncharacterised proline auxotroph, showed that proline auxotrophy fails to exhibit reduced virulence, suggesting that the host tissue contains a relatively abundant source of free proline or proline containing peptides. Furthermore, manipulation of the system resulting in proline overproduction also failed to alter the virulence potential in *L. monocytogenes* [150].

6.2. EnvZ–OmpR

The EnvZ–OmpR two-component regulatory system, originally identified as a regulator of the outer membrane porins OmpC and OmpF, has emerged as a global regulator of virulence potential. Mutating *ompR* dramatically reduces virulence of both *Shigella flexneri* and *S. typhimurium*, suggesting a major role for this locus in both pathogens [235,236]. In *S. typhimurium*, OmpR mutants fail to lyse infected macrophages and so fail to induce a key step in pathogenesis [237]. While mutations in *ompC* and *ompF* alone failed to affect virulence, strains carrying mutations in both porins are significantly attenuated (though not to the same extent as an OmpR mutant [238]). A specific role for these porins in intestinal survival is supported by the fact that double mutants in *ompC* and *ompF* are severely attenuated when administered via the oral route, but only marginally affected when administered intravenously.

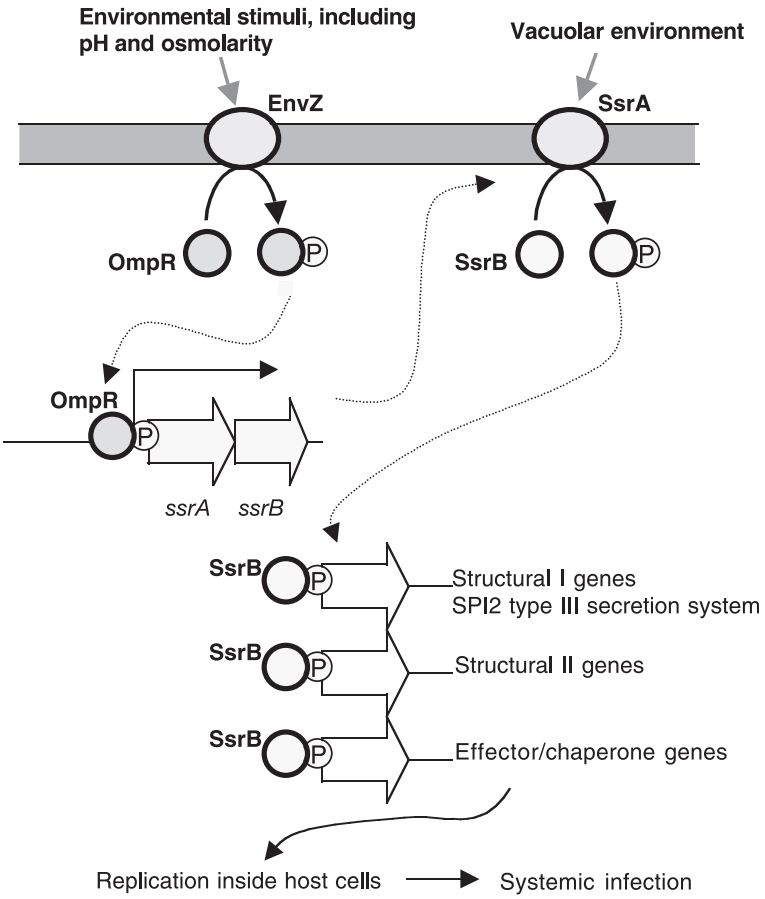

Fig. 6. Model for *Salmonella* SPI2 regulation inside host macrophages. The OmpR–EnvZ system responds to the intracellular environment, possibly stimulated by the acidic pH and osmolarity of the phagosome. OmpR binds to the *ssrA* promoter region to activate transcription of the *ssrAB* genes. Later, SsrA detects a different environmental stimulus in the vacuole. SsrB activates expression of the type III secretion system encoded within SPI2, which then allows for replication inside cells and systemic infection in mice. Adapted from [244].

Since conditions of high salinity (e.g. 0.3 M NaCl in the intestinal lumen [239]) and high temperatures (37°C) favour synthesis of OmpC over OmpF, Nikaido and Vaara [161] conjectured that OmpC may be synthesised preferentially when the pathogen is present in the intestinal tract of the animal host. In this environment the small pore size of OmpC may help to exclude harmful molecules such as bile salts, while facilitating uptake of nutrients present at high concentrations. OmpF, on the other hand, exhibiting a larger pore size than OmpC [240], is most likely expressed outside of the host where temperature and salinity are lower and nutrients are likely to be more dilute.

Given that *ompR* mutants of both *S. typhimurium* and *Salmonella typhi* are significantly more attenuated than OmpC:OmpF double mutants, the influence of EnvZ–OmpR on virulence potential is expected to extend beyond the regulation of outer membrane porins [241]. Other genes regulated by OmpR in *S. typhimurium* include *tppB*, which encodes a tripeptide permease [242], and *aas*, a gene, induced within macrophages, encoding 2-acylglycerolphosphoethanolamine acyltransferase [243]. While mutations in either *tppB* or *aas* had no significant effect on virulence [244], deletion of *sifA* (an OmpR-regulated gene responsible for the formation of *Salmonella*-induced filaments within HeLa cells [245]) results in partial attenuation of virulence, indicating some requirement for filament formation during infection [246]. Bernardini et al. [235] showed that transcription of the *mxi* operon (membrane expression of invasion plasmid antigens) of *S. typhi* is induced at high osmolarity. Furthermore, this osmoregulation was not seen in an *ompR* deletion background. Indeed, expression of the operon was reduced 10-fold in the *ompR* mutant. In addition, Pickard et al. [247] demonstrated that the Vi capsule in *S. typhi* was also affected by mutations in *ompR*. Strains carrying an *ompR* mutation were no longer agglutinated by Vi antiserum. The authors concluded that the mutation was a consequence of reduced production (as opposed to decreased export) of the polysaccharide, a defect that could be complemented by a plasmid containing the *ompR* gene.

Since mutations of individual components of the OmpR regulon have only a marginal effect on virulence potential, researchers have continued the search for the key component of the regulon. In this regard a most interesting recent discovery is the fact that OmpR regulates the two-component system SsrA–SsrB in *Salmonella* pathogenicity

island SPI2, which in turn regulates a type III secretion system required for both murine infection and replication within macrophages (Fig. 6) [244]. Evidence suggests that EnvZ, sensing both the low pH and osmolarity of the phagosome, activates OmpR, which in turn stimulates rapid expression of *ssrA* and *ssrB*. The SsrA–SsrB two-component system then detects another signal (possibly mediated by PhoP–PhoQ) and in turn activates expression of the SPI2 type III secretion system [244,248].

7. Concluding remarks

7.1. Future prospects

The availability of complete genome sequences, in this post-genomic era, has facilitated rapid advances in the field of bacterial (osmo)stress adaptation. Comparative genomics, coupled with a proteomic approach to the study of bacterial 'fitness', facilitates the rapid detection of new and often novel systems employed by bacteria to adapt to their ever-changing environment.

Structural analysis of some of the isolated systems (e.g. MscL) using techniques such as X-ray diffraction studies, coupled with functional reconstitution into artificial membranes, provides important information concerning the structure and function of these isolated systems. Rigorous kinetic analysis of the activation mechanisms, in relation to structural studies of the system components, should indicate whether or not certain structural modifications ('added' loops and/or tails or specific transmembrane segments) have a role in osmosensing.

The next major challenge is thus to elucidate the signals sensed, and untangle the complex interplay that exists, linking cause and effect. Given the diversity of systems involved and the existence of multiple physiochemical signals (internal/external osmolarity, turgor pressure or related parameters such as membrane tension), the molecular mechanisms of osmosensing remain the most challenging and as yet largely unexplored areas of bacterial osmoadaptation.

In relation to (osmo)stress and virulence, the advent of elegant and imaginative techniques for detecting genes expressed both in vivo (IVET: in vivo expression technology) and at elevated osmolarities, allied to the completion of entire genome sequences (gene chip technology), offers the possibility that a more complete understanding of the relationship between (osmo)stress and virulence is within reach.

7.2. Commercial applications

Detailed genetic and physiological analysis of bacterial stress responsive systems, particularly salt stress, as outlined in this review, has provided the basis for a number of recent advances both in the fields of biotechnology and medicine. Taking advantage of the existence of specific solute efflux systems and stretch-activated channels (dedicated to the release of compatible solutes following osmotic down-shock, Section 3.3) Sauer and Galinski [249] developed a technique known as bacterial milking: a novel bioprocess involving alternating hyper- and hypo-osmotic shocks, for the quick and efficient production of large quantities of compatible solutes, which until recently could be produced only in small amounts at significant cost. The stabilising effects of such compounds (e.g. betaine) on enzyme structure under adverse conditions such as elevated temperature and salt concentration (a consequence of their preferential exclusion from protein surfaces, Section 2.2.1) have additionally resulted in the development of a number of in vivo and in vitro biotechnological applications. An example of the former is the development of transgenic drought resistant plants. Heterologous expression of glycine betaine synthesis systems, such as the *E. coli betBA* genes, has facilitated the creation of desiccation resistant varieties of commercially important crops such as tobacco, rice and potatoes [250]. In vitro biotechnological applications of compatible solute function have, on the other hand, focused mainly on the development of improved buffer systems for optimal efficiency of commercially available restriction enzymes and polymerase chain reaction reagents.

A number of potential medical applications for compatible solutes include the development of moisturisers, skin care products and possibly a role as protective compounds for healthy cells during chemotherapy [249]. However, given the increasing incidence of multiple drug resistance amongst microbial pathogens, perhaps the most interesting application of compatible solutes is the development of novel drug delivery systems. Based on smugglin technology [251], the widespread ability of microorganisms to accumulate compatible solutes may be exploited for the delivery of structurally related compounds with anti-microbial activity [252].

In conclusion then, a detailed analysis of the molecular mechanisms governing the salt stress response of bacterial cells provides us not only with a better understanding of the characteristics of bacterial growth and survival in the natural environment, but also facilitates the development of novel and innovative processes in food and biomedicine.

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