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Review

Osmosensing and osmoregulatory compatible solute accumulation by bacteria ☆

Janet M. Wood[a,*], Erhard Bremer[b], Laszlo N. Csonka[c], Reinhard Kraemer[d], Bert Poolman[e], Tiemen van der Heide[e], Linda T. Smith[f]

[a] *Department of Microbiology and Guelph-Waterloo Centre for Graduate Work in Chemistry and Biochemistry, University of Guelph, Guelph, Canada N1G 2W1*
[b] *Department of Biology, Laboratory for Microbiology, University of Marburg, Karl-von-Frisch Str., D-35032 Marburg, Germany*
[c] *Department of Biological Sciences, Purdue University, West Lafayette, IN 47907-1392, USA*
[d] *Institute of Biochemistry, University of Köln, 50674 Köln, Germany*
[e] *Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, Netherlands*
[f] *Department of Agronomy and Range Science, University of California, Davis, CA 95616, USA*

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Abstract

Bacteria inhabit natural and artificial environments with diverse and fluctuating osmolalities, salinities and temperatures. Many maintain cytoplasmic hydration, growth and survival most effectively by accumulating kosmotropic organic solutes (compatible solutes) when medium osmolality is high or temperature is low (above freezing). They release these solutes into their environment when the medium osmolality drops. Solutes accumulate either by synthesis or by transport from the extracellular medium. Responses to growth in high osmolality medium, including biosynthetic accumulation of trehalose, also protect *Salmonella typhimurium* from heat shock. Osmotically regulated transporters and mechanosensitive channels modulate cytoplasmic solute levels in *Bacillus subtilis*, *Corynebacterium glutamicum*, *Escherichia coli*, *Lactobacillus plantarum*, *Lactococcus lactis, Listeria monocytogenes* and *Salmonella typhimurium*. Each organism harbours multiple osmoregulatory transporters with overlapping substrate specificities. Membrane proteins that can act as both osmosensors and osmoregulatory transporters have been identified (secondary transporters ProP of *E. coli* and BetP of *C. glutamicum* as well as ABC transporter OpuA of *L. lactis*). The molecular bases for the modulation of gene expression and transport activity by temperature and medium osmolality are under intensive investigation with emphasis on the role of the membrane as an antenna for osmo- and/or thermosensors.

Keywords: Osmosensing; Osmoregulation; Compatible solute; Volume regulation; Bacteria; Stress; Solute transport; Uptake; Curvature stress; Secondary transporter; ABC transporter; Glycine betaine; Proline; Choline; Ectoine

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* Corresponding author. Tel.: +1-519-824-4120, ext. 3866; fax: +1-519-837-1802.
E-mail address: jwood@uoguelph.ca (J.M. Wood).

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1. Introduction

Bacteria inhabit natural and artificial environments with diverse and fluctuating physical properties, including osmolality, salinity and temperature. They maintain cytoplasmic hydration, growth and survival, despite variations in external water activity. This may be accomplished by accumulating solutes when extracellular osmolality rises and rapidly releasing those solutes when extracellular osmolality declines. Although most, if not all, microorganisms use this strategy, the types of molecules accumulated and the accumulation mechanism(s) differ among species. For instance, halophilic archaea and acetogenic anaerobes accumulate large amounts of salts (KCl). Other bacteria can also accumulate electrolytes (e.g. K^+ glutamate) but accumulation of organic solutes that are more compatible with cell physiology appears to be preferred (Galinski, 1995; Gutierrez et al., 1995; Hagemann et al., 1999; Kempf and Bremer, 1998; Miller and Wood, 1996; Poolman and Glaasker, 1998; Smith et al., 1998; Ventosa et al., 1998; Wood, 1999).

Compatible solutes fall into a few structural classes (e.g. Fig. 1) and diverse organisms accumulate similar solutes. These solutes are often kosmotropes, compounds that structure water and stabilize the native conformations of biological macromolecules (Collins and Washabaugh, 1985). Compatible solute accumulation can be accomplished through biosynthesis and/or transport. Species examined to date possess multiple osmoregulatory transporters with overlapping substrate specificities and energy coupling mechanisms. Recent research has also revealed that mechanosensitive channels mediate solute release from bacteria that are exposed to environments of abruptly decreasing osmolality (Booth and Louis, 1999; Sukharev et al., 1997; Wood, 1999).

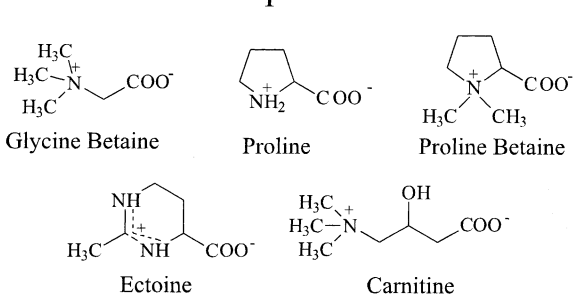

Fig. 1. Structures of selected compatible solutes.

The literature now includes reasonably thorough descriptions of the osmoregulatory systems present in a variety of bacteria (Table 1). Gram-positive and Gram-negative bacteria differ in being bounded by one or by two concentric semipermeable membranes, respectively. Thus, though they lack the membrane-bounded organelles characteristic of eukaryotic cells, Gram-negative bacteria do include two distinct osmotic compartments. The concentration of osmotically active ionic and non-ionic solutes in the bacterial cytoplasm is often maintained above that of the envi-

Table 1
Osmoregulatory compatible solute accumulation by bacteria

Organism	Phylogenetic group	Cell wall	Ref.
Synechocystis	Cyanobacteria	Gram-negative	(Hagemann et al., 1999)
Rhizobium meliloti	Alpha proteobacteria	Gram-negative	(Miller and Wood, 1996)
Escherichia coli	Gamma proteobacteria	Gram-negative	(Wood, 1999)
Salmonella typhimurium	Gamma proteobacteria	Gram-negative	(Wood, 1999)
Halomonas elongata	Gamma proteobacteria	Gram-negative	(Ventosa et al., 1998)
Corynebacterium glutamicum	Firmicutes (high GC)	Gram-positive	(Peter et al., 1998b)
Staphylococcus aureus	Firmicutes (low GC)	Gram-positive	(Gutierrez et al., 1995)
Lactobacillus plantarum	Firmicutes (low GC)	Gram-positive	(Poolman and Glaasker, 1998)
Listeria monocytogenes	Firmicutes (low GC)	Gram-positive	(Smith et al., 1998)
Bacillus subtilis	Firmicutes (low GC)	Gram-positive	(Kempf and Bremer, 1998)

ronment. The resulting water activity gradient will lead to water influx, increased cell volume and/or the development of hydrostatic pressure (turgor pressure) depending upon the mechanical properties of the cell wall (Csonka, 1989; Wood, 1999). Limited measurements indicate that the internal osmotic pressure of Gram-positive bacteria, resulting in turgor pressures (ΔP) of 15–25 atm, is much higher than that of Gram-negative bacteria (ΔP of 1–5 atm), even when the bacteria are growing in media of low osmolality (Csonka, 1989; Mitchell and Moyle, 1956; Poolman and Glaasker, 1998; Wood, 1999). Of course many other characteristics also differentiate the bacteria listed in Table 1. This article summarizes recent advances in our understanding of osmoregulation by *Bacillus subtilis*, *Corynebacterium glutamicum*, *Escherichia coli*, *Lactobacillus plantarum, Lactococcus lactis*, *Listeria monocytogenes* and *Salmonella typhimurium*. The ensuing discussions of osmoregulation by each of these organisms address a number of important questions and raise new ones.

First, how do bacteria sense changes in the osmolality of their environment? What roles do genetic and biochemical mechanisms play in the osmoregulation of solute accumulation? Evidence that medium osmolality modulates the expression of genes encoding compatible solute biosynthetic and transport systems continues to accumulate. Relevant signal transduction mechanisms have been identified in *E. coli*, *S. typhimurium* and *B. subtilis* (Kempf and Bremer, 1998). A soil organism, *B. subtilis* possesses highly integrated stress adaptation mechanisms, including sporulation. Its osmoadaptative mechanisms, including systems for compatible solute synthesis, uptake and efflux, are described below. Expression of these systems involves both the SigB-controlled general stress response network and specific osmostress response systems. Despite extensive descriptions of the osmoregulation of gene expression, the associated sensory mechanisms are not understood and warrant further, intensive study.

Purification and reconstitution in proteoliposomes has revealed that compatible solute transporters ProP of *E. coli* (a proton-solute symporter) (Racher et al., 1999), BetP of *C. glutamicum* (a Na^+-solute symporter) (Ruebenhagen et al., 2000) and OpuA of *L. lactis* (an ABC transporter) (van der Heide and Poolman, 2000b) can both sense and respond to osmotic shifts. Thus, each is an osmosensor, a transducer of the resulting signal and the respondent, which mediates cytoplasmic compatible solute accumulation. Current research is designed to determine whether these transporters sense osmotically-induced membrane changes or whether the proteins detect osmotic shifts directly. This work is complex, since changes in transporter activity (itself subject to changes in membrane structure and the solution environment) are currently the sole indicators of osmosensing.

The responses of transporters BetP (Peter et al., 1996; Ruebenhagen et al., 2000) and OpuA (van der Heide and Poolman, 2000a) to osmotic shifts are sensitive to membrane lipid composition and to membrane-active amphipaths. Thus, these transporters may sense and respond to osmotically-induced membrane changes. Future work will be designed to elucidate which membrane property is influenced by osmotic shifts and correlated with osmosensing.

Deletions, point mutations and domain swapping experiments altering the hydrophilic, N- and/or C-terminal domains of ProP (Culham et al., 2000), BetP (Peter et al., 1998a) and EctP modulate the responses of these transporters to osmotic shifts. In the case of ProP, the C-terminal domain is capable of forming a homodimeric α-helical coiled-coil of limited stability in vitro and mutation R488I, which destabilizes this coiled-coil, renders osmotic activation of the transporter transient in vivo (Culham et al., 2000). Closely related transporters (including OusA of *Erwinia chrysanthemi* and ProP of *C. glutamicum*) lack the coiled-coil motif and soluble protein ProQ is required for full activation of *E. coli* ProP in vivo (Kunte et al., 1999) Thus, regulation of ProP activity in vivo via the C-terminal domain and ProQ may be superimposed on the osmoregulatory response observed in the proteoliposome system. In the case of BetP, a variety of protein modifications have revealed a motif of 12 amino acids or less whose presence and position within the C-terminal domain appears to be crucial for the function of this domain in osmosensing (Peter et al., 1998a). Thus, the C-terminal domain of BetP may sense osmolality changes directly or it may interact with the membrane, detecting osmotically-induced membrane changes.

The majority of the bacteria examined in detail

to date possess structurally and functionally redundant osmoregulatory solute accumulation and efflux systems. For example, *B. subtilis* possesses at least five compatible solute uptake systems, with overlapping substrate specificities, falling into either the secondary or the ABC transporter class. *C. glutamicum* is exceptional in that all uptake systems detected so far are secondary transporters. In contrast, however, inactivation of the genes for OpuA in *L. lactis* makes the organism sensitive to osmotic upshifts, suggesting that there is much less redundancy in osmoregulated systems. This is consistent with the low metabolic flexibility, small genome size and specific ecological niche of this lactic acid bacterium. What purpose is served by the redundancy of osmoregulatory systems? Existing data hint at multiple explanations, including the need to mediate the uptake of structurally diverse compatible solutes available in diverse ecological niches, the involvement of different transporters in response to environments with different osmolalities and the associated need for differential regulation of transporter expression. Future study of this topic may reveal how the selection of compatible solutes by each species is related to its ecology.

Surprisingly, recent research on *S. typhimurium* shows that bacteria cultivated in high osmolality growth media show enhanced thermotolerance (high temperature), at least in part attributable to biosynthetic trehalose accumulation. Furthermore, betaine uptake via ABC transporter Gbu, similar in sequence to ProU of *E. coli* and OpuA of *B. subtilis*, responds to low temperature as well as high osmolality. This system confers both osmotic and chill stress tolerance on *Listeria monocytogenes* (Ko and Smith, 1999). These observations indicate that the molecular bases for osmotolerance and thermotolerance are related, suggesting that compatible solute accumulation may address physiological needs other than cellular hydration.

Though the above discussion emphasizes developments in our understanding of osmoregulatory mechanisms, this research also has considerable practical importance. Research on these mechanisms continues to be motivated by the importance of the subject microorganisms for agriculture, environmental protection and remediation, clinical microbiology, food production and distribution, and/or industrial fermentation.

2. Osmoregulatory compatible solute accumulation and release by *Bacillus subtilis*

2.1. Stress responses to high osmolality habitats

The challenge posed by changing environmental osmolality is vividly illustrated by the common habitat of *B. subtilis*, the upper layers of the soil. Drought and rain drastically alter the osmotic conditions within this ecosystem (Miller and Wood, 1996) and threaten the cell with dehydration or rupture as water permeates across the semipermeable cytoplasmic membrane along the osmotic gradient. Sudden osmotic changes trigger a behavioral response (osmotaxis) such that the *B. subtilis* cells are repelled by both high and low osmolality (Wong et al., 1995), but this reaction is of limited use when the entire habitat undergoes an osmotic change. *B. subtilis* is well known for its ability to produce a highly desiccation-resistant endospore, and one would expect that it would use this cellular differentiation to escape the threat posed by high osmolality media. However, high osmolality actually inhibits spore formation by impeding the signal transduction cascade that activates a number of transcription factors controlling the sporulation process (Kunst and Rapoport, 1995; Ruzal et al., 1998). To survive and grow in its osmotically changing habitats, *B. subtilis* has developed highly integrated cellular stress adaptation reactions that are either part of a general stress response system (Hecker and Völker, 1998; Price, 2000), or are specific to osmotic stress (Bremer and Kraemer, 2000; Kempf and Bremer, 1998).

2.2. The SigB-controlled general stress response network participates in osmostress resistance

The alternative transcription factor sigma B (SigB) controls a general stress regulon that encompasses at least 100 members and provides *B. subtilis* cells in transition from a growing to a non-growing state with protection against a wide variety of environmental insults including pH, oxidative, heat and salt stress. Activity of the master regulator SigB is determined by the anti-sigma factor RsbW. Release of SigB from the SigB-RsbW complex is mediated by the anti–anti sigma factor RsbV, whose activity is determined through

a complex signal transduction network that relies on serine and threonine phosphorylation events to control key protein–protein interactions (Hecker and Völker, 1998; Price, 2000). High salinity is an inducer of the SigB regulon, and *B. subtilis* mutants lacking SigB are highly sensitive to sudden and growth-restricting upshocks with NaCl (Völker et al., 1999). This observation implies that osmotic stress resistance is conferred by members of the SigB regulon and possible contributors are the GsiB and YtxH proteins, both of which resemble plant desiccation proteins (Hecker and Völker, 1998; Price, 2000). The survival of *sigB* mutants is severely diminished following rapid increases (1.2 M NaCl) in medium salinity (Völker et al., 1999), but when propagated in a synthetic medium containing 1.2 M NaCl their growth curve is like that of their *sigB*$^+$ parent strain (Spiegelhalter and Bremer, unpublished data). The induction of the SigB regulon following an osmotic upshock is only transient; therefore, SigB-controlled proteins cannot adequately protect actively growing cells against prolonged high osmolality. Under these conditions, synthesis and uptake of compatible solutes is crucial for cell survival and growth.

2.3. Control of cellular water content in response to osmotic up- and down-shifts

For *B. subtilis*, turgor has been estimated at 19 atm (1.9 MPa) (Whatmore and Reed, 1990), which is approximately ten times the pressure within an ordinary car tire. Like most other bacteria, *B. subtilis* copes with the problem of an unfavorable osmotic gradient across the cytoplasmic membrane by actively modulating its intracellular solute pool (Booth and Louis, 1999; Bremer and Kraemer, 2000; Kempf and Bremer, 1998). It amasses ions and organic osmolytes when it is challenged by high osmolality environments and expels them when it faces hypoosmotic conditions. A sudden osmotic upshock with 0.4 M NaCl triggers potassium uptake, raising the potassium pool from a basal level of approximately 350 to 650 mM (Whatmore et al., 1990). Despite the importance of potassium in the initial osmostress reaction, high intracellular concentrations of this ion interfere with many important cellular functions. The cell, therefore, accumulates large quantities of organic compounds, the so-called compatible solutes, which are more congruous with its physiology and allow it to reduce its potassium pool as the cell grows under high osmolality conditions (Whatmore et al., 1990).

2.4. Accumulation of proline and glycine betaine through synthesis

The compatible solute proline is synthesized by a wide variety of microbial and plant species as protection against osmotic stress. It has been known for over 25 years that *B. subtilis* belongs to this group of proline producers (Measures,

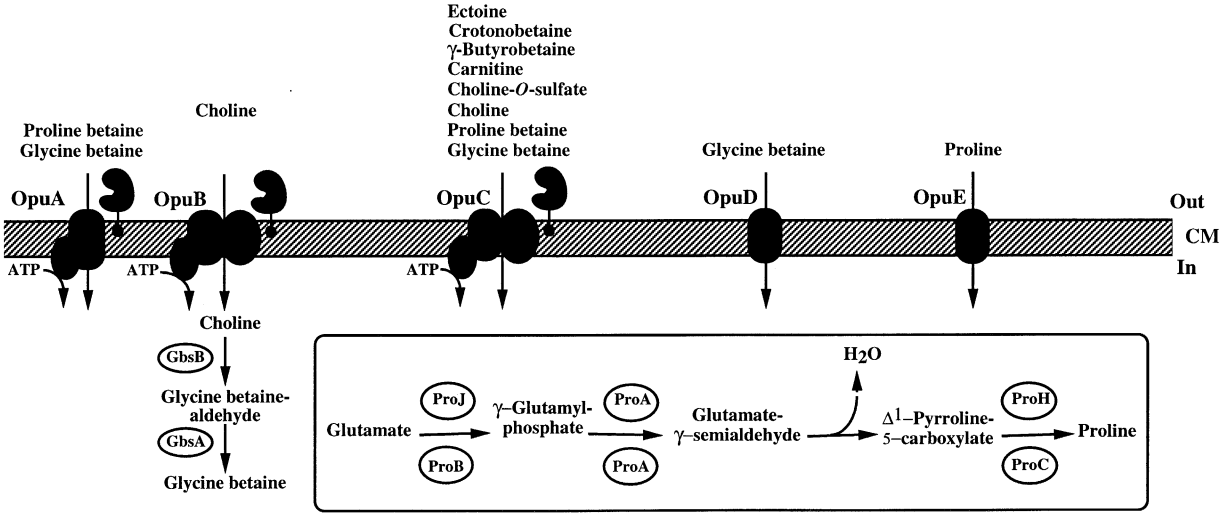

Fig. 2. Osmoprotectant transporters and compatible solute synthesis pathways in *B. subtilis*. This figure was adapted from a review by Bremer and Kraemer (2000).

1975). Recent studies revealed that a dedicated pathway exists in *B. subtilis* for the synthesis of proline under osmotic stress and that this pathway is connected with the proline synthesis pathway used by this bacterium for anabolic purposes (Brill and Bremer, unpublished data). The anabolic pathway proceeds from glutamate and is catalyzed by the sequential reactions of the ProB, ProA and ProC enzymes (Fig. 2). In the osmostress-controlled proline biosynthetic pathway, the precursor and the intermediates are identical but the ProB and ProC proteins are replaced by the sequence-related ProJ and ProH isoenzymes, respectively (Fig. 2). ProJ and ProH are encoded by an operon (*proHJ*) whose expression is enhanced when *B. subtilis* is grown in high-osmolality media. Disruption of the *proHJ* gene cluster does not cause proline auxotrophy, but it abolishes osmotically stimulated proline synthesis and causes a growth defect in high osmolality media (Brill and Bremer, unpublished data).

Glycine betaine (Fig. 1) is one of the most potent and widely used compatible solutes found in nature. *B. subtilis* can achieve a considerable degree of osmoprotection by synthesizing this compound, but the precursor choline must be acquired from exogenous sources (Boch et al., 1994). *B. subtilis* possesses two high-affinity and osmotically regulated choline transporters (OpuB and OpuC) (Fig. 2), which are members of the ABC superfamily (Kappes et al., 1999). OpuB functions only for choline transport, whereas OpuC also recognizes a large number of preformed compatible solutes, including glycine betaine (Jebbar et al., 1997; Kappes et al., 1996, 1999; Kappes and Bremer, 1998; Lin and Hanson, 1995; Nau-Wagner et al., 1999) (Fig. 2). Glycine betaine synthesis proceeds via a two-step oxidation process involving the GbsB (a type-III alcohol dehydrogenase) and GbsA (a glycine betaine aldehyde dehydrogenase) enzymes. The *gbsAB* structural genes form an operon whose expression is increased when choline is present in the environment, but high osmolality does not directly stimulate *gbsAB* transcription (Boch et al., 1996). The GbsR repressor protein, whose structural gene is located upstream of the *gbsAB* gene cluster, mediates induction of the *gbsAB* operon by choline (Nau-Wagner et al., unpublished data). GbsR also controls expression of the *opuB* operon in response to choline, but it does not regulate the *opuC* gene cluster. In contrast to *gbsAB* expression, transcription of *opuB* and *opuC* is increased when the *B. subtilis* cell experiences high osmolality environments (Kappes et al., 1999). GbsR is a novel type of choline-sensing protein and is not related to the BetI repressor, which controls the choline to glycine betaine synthesis pathway in *E. coli* (Lamark et al., 1996).

2.5. Acquisition of preformed compatible solutes from environmental resources

The varied habitats of *B. subtilis* offer a wide variety of preformed compatible solutes, which are released into ecosystems by primary producers from osmotically downshocked microbial cells, by root exudates and by decaying microbial, plant and animal cells (Welsh, 2000). *B. subtilis* can take up these osmoprotectants via multiple transport systems that have overlapping substrate specificity and energy coupling mechanisms (Fig. 2). The proline transporter OpuE is a member of the sodium/symporter family and, surprisingly, is evolutionarily related to the PutP transporters, which are used by a variety of microorganisms to acquire proline for catabolic purposes (von Blohn et al., 1997). The glycine betaine transporter OpuD is a member of the BCCT (betaine-choline-carnitine-transporters) family of secondary uptake systems which also includes compatible solute transporters from a variety of Gram-negative and Gram-positive bacteria (Kappes et al., 1996). Like the glycine betaine transporter BetP from *C. glutamicum* (Peter et al., 1998a), OpuD transport activity is enhanced by osmotic upshifts (Kappes et al., 1996), but the activity of OpuE is not regulated by medium osmolality (von Blohn et al., 1997). The OpuA and OpuC transport systems are members of the ABC family of transporters (Kappes et al., 1999; Kempf and Bremer, 1998) and, like OpuB, they are related to the binding-protein-dependent compatible solute transporter ProU from *E. coli* (Gowrishankar, 1989). Their extracellular substrate binding proteins are tethered to the cytoplasmic membrane via a lipid modification at their amino-terminal cysteine residues (Kempf et al., 1997). Collectively, the Opu transporters can provide *B. subtilis* with at least eight preformed osmoprotectants as well as the glycine betaine

biosynthetic precursor choline. The complex arsenal of osmoprotectant transporters operating in *B. subtilis* (Fig. 2) highlights the important role of compatible solute acquisition in this bacterium's adaptation to high osmolality surroundings.

Unlike *opuB* expression, which is induced by choline, expression of *opuA, opuC, opuD* and *opuE* is not enhanced when a substrate for the encoded transporter is present in the growth medium. However, transcription of the structural genes for each of the *B. subtilis* Opu transporters is induced when cells are exposed to increased medium osmolality, and the level of expression is sensitively linked to the degree of osmotic stress. The mechanism(s) through which *B. subtilis* senses osmotic changes and communicates this information to the transcription apparatus of the cell is completely unknown. Both the *opuD* and *opuE* genes are part of the SigB-controlled general stress regulon but each has an additional, independently controlled promoter that responds to increases in medium osmolality (Spiegelhalter and Bremer, 1998; von Blohn et al., 1997, Spiegelhalter and Bremer, unpublished data). Thus, at least two different pathways for the transduction of osmotic signals must operate in *B. subtilis*.

2.6. Expulsion of compatible solutes: protection against extreme turgor

Rain, flooding and washout into freshwater sources expose *B. subtilis* to rapid and severe osmotic downshocks that cause a sudden entry of water into the cell and a drastic increase in turgor. The severity of this effect is exemplified in *E. coli*, where an osmotic down shift equivalent to 0.3 M salt was calculated to increase turgor from a basal level of approximately 4 atm to approximately 11 atm (Booth and Louis, 1999). To a certain extent, elevated turgor can be accommodated by the elasticity of the murein sacculus (Wood, 1999), but when this capacity is exceeded the cell must eliminate water-attracting osmolytes to avoid bursting. Mechanosensitive channels act as safety valves for the rapid release of these compounds when turgor rises beyond a threshold level (Blount and Moe, 1999; Booth and Louis, 1999). Two such channels, MscL and MscS (YggB) have been characterized in *E. coli*, and their simultaneous disruption resulted in cell death following an osmotic downshock (Levina et al., 1999). In *B. subtilis*, the presence of ion-conducting pores that respond to mechanical forces has been demonstrated through electrophysiological studies. These channels display conductances ranging from a few hundred pS to more than 3 nS and most exhibit complex gating kinetics, suggesting that at least some are composed of subunits that function cooperatively (Alcayaga et al., 1992; Szabó et al., 1992). The MscL protein is evolutionarily well conserved, and a single copy of the *mscL* gene is present in the *B. subtilis* genome. When this *mscL* gene was heterologously expressed in *E. coli*, its product formed an ion-conducting mechanosensitive channel with a conductance of 3.6 nS (Moe et al., 1998). Three YggB homologues that might function as MscS-type channels are also present in *B. subtilis* (Levina et al., 1999). Taken together, these data and observations strongly suggest that mechanosensitive channels play a pivotal role in managing the transition of *B. subtilis* from high- to low-osmolality environments.

2.7. Perspectives

A solid framework for understanding how *B. subtilis* adapts to changing osmolality has been laid by the characterization of biosynthetic pathways for compatible solutes, the analysis of a complex set of compatible solute transporters and the initial characterization of mechanosensitive channels. Furthermore, the participation of the SigB-controlled general stress response network in this adaptation process has been demonstrated and it is clear that general and specific osmostress response systems are interwoven. This is illustrated by the inclusion of the OpuD and OpuE compatible solute transporters in the general stress response network (Spiegelhalter and Bremer, 1998; von Blohn et al., 1997, F. Spiegelhalter and E. Bremer, unpublished data). Many of the genes described above are controlled by increases in medium osmolality at the level of transcription, but it is unknown which parameter (osmolality, turgor, ionic conditions in the cytoplasm) is actually sensed by the cell and how this information is processed into a genetic signal that finally results in altered gene expression. Answering these questions poses a considerable challenge for future research efforts.

3. Osmosensing and osmoregulatory compatible solute accumulation by *Corynebacterium glutamicum*

Corynebacterium glutamicum is a Gram-positive soil bacterium and, in contrast to *B. subtilis*, a member of the high G/C group. *C. glutamicum* is well-known for its intensive use in biotechnology for the production of amino acids, e.g. glutamate and lysine. Mainly due to this fact, it is among the best-studied bacteria in terms of metabolism and regulatory properties. *C. glutamicum* is equipped with effective mechanisms to cope with osmotic challenges, this refers to the stresses imposed by both high and low osmolality media.

During growth in high osmolality media, *C. glutamicum* is able to synthesize several osmoprotectants and compatible solutes such as trehalose, proline, glutamate and glutamine. However, *C. glutamicum* achieves protection against high osmolality conditions primarily by the uptake of external compatible solutes, in particular, glycine betaine, ectoine, and proline (Farwick et al., 1995; Peter et al., 1998b). In response to a sudden osmotic upshift, *C. glutamicum* prefers uptake of betaine to ectoine and proline, followed by synthesis of proline and finally trehalose.

C. glutamicum is equipped with four uptake systems for compatible solutes, three of which are osmoregulated (Table 2). BetP is a high-affinity system, specific for glycine betaine; ProP a medium-affinity system for proline and ectoine; and EctP, a low affinity system for all three compounds. All three systems are effectively regulated at the level of activity (see below), and the former two are also controlled at the level of gene expression (Peter et al., 1996, 1997, 1998b). An additional proline uptake system (PutP) is present in *C. glutamicum*, but it is used for anabolic purposes and its physiological function is unrelated to osmoprotection (Peter et al., 1997). Different from other bacteria, all osmoregulated transport systems are secondary transporters and coupled to either the electrochemical Na^+ potential (BetP, EctP) or the proton potential (ProP).

Among the osmoregulated solute uptake systems in *C. glutamicum,* BetP is the best-studied transporter. It is highly active [V_{max} up to 100 μMol (g dw min)$^{-1}$] and specific for glycine betaine ($K_M = 8$ μM). By catalyzing cotransport of betaine and 2 Na^+ ions it leads to extremely high steady state betaine accumulation of up to 4×10^6 (in/ex) (Farwick et al., 1995). Expression of the *betP* gene depends on the osmolality of the growth medium, a 20-fold increase in the measured maximum rate of betaine uptake has been observed when comparing cells grown in media with low and high osmolality, respectively. The regulation of BetP on the level of protein activity is even more impressive (Fig. 3). The change from the inactive state at low osmolality to high activity at increased osmolality takes place on a time scale of less than 1 s.

Table 2
Functional and structural properties of BetP, ProP, and EctP of *C. glutamicum*

Property	BetP	ProP	EctP
Size (amino acids)	595	504	615
N-terminal domain (aa)	62	52	25
C-terminal domain (aa)	55	57	108
Expression	Inducible	Inducible	Constitutive
Activity regulation	+	+	+
Coupling ion	Na^+	H^+	Na^+
Substrates	Glycine Betaine	Proline > Ectoine	Ectoine > Proline > Betaine

There are a two major arguments demonstrating that BetP itself is both an osmosensor for signal(s) related to osmotic stress, and an osmoregulator, adapting its catalytic activity (betaine uptake) to the extent of osmotic stress. First, it has been shown that BetP from *C. glutamicum* fully retains its regulatory properties

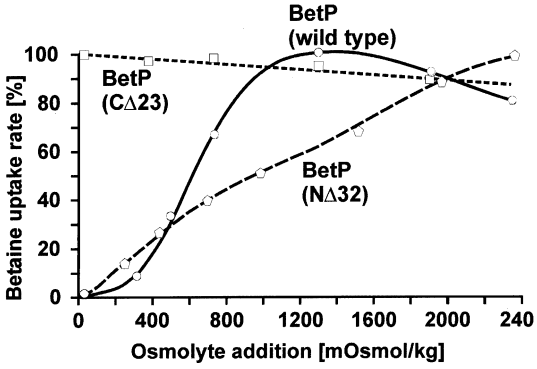

Fig. 3. Activity of BetP and recombinant proteins in intact *C. glutamicum* cells in dependence of added sorbitol. The basic buffer (250 mOsmol/kg) contains 50 mM NaCl. Wild type protein (circles), recombinant BetP, truncated by 23 amino acids in the C-terminal domain (squares), and recombinant BetP, truncated by 32 amino acids in the N-terminal domain (pentagons). Maximum uptake at optimum conditions of external osmolality was set to 100% for each strain.

when heterologously expressed in *E. coli* cells (Peter et al., 1996). Second, after expression in *E. coli* membranes, solubilization, purification and reconstitution into proteoliposomes, BetP is both functionally active in kinetic terms as well as fully competent in its regulatory response to osmotic stress (Ruebenhagen et al., 2000). Attempts were made, to assign the sensory activity of BetP to particular domains of the protein. BetP belongs to the BCCT subfamily of the SSS superfamily of transporters and is characterized by 12 transmembrane segments as well as a highly negatively charged hydrophilic N-terminal domain of approximately 62 amino acids and a highly positively charged hydrophilic C-terminal domain of 55 amino acids. By selective truncations of the two terminal domains individually and in combination, it has been shown that they are involved in sensing the signal related to osmotic stress and/or in its transduction to the catalytic domains of the protein (Peter et al., 1998a). Truncations of the N-terminal domain lead to a shift of the activation threshold, whereas truncations of the C-terminal domain result in a catalytically active carrier that is completely deregulated being active without any osmotic stress (Fig. 3). By a series of constructs, it was furthermore, shown that the presence and the correct location of a particular motif of approximately 12 amino acids or less within the C-terminal domain of BetP seems to be crucial for the function of this domain in osmosensing.

Whereas regulatory effects due to modifications of the hydrophilic terminal domains indicate a signal input from the hydrophilic surrounding, other results suggest signal input pathways from the membrane surrounding. As mentioned above, BetP is fully active and regulatory competent when inserted in *E. coli* membranes. Under these conditions, however, we observed a drastic shift of the activation threshold to lower osmolalities (Peter et al., 1996). It is suggestive to relate this finding to the significantly lower turgor of Gram-negative in comparison to Gram-positive cells. Turgor as the parameter triggering BetP activity was ruled out, however, by the finding that BetP is fully competent in proteoliposomes. On the other hand, experiments in the reconstituted system showed a direct influence of the membrane composition on the regulatory properties of BetP. When the phospholipids of the proteoliposomes were changed from an *E. coli* type to a *C. glutamicum* type of composition, the threshold of activation shifted correspondingly as observed in the case of intact *E. coli* and *C. glutamicum* cells, respectively. Furthermore, it was possible to modulate the regulatory response of BetP both in intact cells as well as in proteoliposomes by addition of the local anesthetic tetracaine, which is thought to act directly by influencing the physical state of the membrane in which the carrier protein is embedded.

Further insights into general and protein-specific aspects of osmosensing and regulation were achieved by comparative studies on the EctP carrier of *C. glutamicum*. EctP belongs to the same protein subfamily as BetP and responds to osmotic stress in a closely similar manner. The basic structural properties of BetP and EctP are similar as well, however, the terminal domains differ significantly, both in terms of charge and size (Fig. 4). The N-terminal domain of EctP is

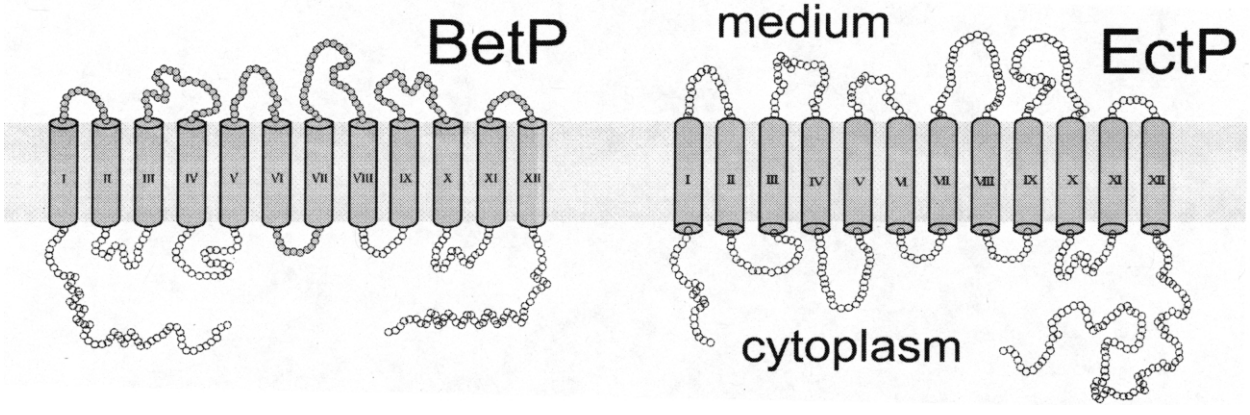

Fig. 4. Putative topology of BetP and EctP from *C. glutamicum.*

short (25 amino acids) and lacks a significant net charge, whereas the C-terminal domain is twice as long as that of BetP (108 amino acids) and is predominantly negatively charged. Nevertheless, truncations of the N- and the C-terminal domain of EctP lead to similar effects as in the case of BetP, i.e. to a shift of the activation threshold and to deregulation, respectively. Most interestingly, hybrid constructs of the individual membrane parts of BetP and EctP each with combinations of the corresponding terminal domains show that these domains can to a large extent fulfill their regulatory function even when fused to the heterologous membrane parts.

In summary, the experiments using BetP and EctP both in intact cells and in proteoliposomes indicate: (i) that the two carrier proteins combine both osmosensing and osmoregulatory activity; (ii) that the input of the signal related to osmotic stress possibly occurs via the hydrophilic as well as hydrophobic surrounding of the membrane-embedded carrier protein; and (iii) the N- and C-terminal domains of at least BetP and EctP of *C. glutamicum* are directly or indirectly involved in osmosensing.

4. Osmosensing and osmoregulatory compatible solute accumulation by *Escherichia coli*

Our understanding of bacterial osmoregulation advanced rapidly during the 1980s as genetic, molecular biological and biochemical tools were used to elucidate the osmoregulatory mechanisms present in the enteric, Gram-negative bacteria, *E. coli* K-12 and *Salmonella typhimurium* LT-2 (Csonka, 1989; Csonka and Hanson, 1991). Advances made during that period included the identification of osmoregulatory transporters ProU (an ABC transporter), ProP and BetT (secondary transporters). These systems serve as prototypical mediators of osmoprotection by such exogenous organic solutes as proline, glycine betaine and proline betaine (ProP and ProU) and for the uptake of choline, the biosynthetic precursor of glycine betaine (BetT and ProU). More recently, mechansosensitive channels MscL and MscS were shown to be essential mediators of solute release in response to osmotic down-shifts (Levina et al., 1999). The roles of these and other systems in the physiology of osmotic stress adaptation by *E. coli* have been discussed extensively (Blount and Moe, 1999; Booth and Louis, 1999; Csonka and Epstein, 1996; Kempf and Bremer, 1998; Wood, 1999). Current research foci include the mechanism by which transporter ProP senses and responds to osmotic upshifts and the role of osmoregulatory compatible solute accumulation in virulence for uropathogenic *E. coli*.

4.1. A model for the osmotic activation of ProP

The recent purification and reconstitution of transporter ProP in proteoliposomes led to the

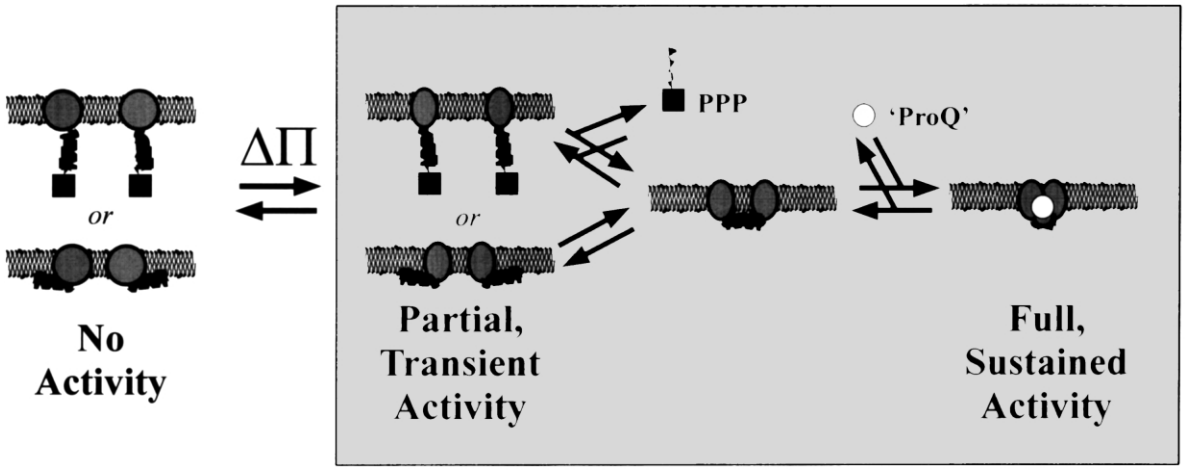

Fig. 5. Model for the osmotic activation of ProP*Ec.* Black-rimmed grey circle or ellipse, ProP; Black Box, PPP (the Putative Partner Protein); Black-rimmed white circle, 'ProQ' (ProQ, or a protein whose presence requires ProQ). At an unidentified, low medium osmolality, ProP is inactive. The C-terminus of ProP is free, associated with PPP and/or associated with the membrane surface. Osmotic upshifts change ProP from an inactive to a more active conformation. This potentiates formation of a homodimeric α-helical coiled-coil by two adjacent ProP proteins and partial activation. 'ProQ' binds to the resulting ProP homodimers causing a further conformational change and stabilizing a fully active conformation of ProP (see text for further details and supporting data).

demonstration that ProP, alone, can sense and respond to osmotic upshifts (Racher et al., 1999). Nevertheless, soluble protein ProQ is required for full osmotic activation of ProP, in vivo (Kunte et al., 1999). These observations and others must be accommodated as we formulate complete mechanisms for both osmosensing and osmotic activation. The critical observations are summarized below, as are the features of a working model for the osmoregulation of ProP activity (Fig. 5).

Different conditions are required to attain maximal activation of ProP in the intact cell, membrane vesicle and proteoliposome systems. In vivo an osmotic upshift of 0.2 mol/kg (NaCl or sucrose) yields optimal activity for wild type ProP and its His-tagged variant [ProP(His)$_6$, to which six C-terminal histidine residues have been added] (Culham et al., 2000; Milner et al., 1988; Pelletier et al., 1999). In contrast, an osmotic upshift of 0.8 mol/kg (NaCl or sucrose) is required to activate wild type ProP in cytoplasmic membrane vesicles (Marshall, 1996). Larger osmotic upshifts yield lower activities in both of these systems. This may occur because large osmotic upshifts impair respiration, which provides the proton motive force in these systems. In the presence of a constant membrane potential, ProP(His)$_6$ in proteoliposomes is activated maximally by an upshift of approximately 0.3 mol/kg (NaCl or sucrose). Larger osmotic upshifts neither increase nor decrease activity (Racher and Wood, unpublished data). The half time for activation of ProP or ProP(His)$_6$ in vivo and in membrane vesicles is approximately one min (Culham et al., 2000; Milner et al., 1988). In proteoliposomes it is less than 10 s (too short to be more precisely defined by existing methods). These differences in osmotic

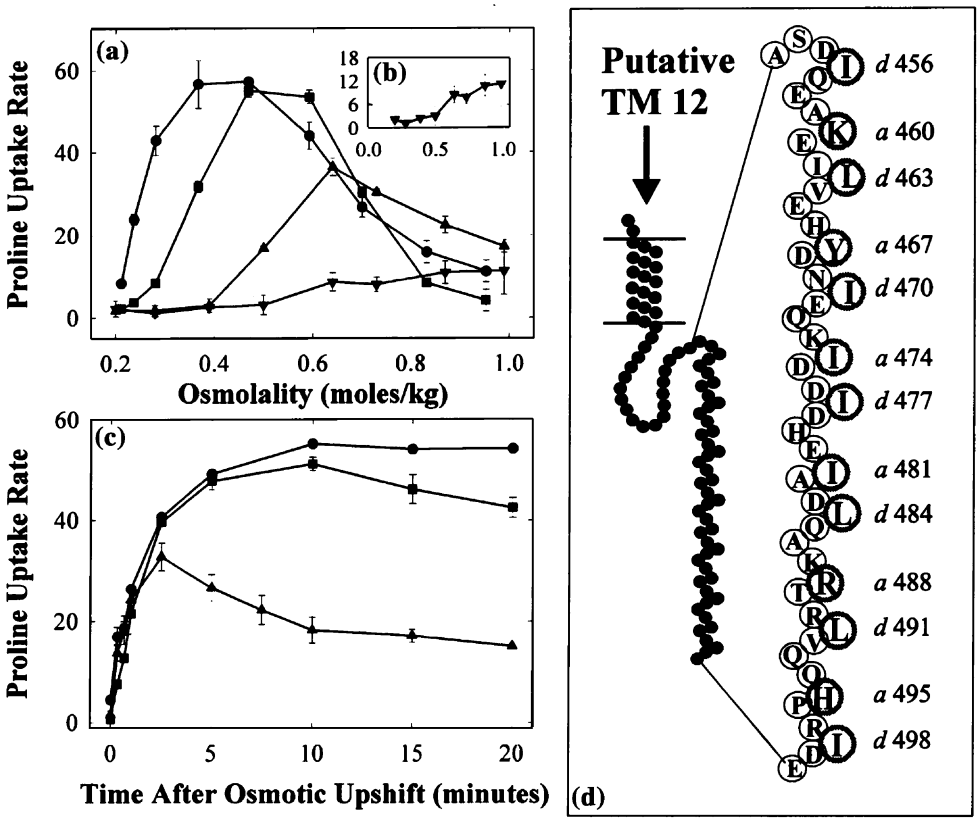

Fig. 6. Impact of C-terminal amino acid replacements on the osmotic activation of ProP*Ec.* Initial rate of proline uptake are reported in nmoles/min/mg cell protein. (a) Osmotic activation profile for transporter variants ProP (circles), ProP-I474 (b) (inverted triangles), ProP-R488I (triangles) and ProP-K460I Y467I H495I (squares) (reproduced from (Culham et al., 2000). (b) inset showing profile for ProP-I474P. (c) Osmotic activation kinetics for transporter variants ProP (circles), ProP-R488I (triangles) and ProP-K460I Y467I H495I (squares) (reproduced from (Culham et al., 2000). (d) Structure of the carboxyl terminal domain of ProP, showing the interfacial '*a*' and '*d*' positions of the homodimeric α-helical coiled-coil formed by a peptide replica (Culham et al., 2000).

upshift optimum and activation kinetics may result from structural differences among whole cells, cytoplasmic membrane vesicles and proteoliposomes (see below). It is also possible that the turnover number attained by ProP$(His)_6$ in proteoliposomes falls far short of that reached in intact cells.

ProP includes an extended, hydrophilic carboxyl terminal domain (46 amino acids) (Fig. 6d). A peptide replica of that structure can form a homodimeric α-helical coiled-coil of moderate stability in vitro (Culham et al., 2000). This behaviour was expected since residues I456 through E500 of ProP show the heptad repeat sequence characteristic of coiled-coil structures (Culham et al., 1993) (Fig. 6d). It is not yet known whether formation of an analogous structure mediates dimerization of the intact transporter, in vitro or in vivo. ProP variants lacking 26 C-terminal amino acids or including amino acid substitution I474P are expressed but very poorly active, in vivo (Culham et al., 2000) (Fig. 6a,b). These modifications would completely disrupt coiled-coil formation. Thus, the C-terminal domain (and perhaps homomeric coiled-coil formation) appears to be required for activation of ProP.

ProP residues K460, Y467, I474, I481, R488 and H495 occupy the critical heptad '*a*' positions of the C-terminal coiled-coil (Fig. 6d). Isoleucine or leucine at the '*a*' position is expected to promote coiled-coil formation and stability. Amino acid replacement R488I elevated the ProP activation threshold and rendered activation transient (Fig. 6a and c). The same replacement disrupted coiled-coil formation by a peptide corresponding to the ProP C-terminal domain (Culham et al., 2000). This unexpected result suggests that the coiled-coiled formed by the ProP C-terminal domain, in vitro, is unusual in structure. Mutations K460I Y467I H495I stabilized and extended the coiled-coil formed by a peptide corresponding to the ProP C-terminus. This stabilization is consistent with studies based on model peptides that form, parallel homodimeric coiled-coils. ProP-K460I Y467I H495I showed a moderately elevated activation threshold and a slightly reduced duration of activation compared to the wild type transporter (Fig. 6a and c). A transporter incorporating all four replacements (ProP-R488I K460I Y467I H495I) was similar in activity to that harbouring the R488I replacement, alone. In addition to modulating homodimeric coiled-coil formation, these amino acid replacements may influence interactions between ProP and heterologous regulatory elements, perhaps including heteromeric coiled-coil formation.

Mutation *proQ220*::Tn*5* impairs the extent and rate of ProP activation, in vivo, without influencing its transcription or level of expression (Kunte et al., 1999; Milner and Wood, 1989). A hydrophilic protein (calculated MW 26 kDa) with acidic and basic ends and a calculated pI of 9.7, ProQ co-purifies during ion exchange, exclusion and DNA-cellulose chromatographies with DNA binding proteins (HU and a histone-like protein) (Crane and Wood, unpublished data). It has no strong sequence homologues and no obvious DNA binding motifs. Since ProP can undergo osmotic activation after purification and reconstitution in proteoliposomes, ProQ is not absolutely required for ProP activation. Nevertheless, maximal and/or sustained activation of ProP, in vivo, may require interaction with ProQ or a protein whose expression depends upon ProQ.

The working model illustrated in Fig. 5 is designed to account for these observations. At an unidentified, low medium osmolality in vivo or in the absence of an osmotic upshift (in vitro), ProP is inactive. Under these conditions the C-terminus of ProP is free, it forms a heteromeric α-helical coiled-coil with an unidentified partner protein (the Putative Partner Protein or PPP) and/or it associates with the membrane surface. Osmotic upshifts trigger a conformational change in ProP (from an inactive to a more active conformation) by acting directly on the protein or by causing changes in the membrane that are transmitted to ProP.

The conformational change triggered by an osmotic upshift potentiates release of the ProP C-terminal domain from other associations (if present) and formation of a homodimeric α-helical coiled-coil by the C-terminal domains of two adjacent ProP proteins. Activation to this stage is partial. The resulting homo-dimers remain unstable in whole cells, but they are reasonably stable in proteoliposomes, perhaps due to confinement. The activation of ProP in vivo is slower than the activation of ProP in proteoliposomes because activation in vivo requires structural rearrangements that are absent from the proteoliposome system.

ProQ [or a protein ('ProQ') whose expression depends upon ProQ] binds to ProP homodimers

created through C-terminal homodimeric coiled-coil formation, but not to the ProP monomer. This interaction causes a further conformational change that stabilizes a fully active conformation of ProP. C-terminal truncation of ProP and the I474P replacement prevent formation of this fully active ProP dimer. Replacement R488I disrupts homodimerization (and may favour heteromerization with PPP or association with the membrane surface), hence inhibiting homodimerization and the association with 'ProQ'. It impairs the transition to the partially active conformation and prevents stabilization of the fully active conformation by 'ProQ'. Replacements K460I Y467I H495I favor homodimerization but also disrupt 'ProQ' association with homodimeric ProP. Thus full activation occurs, but activation is not sustained as it is with wild type ProP. Elimination of 'ProQ' prevents the full and stable activation of ProP. This model focuses attention on a number of important questions that are now being addressed experimentally.

4.2. Compatible solute accumulation by uropathogenic E. coli

Since mammalian urine undergoes broad diurnal variations in salinity and osmolality, it is no surprise that both kidney cells and uropathogenic bacteria (usually *E. coli*) possess powerful osmoregulatory mechanisms (Burg, 1997; Chambers and Lever, 1996). Glycine betaine and proline betaine are present in human urine as a result of osmoregulation by kidney cells and dietary intake, respectively. Chambers and Kunin were the first to propose that betaine-based osmoregulatory mechanisms promote the growth of uropathogenic *E. coli* in mammalian urinary tracts (Chambers and Kunin, 1985). In an effort to test that hypothesis, the genes encoding transporters ProP and ProU were deleted from two human pyelonephritis isolates. The effects of these lesions on bacterial growth in high osmolality human urine and on colonization of the murine urinary tract were more limited than had been anticipated (Culham et al., 1994, 1998, 2001; Culham et al., unpublished data). Subsequent osmoprotection studies and transport measurements have revealed that one isolate possesses an additional transporter, distinct in K_M for glycine betaine (22 μM) and substrate specificity (glycine betaine and proline betaine but not proline) from transporters ProP and ProU (Culham et al., unpublished data). Although the other isolate appears to lack that system (designated BetU), it utilizes another, unidentified osmoprotectant (or osmoprotectants) also present in human urine. Sequence analysis has now revealed that the genome of *E. coli* K-12, the laboratory isolate that was the object of these early studies, is smaller than those of various wild-type *E. coli* strains (Bergthorsson and Ochman, 1995). DNA insertions, deletions and polymorphisms are now known to differentiate *E. coli* K-12 and various *E. coli* virotypes (Culham and Wood, 2000; Hurtado and Rodríguez-Valera, 1999; Rode et al., 1999). Future studies will reveal whether the additional osmoregulatory transport activities found in the pyelonephritis isolates are parologues of systems now identified in other organisms, and whether their apparent expression by only some *E. coli* strains results from differences in gene complement or in the regulation of gene expression. They will also allow further testing of the hypothesis that osmoregulatory compatible solute accumulation contributes to the growth of *E. coli* in mammalian urinary tracts.

5. Osmosensing and osmoregulatory compatible solute accumulation by *Lactobacillus plantarum* and *Lactococcus lactis*

The Gram-positive bacteria *Lactobacillus plantarum* and *Lactococcus lactis* predominantly use the organic compatible solutes, glycine betaine or carnitine, to respond to hyperosmotic stress. The high turgor pressure (ΔP) in *Lb. plantarum* and *L. lactis* stems to a large extent from the high concentrations of potassium (> 1 M) plus corresponding counterions inside the cell even at low medium osmolarities. In fact, the concentrations of potassium do not change very much as a function of medium osmolarity or upon imposition of an osmotic upshock. The growth of *Lb. plantarum* and *L. lactis* is severely inhibited by hyperosmotic stress when a suitable organic compatible solute, such as glycine betaine or carnitine, is not available. In the presence of either of these organic compatible solutes, the growth inhibition occurs at much higher stresses (Glaasker et al., 1996b, 1998b; Molenaar et al., 1993). Since lactic acid bacteria have limited biosynthetic capacities, the organic compatible solutes need in almost all

cases to be taken up from the medium. Quaternary ammonium compounds, like glycine betaine or carnitine, are preferred by *Lb. plantarum* and *L. lactis* but proline, glutamate and a few others are also used by these bacteria to raise their internal osmolarity.

5.1. Response to hyperosmotic stress

Gene inactivation studies have shown that single hyperosmotic stress-activated transport systems protect *Lb. plantarum* and *L. lactis* against a low water activity (hyperosmotic stress). In *Lb. plantarum*, system QacT accepts various quaternary ammonium compounds with high affinity and proline with low affinity (Glaasker et al., 1998a). Activation by hyperosmotic stress is mainly at the enzyme level, increased activity (V_{max}), and not so much at the level of gene expression. Since the rate of accumulation of glycine betaine by *Lb. plantarum* decreases with increasing internal concentration of the compatible solute, and this apparent inhibition of uptake by *trans*-substrate is diminished upon osmotic upshock, the 'activation mechanism' most likely involves regulation of the transporter through conformational changes at an internal binding site (Glaasker et al., 1998a). A similar mechanism of osmotic regulation is thought to be operative in *Listeria monocytogenes* (Verheul et al., 1997), whereas the phenomenon of *trans*-inhibition is not evident from studies on the osmotic activation of the transporter (OpuA) that mediates glycine betaine accumulation in *L. lactis* (van der Heide and Poolman, 2000a). How osmotic stress is sensed by these transporters and how the signal(s) are converted into an activity change of the transporters is not known. These issues may soon be resolved for system OpuA, as the corresponding genes have been isolated and the proteins purified (Obis et al., 1999; van der Heide and Poolman, 2000b). In *L. lactis* IL1403, hyperosmotic conditions not only result in a stimulation of the activity of individual OpuA molecules, but also in a more than 10-fold increase in the expression of the genes encoding OpuA (Obis et al., 1999; van der Heide and Poolman, 2000a).

The glycine betaine transport system of *L. lactis* belongs to the superfamily of ATP-binding cassette (ABC) transporters, but it has unique structural features that had not yet been recognized among its members. The OpuA system consists of an ATP-binding/hydrolyzing subunit (OpuAA) and a protein (OpuABC) that contains both the translocator and the substrate-binding domain (Fig. 7). Typical prokaryotic binding protein-dependent transporters of the ABC-type are composed of five protein(s) (domains), i.e. an

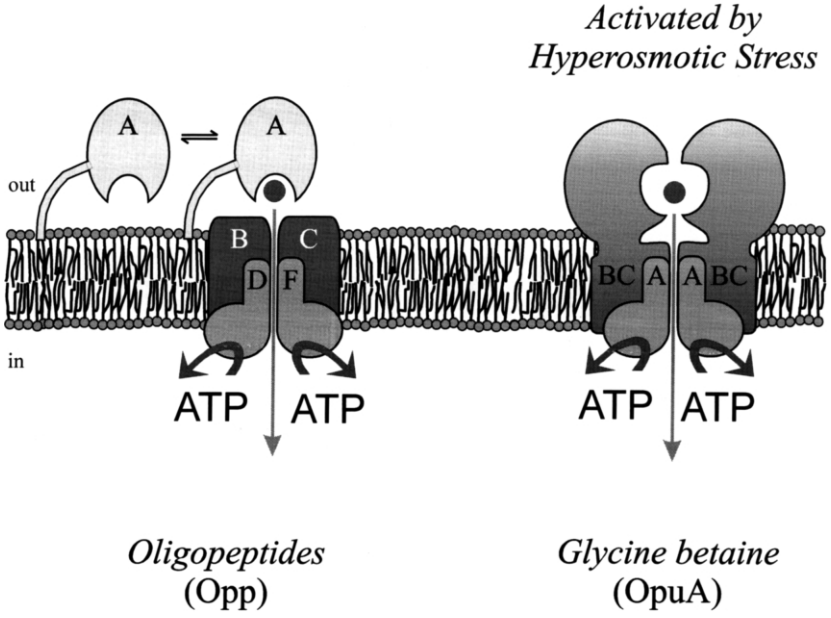

Fig. 7. Schematic representation of the domain organization of the OpuA transporter of *L. lactis*. The oligopeptide transporter (Opp) of *L. lactis*, which also belongs to the ABC superfamily, is shown for comparison.

extracellular binding protein (receptor), two ATP-binding subunits and two integral membrane subunits. Except for the substrate binding protein, the other subunits can be present as distinct polypeptides or fused to one another but always each entity is present twice (Fig. 7). In analogy with other ABC-transporters, functional OpuA will most likely be composed of two transmembrane subunits and two ATP-binding subunits. Since OpuABC has the translocator fused to the substrate-binding domain, the oligomeric structure implies that also two receptor domains are present. This raises questions regarding the observations that only a single substrate binding-protein interacts with the dimeric membrane complex, and that two lobes of a single substrate binding protein interact with different integral membrane protein(s) (domains) (van der Heide and Poolman, 2000a). The oligomeric structure of OpuA of *L. lactis* is not unique as database searches indicate that similar gene clusters, encoding putative glycine betaine ABC-transporters, are present in *Streptomyces coelicolor, Streptococcus pneumonia, Chlamidia pneumoniae,* and *Helicobacter pylori.* At present, it is not known whether the unusual domain rearrangement of OpuA is related to its osmosensing/regulatory activity.

The mechanism of osmotic activation of OpuA has been studied in proteoliposomes, that is, after purification of the proteins and incorporation of OpuA into liposomes with an ATP-regenerating system inside the vesicle lumen (van der Heide and Poolman, 2000b). These studies indicate that a transmembrane osmotic gradient (outside hyperosmotic relative to inside) of both ionic and non-ionic compounds activates OpuA by affecting some physical property of the lipid bilayer in which the system is embedded. Moderate hypo-osmotic medium conditions, that do not lyse the proteoliposomes, inhibit the basal activity of the system, which is in line with observations made for the glycine betaine transporter of *Lb. plantarum* (Glaasker et al., 1996a). Thus, OpuA is sufficient for osmoregulated transport and the actual activity is set by the difference in external and internal osmolarity, indicating that the system can act both as osmosensor and osmoregulator without the need for additional extramembranous factors. Strikingly, OpuA is also activated by low concentrations of cationic and anionic amphipaths, interfacially active solutes that interact with the membrane. This indicates that the activation by a *trans*-membrane osmotic gradient is mediated by changes in membrane properties/protein-lipid interactions. Further support for this notion comes from an analysis of the morphology and volume of proteoliposomes under conditions of osmotic stress. Since proteoliposomes do not have a rigid wall or skeleton to set off differences in internal external osmolarity, the vesicles shrink/swell in response to the osmotic gradient and behave as (semi-)perfect osmometers. Shrinkage of the proteoliposomes imposed by hyperosmotic stress results in a decrease in the internal volume of the vesicles, which reduces/abolishes the imposed transmembrane osmotic gradient (unpublished results). As membrane-reconstituted OpuA remains activated for much longer periods of time than required for the vesicles to shrink upon osmotic upshock, the transmembrane osmotic gradient cannot be the trigger for activation of OpuA. It suggests that a global bilayer property such as the curvature of the membrane or related physical factor changes in response to the initial difference in the external and internal osmolarity, and that this parameter is pivotal in the activation mechanism.

Recent theoretical studies suggest that the distribution of the lateral pressure in a lipid bilayer is strongly affected by the incorporation of amphipaths into the membrane as well as by an altered lipid composition (Cantor, 1997). In these studies, lateral pressure or curvature stress is a function of the depth normal to the protein in the membrane plane, and it is speculated that variations in this parameter may be coupled to conformational changes in proteins (Cantor, 1997). Our recent studies indicate that the activation of OpuA can be manipulated, amongst others, by varying the headgroup composition of the lipids in the liposomes. In fact, the threshold value for osmotic activation can be at zero ('constitutively active'), intermediate or infinite ('no activation') value as a function of 'apparently' modest changes in the lipid composition of the membrane. Future work should indicate how transmembrane osmotic gradients, lateral pressure profiles, membrane curvatures, protein (OpuA) conformational changes and transport activities all relate to each other.

5.2. Response to hypo-osmotic stress

It has been shown for a number of microorgansims that compatible solutes are rapidly released

from the cells upon a hypoosmotic shock. For instance, in *E.coli* a rapid release of K^+, glutamate and trehalose is observed upon an osmotic downshock, whereas solutes such as alanine, lysine, arginine and sucrose are fully retained by the cells (Schleyer et al., 1993). When *Lb. plantarum* is subject to an osmotic downshock, a rapid efflux of glycine betaine, proline and some glutamate occurs whereas the pools of other amino acids remain unaffected (Glaasker et al., 1996b). Although the molecular nature of these efflux activities is still not precisely known, the systems exhibit properties that mimic mechanosensitive channels (Martinac et al., 1990; Sukharev et al., 1997). Some features that discriminate the systems from 'ordinary' carrier proteins, driven either by ATP or electrochemical ion gradients, are the following: (i) efflux is extremely fast and effected by an osmotic downshock as well as amphipaths that insert into the membrane; (ii) efflux is independent of metabolic energy; (iii) efflux is unaffected by substrate at the trans site of the membrane; and (iv) in many cases efflux is inhibited by gadolinium ions (Gd^{3+}), an unspecific channel blocker.

The efflux of glycine betaine and proline by *Lb. plantarum* upon osmotic downshock is characterized by two kinetic components, i.e. a fast one with a $t_{0.5} < 1$ s and a slow one with a $t_{0.5}$ of 4–5 min (Glaasker et al., 1996a). Similar observations have been made for the efflux of glycine betaine and carnitine in *L. monocytogenes* and *L. lactis* (van der Heide and Poolman, 2000a; Verheul et al., 1997). The component with the slow kinetics is affected by the metabolic state of the cell and may represent a specific efflux system. The kinetics of the rapid efflux component is too fast to be analyzed accurately, but an estimate of the turnover number of the putative channel in *Lb. plantarum* indicates that it releases glycine betaine with a rate of more than 10^5–10^6 s^{-1}, which is orders of magnitude faster than the turnover number of known carrier proteins (10^{-1} to at most 10^3 s^{-1}). It thus seems likely that the observed rapid efflux of compatible solutes upon hypoosmotic shock corresponds to that of one or more mechanosensitive channel proteins. In *L. lactis* there are most likely only two mechanosensitive channel proteins, that is MscL and a homologue of the *E. coli* YggB (Levina et al., 1999). MscL of *L. lactis* has been purified to homogeneity and its role in osmoregulation and molecular properties are currently being analyzed (unpublished results).

6. Osmosensing and osmoregulatory compatible solute accumulation by *Listeria monocyogenes*

Despite fundamental differences between Gram-positive and Gram-negative bacteria, many Gram-positive bacteria seem to osmoregulate much like *E. coli*. One example is *Listeria monocytogenes*, a food-borne pathogen that grows well over a wide range of temperatures, salt concentrations and pH (Farber and Peterkin, 1991; Walker et al., 1990). This species has been isolated from a variety of foods, particularly fresh and processed meats and dairy products (Farber and Peterkin, 1991). It is an especially insidious pathogen because it is both highly salt tolerant and psychrotrophic and thus can survive and grow under refrigeration (Walker et al., 1990). That *L. monocytogenes* utilizes an osmoregulatory mechanism to adapt to environments of elevated osmolarity was determined using natural abundance

Table 3
Effect of osmolytes on the growth of stressed *L. monocytogenes*

Temp. (°C)	NaCl (%)	Growth Rate (h/gen) with the following added		
		None	GB	CAR
30	0	3.0	3.2	3.1
	4	9.7	4.8	5.5
	8	> 150	7.4	15
7	0	51	31	38
	4	NG	40	48

Cultures were grown in modified Pine's medium (Ko et al., 1994) with 1 mM glycine betaine (GB) or carnitine (CAR) where shown. NG indicates that no growth was observed.

^{13}C-NMR spectroscopy. In this way osmolytes in the cytosol could be identified and quantified. Extracts of *L. monocytogenes* cultures grown in 8% NaCl were found to contain significant levels of either glycine betaine or carnitine or both and to a lesser degree glutamate (Ko et al., 1994; Smith, 1996). Also, the presence of glycine betaine or carnitine in the growth medium was found to confer salt tolerance upon the cell (Table 3). Not only are osmolytes accumulated in cultures grown in laboratory media, but this accumulation also occurs in *L. monocytogenes* grown in foods (Smith, 1996), which suggests that the osmoregulatory mechanism is as important in foods as it is in laboratory growth media. Although this mechanism of osmotic adaptation is similar to that in *E. coli*, a unique property of *L. monocytogenes* is that it accumulates osmolytes in response to chill stress, as well. More importantly, these osmolytes protect the cell against chill stress (Table 3).

Intracellular osmolyte accumulation is not observed if glycine betaine or carnitine is omitted from the growth medium, indicating that their accumulation is due to stress-activated transport, an examination of which revealed two uptake systems for glycine betaine. The first, Porter I, was characterized in vesicles prepared by a modification of the method described by Konings and coworkers for Gram-positive bacteria (Konings et al., 1973). Uptake of ^{14}C glycine betaine could be driven in these vesicles by addition of ascorbate and catalytic amounts of PMS (Gerhardt et al., 1996). Moreover, the active transport observed generated approximately a 1000-fold gradient (inside:outside) in glycine betaine concentration (Gerhardt et al., 1996). The K_M value for glycine betaine (4.4 μM) was comparable to that observed in whole cells. Glycine betaine transport in these vesicles was found to be dependent upon the presence of a hyperosmotic gradient and Na^+. The dependence on Na^+ followed Michaelis–Menten kinetics and yielded K_M values for NaCl (presumably Na^+) of 75 and 200 mM, when the osmotic gradient was provided by sucrose or KCl, respectively (Gerhardt et al., 1996).

The fact that transport generated a 1000-fold concentration gradient of glycine betaine, which is thermodynamically unfavorable, indicates that transport must be coupled to a favorable process. To determine the nature of the energy coupling under these conditions, artificial gradients were employed (Gerhardt et al., 1996). By using K^+/valinomycin in combination with dilution into buffers of different pH, it was possible to generate $\Delta\Psi$, ΔpH, or $\Delta\Psi$ plus ΔpH, and by monitoring the accumulation of ^{14}C-glycine betaine under these conditions it was found that the $\Delta\Psi$ component, positive on the outside, was required for transport. Further experiments using other ion-selective ionophores offered support for this conclusion (Gerhardt et al., 1996). This information clarified the role of Na^+ in glycine betaine transport: coupling glycine betaine transport to the $\Delta\Psi$-driven influx of Na^+ ion allows the electric gradient to power transport of the neutral solute glycine betaine, as well. Thus, the system is not only membrane-potential driven because of the charge on the sodium, but the sodium gradient itself also provides a driving force.

To function effectively in osmotic protection, the incoming Na^+ ion would have to be removed from the cell, most likely by a system such as the Na^+/H^+ antiporters found in *E. coli* (Shimamoto et al., 1994) and *Bacillus* sp (Kitada et al., 1994). The protons that exchange for Na^+ would presumably be exported by the electron transport system, which is responsible for generating the protonmotive force.

It is clear that this transport system is relatively effective only when the osmotic stress is provided by or accompanied by sodium ion. Furthermore, the Na^+-coupled glycine betaine transport system could not be shown to be activated by cold: an Arrhenius plot over the range of 4–15°C indicated normal behavior (Gerhardt et al., 1996). Hence, this transport system cannot explain either the osmotically-activated transport observed at low Na^+ concentrations or the chill-activated transport found in whole cells.

The possibility of an additional glycine betaine transport system was investigated in vesicles prepared using a method devised for Gram-negative bacteria (Prossnitz et al., 1989). Vesicles from cells grown under osmotic stress provided by sucrose, and assayed in the absence of Na^+revealed the presence of a second glycine betaine transporter, Porter II (Gerhardt et al., 2000). This system could be energized by using ascorbate/PMS, and it could be activated by a hyperosmotic gradient of sucrose or KCl. In contrast to Porter I, a requirement for Na^+ could not be demonstrated in Porter II. Under osmotic activation by KCl or sucrose, Porter II-mediated glycine be-

taine transport decreased with decreasing temperature. An Arrhenius plot of the temperature dependence data under osmotic stress was triphasic, with breakpoints at approximately 18 and 10°C. Above 18°C, the temperature dependence is different for KCl and sucrose activation, and is difficult to interpret. From 18–10°C and 10–1°C, separate activation energies of 18 and 48 kcal/mole, respectively, were observed, regardless of the stressing solute. The temperature dependence of the kinetic behavior closely paralleled the phase transition of the membrane, which was also triphasic as measured by FTIR (Gerhardt et al., 2000).

In the absence of osmotic activation, the temperature dependence of transport was strikingly different. The transport rate was barely detectable at 30°C, but increased steadily as the temperature decreased from 15 to 4°C. An Arrhenius plot of these data shows a negative activation energy over this temperature range, indicating activation of the transport activity by cold. Thus, Porter II is the cold-activated glycine betaine transport system we previously observed in whole cells.

The genes encoding Porter II activity were characterized utilizing a salt and chill sensitive Tn917-LTV3 mutant which was found to be deficient in glycine betaine Porter II activity (Ko and Smith, 1999). When a DNA sequence analysis of the region flanking the transposon insertion was carried out, three closely-spaced open reading frames were identified. Also, a region upstream from the first reading frame was identified which showed homology with promoters from other bacterial glycine betaine transporter genes (Gowrishankar, 1989; Kempf and Bremer, 1995), and a palindromic region 7–59 bp downstream of the last stop codon was found that could function as a transcription terminator. This organization strongly suggested that the three genes, designated *gbuA*, *gbuB* and *gbuC* are arranged in an operon. Furthermore, the protein sequences deduced from these genes showed strong homology to OpuAA, OpuAB and OpuAC and somewhat less homology to ProU, ProV and ProX, the glycine betaine transport systems of *B. subtilis* (Kempf and Bremer, 1995) and *E. coli* (Gowrishankar, 1989), respectively. These transport systems have been shown to belong to the superfamily of ATP-dependent (ABC) transporters. As with other ATP-dependent glycine betaine transporters in Gram-positive organisms, all three polypeptides encoded by the *gbu* genes are thought to be associated with the membrane. The observation that the chill-activated transport activity in vesicles is Na^+-independent and ATP-dependent supports this assumption.

A summary of the current understanding of the

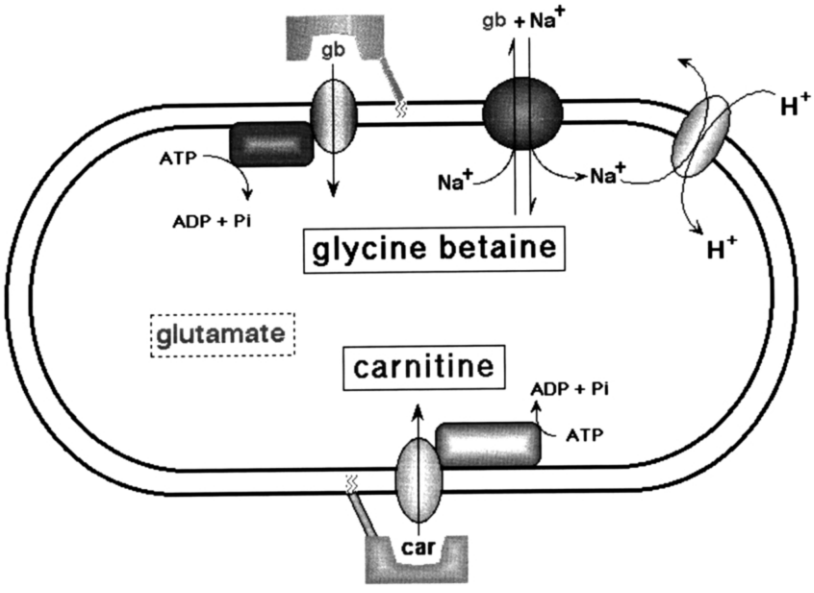

Fig. 8. Schematic representation of the mechanism of osmotic and chill stress adaptation in *L. monocytogenes*.

mechanism of osmotic stress and chill stress adaptation is given in Fig. 8. In addition to the two porters discussed above, recent evidence (Medum and Smith, unpublished data) indicates that a third stress-activated transporter occurs, but it transports carnitine rather than glycine betaine (Fig. 8). Multiple osmolyte transport systems seem to be the rule, rather than the exception in bacteria. However, each transporter functions optimally under a unique set of conditions. Hence, these systems are not merely redundant, but in fact may account for the ability of the cell to prevail in diverse, stressful environments.

7. High osmolality induces increased thermotolerance in *Salmonella*

As discussed in Section 1, exposure of bacteria to high osmolality triggers an array of adaptive responses, including compatible solute accumulation, changes in gene expression, and alterations in cell morphology. Some of these responses, such as the acquisition of compatible solutes, can be easily understood as being beneficial for growth or survival at high osmolalities. However, exposure to high osmolality has another more unexpected consequence: it can confer a marked increase in thermotolerance. The observation that high osmolality confers increased thermotolerance has been seen not only in bacteria (Tesone et al., 1981), but also in animal cells (Sheikh-Hammad et al., 1994), and induction of heat shock proteins by osmotic stress has also been reported in plants (Parek et al., 1995). This response has now been characterized in the bacterium, *Salmonella typhimurium*.

There are at least two experimental ways that thermotolerance can be quantified: as growth rate at non-lethal high temperatures, and as survival at lethal high temperatures. High osmolality, imposed by $\geq$ 0.2 M NaCl or equivalent concentrations of sucrose, increases the heat resistance of wild type *S. typhimurium* LT2 by both of these criteria (Fletcher and Csonka, 1998). This organism is unable to grow in a standard minimal medium (Medium 63) above 43.5°. However, it can grow well (doubling time of 1.5 h) at 45°C if this medium is supplemented with 0.2 M NaCl. Upon exposure to the lethal temperature of 53°, this strain loses viability (colony forming units) with a half-time of approximately 1 min. However, the addition of 0.2–0.3 M NaCl results in a 40-fold increase in the half-time of survival at 53°C.

The 'heat shock response' is a universal adaptive mechanism which involves the induction of thermoprotective proteins by a challenge with sub-lethal high temperatures (Gross, 1996). It could be conjectured that high salinity imparts increased thermotolerance by inducing the heat shock response at low temperature. However, we found that exposure to high osmolality did not result in a detectable induction of one of the main heat shock regulated proteins, GroEL (HSP-60), at 30 or 42°C (Kovar and Csonka, unpublished results). This result suggests that high osmolality induces thermotolerance by some mechanism that is independent of the heat shock response.

In order to probe the connection between osmotic adaptation and increased thermotolerance, we isolated mutants that no longer responded to growth stimulation by high salinity at elevated temperatures. These mutants were generated by random mutagenesis of wild type *S. typhimurium* LT2 with the transposable element Mu*d*I1734, followed by screening by replica plating for derivatives that could not grow at 45°C with 0.3 M NaCl but could grow normally at $\leq$ 42°C with or without NaCl (Cánovas and Csonka, unpublished data). We obtained a total of 11 such mutants. The genes disrupted by the insertions were identified by cloning the regions surrounding the Mu*d*I1734 elements and determining their nucleotide sequences. Each of these disrupted genes matched orthologues in the *E. coli* genome, but the function of 8 of the target genes is not yet known. The remaining 3 insertions are at easily identifiable targets. Two of them are in the *polA* gene, which encodes a protein with $5' \rightarrow 3'$ and $3' \rightarrow 5'$ exonuclease and DNA polymerase I activities required primarily for DNA repair; these two mutations are at independent sites in the DNA polymerase domain. The occurrence of these two mutations indicates that DNA polymerase I is required as a repair enzyme, possibly because the incidence of DNA errors is higher at elevated temperature. The third MudI1734 insertion that we could identify was in the *pgm* (phosphoglucomutase) gene, whose product catalyzes the reversible interconversion of glucose-6-phosphate and glucose-1-phosphate. During growth on glucose, this enzyme is required for the formation of

glucose-1-phosphate, which is the biosynthetic precursor of a large number of sugar moieties in polysaccharides. One of the these is trehalose (*O*-α-D-glucosyl[1 → 1]-α-D-glucoside), which has been shown to be accumulated under conditions of high temperature or dehydration in a number of desiccation-tolerant plants and animals (Crowe and Crowe, 1992). We found that an *otsA* insertion mutation, which inactivates the gene that specifies the first unique enzyme of trehalose biosynthesis, resulted in similar defect in the ability to grow at 45°C as did the *pgm* mutation (L. Csonka, unpublished data), providing direct evidence that the accumulation of trehalose is beneficial for growth at high temperature.

We have also investigated the connection between osmotic adaptation and increased thermotolerance by examining the effects of high osmolality and elevated temperature on protein synthesis. Exposure of the cells to 45°C and 0.2 M NaCl induced high level accumulation of two proteins, with masses of 20 and 35 kDa. We have no information yet about the 35 kDa protein, but we obtained the N-terminal amino acid sequence of the 20 kDa protein (Van Bolgelen and Csonka, unpublished data). Comparison of this sequence with the *Salmonella* genome data-base revealed that this protein belongs to a class of '20 kDa heat shock proteins' whose prototype is the *ibp* gene product first identified in *Buchneria aphicola* (van Ham et al., 1997). We cloned the gene for the *S. typhimurium* orthologue, and we are currently carrying out constructions to disrupt the gene and to test the effect of the disruption on thermotolerance.

It is not clear from our research or from the data in the literature why it is beneficial for cells to be able to acquire increased thermotolerance in response to high osmolality. However, the fact that bacteria acquire increased thermotolerance upon osmotic challenge has implications for the area of food microbiology. Treatment of foods with high concentrations of salt or sugar has been a traditional practice of preservation since ancient times. However, these treatments could antagonize the beneficial effects of cooking for the inactivation of food pathogens, because the increased osmolality could promote the survival of contaminating organisms at high temperatures.

At present, there is no information about the regulatory mechanism that connects the induction of thermotolerance to osmotic adaptation. There have been recent thought-provoking reports about the resuscitation of halotolerant bacteria that may have been trapped for up to 250 million years in a non-growing, dormant form in ancient salt crystals (Grant et al., 2000). Thus, exposure to high salinity might be able to confer cross-protection against not only high temperature stress but also against the deleterious effects of long term starvation. Discovering the regulatory connection between osmotic adaptation and thermotolerance will be one of the main experimental aims of our laboratory in the near future.

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