7 Physiological and Molecular Responses of *Bacillus subtilis* to Hypertonicity: Utilization of Evolutionarily Conserved Adaptation Strategies

GUDRUN HOLTMANN, CLARA D. BOIANGIU, JEANETTE BRILL, TAMARA HOFFMANN, ANNE U. KUHLMANN, SUSANNE MOSES, GABRIELE NAU-WAGNER, NATHALIE PICA, ERHARD BREMER

7.1 Introduction

Variations in the supply of water and the concomitant changes in salinity and osmolality are among the most significant environmental parameters affecting the survival and growth of microorganisms (Galinski and Trüper 1994; Csonka and Epstein 1996; Wood 1999; Bremer and Krämer 2000). Microorganisms can colonize a wide variety of ecological niches with a considerable spectrum of salinitiFs and osmotic conditions that range from concentrated salt brines to fresh water sources (Ventosa et al. 1998). Furthermore, within a single bacterial habitat, there also can be drastic fluctuations from the prevalent osmotic milieu.

Here, we focus on the adaptation of *Bacillus subtilis*, the model organism for Gram-positive bacteria, to changing osmolalities (Kempf and Bremer 1998; Bremer and Krämer 2000). *B. subtilis* is certainly not a halophile, but it can grow over a considerable range of osmotic conditions (Boch et al. 1994) and hence needs effective genetic and physiological adaptation mechanisms to cope with changing osmotic conditions in its habitat. The challenge posed to *B. subtilis* by variations in the environmental osmolality and salinity is vividly illustrated by envisioning the conditions in its common ecological niche, the upper layers of the soil. Drought and rain drastically alter the water supply in this ecosystem (Miller and Wood 1996), thereby threatening the cell with dehydration under hypertonic conditions or rupture under hypotonic circumstances as water permeates across the cytoplasmic membrane along the osmotic gradient. Like many other bacteria (Booth and Louis 1999), *B. subtilis* avoids these devastating alternatives by actively modulating its ion and organic solute pool to retain a suitable level of cytoplasmic water and turgor. These adaptation reactions allow *B. subtilis* to readjust its cellular water content by osmosis and thereby readjust its physiology to the prevalent osmotic conditions in its immediate surroundings.

A. Ventosa (Ed.)
Halophilic Microorganisms

7.2 The Cell and the Surrounding Solvent

Each bacterial cell is enclosed by a semipermeable cytoplasmic membrane that restricts the free movement of most ions and metabolites, but not of water. The total concentration of osmolytes within a cell is generally higher than that in the environment, causing water to flow down its chemical potential into the cell thereby building up a hydrostatic pressure, the turgor (Wood 1999). Maintenance of turgor within physiologically acceptable boundaries is considered essential for cell viability and critical for growth and is thought to provide the mechanical force for expansion of the cell wall (Höltje 1998). In *B. subtilis*, turgor has been estimated at 19 atm (1.9 MPa; Whatmore and Reed 1990).

Microorganisms lack systems for active water transport. Hence, water fluxes across the cytoplasmic membrane are determined by osmotic processes (Booth and Louis 1999) and are accomplished by two distinct mechanisms. Simple diffusion of water across the lipid bilayer is usually sufficient to balance intracellular solute levels, but a much accelerated water transit is achieved by diffusion through water-selective channels, the aquaporins. The *Escherichia coli* aquaporin (AqpZ) serves as a model for bacterial water channels (Calamita et al. 1995) and is a member of the ubiquitous major intrinsic protein family (MIP). Expression studies in *Xenopus* oocytes rigorously established the functions of AqpZ as a water channel (Calamita et al. 1995). Cryoelectron microscopy examinations revealed that AqpZ mediates rapid and large water fluxes in both directions in response to sudden osmotic up- or downshifts (Delamarche et al. 1999), suggesting an important role for this channel-forming protein in the cell's management of water flow across the cytoplasmic membrane. Inspection of the *B. subtilis* genome revealed that it does not encode an AqpZ-related protein (Calamita 2000). Hence, either *B. subtilis* contains no aquaporins at all or it possesses water channels of a yet unidentified type.

7.3 Microbial Strategies for Coping with Hyperosmotic Environments

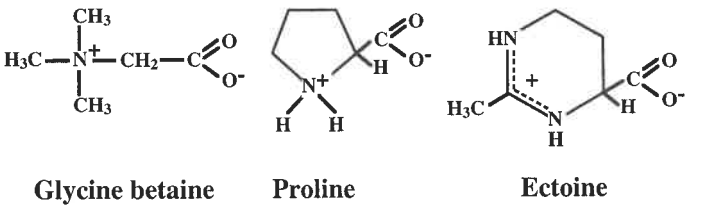
Microorganisms have developed two fundamentally different schemes for maintaining turgor under hyperosmotic conditions. These are frequently referred to as the "salt-in" and "salt-out" strategies (Galinski and Trüper 1994). Microorganisms whose entire physiology has been adapted to a permanent life in high salinity surroundings have adopted the former strategy. They take up molar concentrations of K^+ and Cl^- and they usually actively extrude the cytotoxic Na^+ ions, resulting in a cytoplasmic ion composition substantially

different from the surroundings (Galinski and Trüper 1994; Ventosa et al. 1998). As a result, extremely halophilic microorganisms require high salt concentrations (frequently K^+) for most biochemical reactions, and charge repulsion of the many acid amino acids present in proteins of extreme halophiles frequently leads to cell disintegration at lower salt concentrations. Furthermore, the high ion content of the cytoplasm forced evolutionary changes in essentially all cellular proteins (Dennis and Shimmin 1997). This "salt-in" strategy turns out to be remarkably effective for colonizing of habitats with permanent high salinities, but is far less useful in coping with habitats of moderate salinity or environments that experience drastic fluctuations in their osmotic conditions. Ultimately, the "salt-in" strategy confines to very high saline surroundings.

A more versatile and flexible osmostress response is provided by the "salt-out" strategy (Galinski and Trüper 1994; Ventosa et al. 1998). Bacteria and Archaea that live either in environments of moderate salinity or in ecological niches that are only periodically subjected to conditions of low water activity and increased salinity ubiquitously use this adaptation response. These microorganisms avoid high ionic conditions in their cytoplasm and instead amass large amounts of specific organic osmolytes, the so-called compatible solutes (Brown 1976). These compounds are highly congruous with cellular functions and can be accumulated up to molar concentrations in the cytoplasm without disturbing essential cellular processes and the functioning of cell components. Consequently, they can be effectively used to maintain turgor under hyperosmotic conditions. Because the "salt-out" adaptation mechanism does not require evolutionary adaptation of proteins and cellular processes to high salt concentrations, this response to osmotic stress is prevalent not only in Bacteria (Csonka and Epstein 1996; Ventosa et al. 1998; Wood 1999; Bremer and Krämer 2000) and Archaea (Martin et al. 1999; Roberts 2000), but also in fungal, plant, animal and human cells (Burg et al. 1997; Hohmann 1997; McNeil et al. 1999).

7.4 Compatible Solutes: Characteristics and Physiological Functions

By definition, compatible solutes are compounds that can be accumulated to very high intracellular levels (several moles per liter) without disturbing cellular physiology and protein folding (Brown 1976). Because only a limited number of compounds meet these criteria, it is not surprising that certain compatible solutes have been widely adopted as osmostress protectants across the kingdoms (Le Rudulier et al. 1984). The spectrum of known compatible solutes comprises amino acids, amino acid derivatives, small peptides, methylamines and their sulfonium analogs, sulfate esters, polyols, and sugars



Glycine betaine **Proline** **Ectoine**

Fig. 7.1. Chemical structures of the compatible solutes glycine betaine, proline and ectoine

(da Costa et al. 1998). In the past few years, as more organisms have been examined, in particular those that thrive in extreme environments, additional organic osmolytes have been identified (Martins et al. 1997; Martin et al. 1999). In general, the compatible solutes used by Bacteria are polar, highly soluble molecules, and they usually do not carry a net charge at physiological pH. Glycine betaine, proline, ectoine (Fig. 7.1) – and their structural analogs – are the most important compatible solutes used by *B. subtilis* and taxonomically closely related species (Boch et al. 1994; Kempf and Bremer 1995; Jebbar et al. 1997; von Blohn et al. 1997; Kappes and Bremer 1998; Nau-Wagner et al. 1999; Kuhlmann and Bremer 2002).

Although the exact biochemical mechanism(s) through which compatible solutes act is not completely understood, their functioning is generally explained in terms of the preferential exclusion model (Arakawa and Timasheff 1985). This predicts that compatible solutes are excluded from the immediate hydration shell of proteins because of unfavorable interactions with the polypeptide backbone (Bolen and Baskakov 2001), resulting in a nonhomogeneous distribution of these solutes within the cell water and a preferential hydration of protein surfaces. This disequilibrium provides a thermodynamic force driving the protein to occupy a smaller volume in order to reduce the amount of excluded water and thereby stabilizes the native structure of proteins. In addition to maintaining cellular water content at high external osmolalities, compatible solutes also protect proteins in vitro from denaturation caused by freezing, heating, desiccation and high ionic conditions and they have beneficial effects on membrane integrity, protein stability and folding (Lippert and Galinski 1992; Canovas et al. 1999). Recent in vivo studies support the role of compatible solutes in stabilizing proteins (Bourot et al. 2000) and in protecting cells from the detrimental effects of high salinity and from high and low temperatures (Caldas et al. 1999; Canovas et al. 2001; Mendum and Smith 2002).

7.5 The Initial Stress Response of *B. subtilis*: Uptake of K^+

When *B. subtilis* experiences a sudden osmotic upshift and the resulting loss of cell water, it first responds by rapidly accumulating K^+ from environmental

sources. In cells subjected to an osmotic upshock with 0.4 M NaCl, the K^+ level rises from a basal value of approximately 350 mM to approximately 650 mM within 1 h (Whatmore et al. 1990).

Recently, a new type of potassium transport system (Ktr) has been recognized both in microorganisms and plants. These systems consist of two components: a membrane-spanning subunit (KtrB) that is evolutionarily related to the KcsA potassium channel (Durell and Guy 1999; Durell et al. 1999; Tholema et al. 1999) and a membrane-associated polypeptide (KtrA) that binds both NAD^+ and NADH in different conformations. The interaction of these dinucleotides with KtrA has been proposed to regulate KtrB activity through a ligand-mediated conformational switch mechanism (Roosild et al. 2002). Inspection of the entire *B. subtilis* genome sequence revealed the presence of genes that potentially could encode two Ktr-type K^+-uptake systems (Nakamura et al. 1998). We have investigated the physiological role of the K^+-uptake systems operating in *B. subtilis* and found that there are indeed two major K^+ transporters: the KtrAB transporter with a K_m value of approximately 1.2 mM and the KtrCD transporter with a K_m value of approximately 12 mM (Holtmann et al. 2003). Northern analyses have demonstrated that the structural genes (*ktrAB*) for the KtrAB system are organized as an operon, whereas the genes (*ktrC, ktrD*) encoding the subunits for the KtrCD transporter are positioned at different locations on the *B. subtilis* chromosome and are expressed as single transcription units. Transcription of the various *ktr* genes is not up-regulated in response to K^+ limitation, nor is their expression enhanced when the cells are subjected to high salinity. Gene disruptions that destroy the KtrAB or KtrCD transporters exhibit major defects in K^+ uptake, and a double mutant is almost entirely defective in K^+ acquisition, demonstrating that the two Ktr transporters are the major K^+-uptake systems operating in *B. subtilis*. In contrast to the *B. subtilis* wild-type strain, mutants defective in the KtrAB systems cannot resume growth in a basal minimal medium containing 2 mM K^+ when they are subjected to a sudden osmotic upshock with 0.6 M NaCl. They also exhibit a growth defect when they are continuously cultured in media of increased salinity. These data therefore illustrate the fundamental importance of K^+ acquisition for both initial and sustained adaptation of *B. subtilis* to high salinity and high osmolality surroundings.

Despite the importance of K^+ in the osmostress reaction of *B. subtilis*, high intracellular concentrations of this ion interfere with many important cellular functions (e.g. protein synthesis). Therefore, in the second phase of its osmoadaptation, *B. subtilis* accumulates large quantities of compatible solutes through synthesis and uptake. This permits the reduction of the K^+ pool as the cell expands and proliferates under high osmolality conditions (Whatmore et al. 1990).

7.6 Accumulation of Compatible Solutes Through Biosynthesis in *Bacillus* spp.

7.6.1 Osmoregulatory Synthesis of Proline

Proline plays a crucial role in osmotolerance in many bacteria. Its effectiveness as an osmostress protectant is also demonstrated by plant cells that produce it as a defense against high salinity and drought (Delaunay and Verma 1993). It has been known for over 25 years that *B. subtilis* belongs to the group of microorganisms that accumulate large amounts of proline in response to increased medium salinity (Measures 1975). Whatmore et al. (1990) investigated proline accumulation via de novo synthesis in *B. subtilis* cultures growing in a chemically defined medium. When the cells were subjected to a sudden but relatively mild osmotic upshift by the addition of 0.4 M NaCl, the cellular proline pool increased from a basal level of 16 mM to approximately 700 mM within 7 h. Our own investigations into the pattern of proline synthesis in osmotically stressed cells revealed that there is essentially a linear relationship between the proline content of the cells and the salinity of the growth medium (J. Brill and E. Bremer, unpubl. results). This allows *B. subtilis* to accumulate proline levels well above 1 M when they are grown in a basal minimal medium containing 1 M NaCl.

Recent studies in our laboratory have revealed the molecular details of this osmoregulatory proline synthesis. *B. subtilis* possesses a dedicated pathway for the synthesis of proline under osmotic stress conditions and this route is connected with that used for anabolic purposes (J. Brill and E. Bremer, unpubl. results). The anabolic pathway proceeds from glutamate and is catalyzed by the sequential reactions of ProB (γ-glutamyl kinase), ProA (γ-glutamyl phosphate reductase) and ProI/ProG (Δ^1-pyrroline-5-carboxylate-reductases; Belitsky et al. 2001; Fig. 7.2). The *proHJ* operon, encoding orthologues of the proline biosynthetic *proB* and *proI* genes, is specifically needed for high-level proline synthesis under osmotic stress conditions. In contrast, the ProA protein is used for both proline anabolism and proline-mediated osmoprotection (Fig. 7.2). The *proHJ* operon is transcribed from a SigA-type promoter; its level of expression rapidly increases upon osmotic upshock and is linked in a linear fashion to the degree of osmotic stress. Disruption of the *proHJ* operon does not cause a proline auxotrophic phenotype, but it completely prevents proline buildup in osmotically challenged cells. The *proHJ* mutant exhibits a severe growth defect under high osmolality growth conditions, attesting to the importance of de novo proline synthesis for the osmostress response of *B. subtilis*. Gene fusion studies with the *proHJ* operon have demonstrated that a 153-bp DNA fragment comprising the *proHJ* promoter contains all DNA sequences required in *cis* for full osmoregulated gene

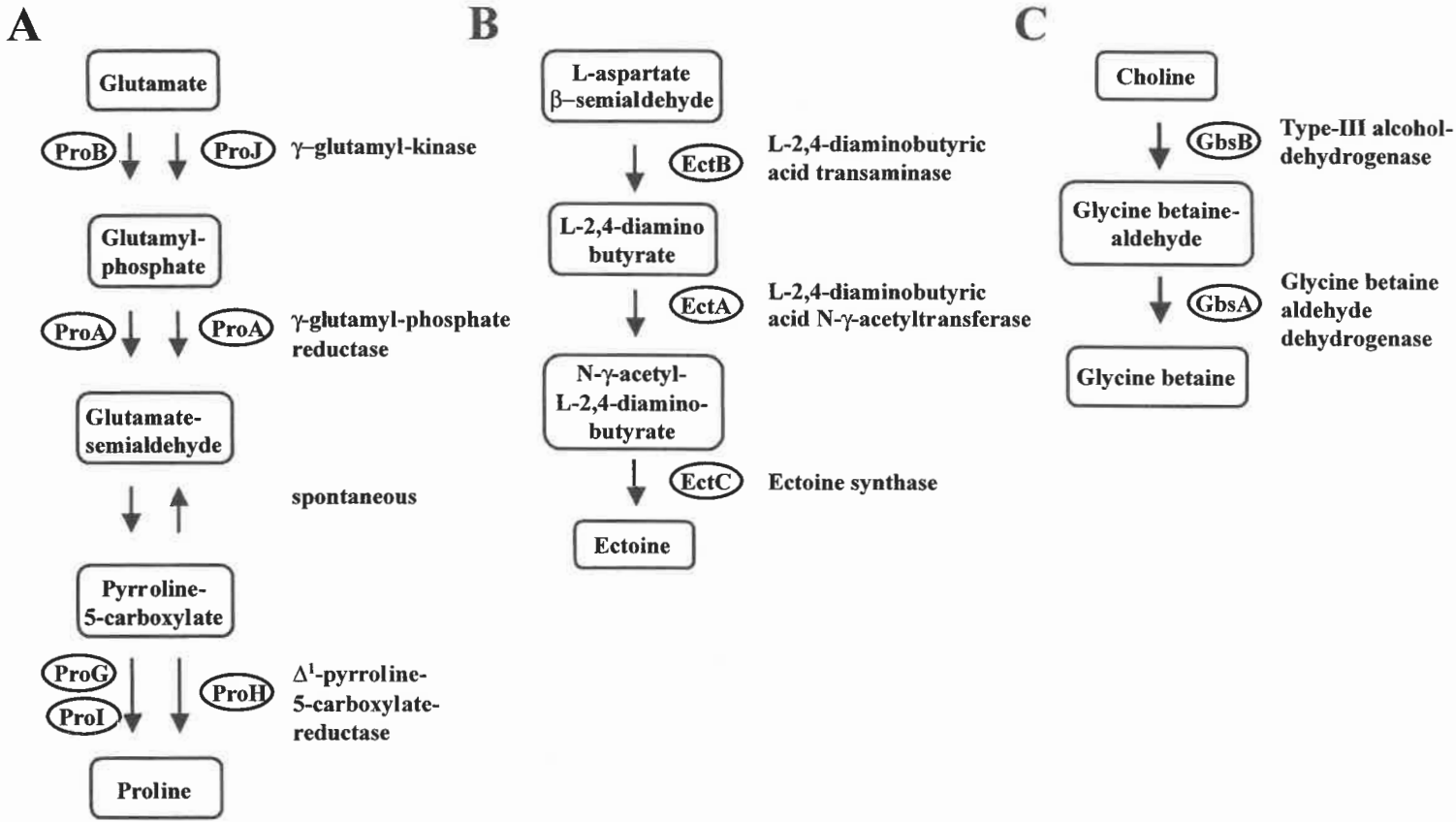


Fig. 7.2. Synthetic pathways for proline, glycine betaine and ectoine in *Bacillus* species

expression; however, the signal transduction cascade that leads to osmoregulated *proHJ* expression is not understood.

7.6.2 Osmoregulatory Synthesis of Ectoine

The identification of *B. subtilis* as a proline producer under conditions of osmotic stress prompted the question of whether most other members of the genus *Bacillus* and closely related species synthesize proline as their dominant compatible solute. We therefore used natural-abundance ^{13}C-nuclear magnetic resonance (NMR) spectroscopy and high-performance liquid chromatography (HPLC) to investigate the types of compatible solutes that are synthesized de novo in a wide variety of *Bacillus* species under high osmolality growth conditions (Kuhlmann and Bremer 2002). These experiments revealed that in addition to proline, ectoine and its hydroxylation derivative, hydroxyectoine, are the dominant compatible solutes synthesized within the genus *Bacillus*. Among the 22 *Bacillus* species investigated so far, we detected five patterns of endogenously synthesized compatible solutes: (1) strains that produce only glutamate; these species are the most salt-sensitive *Bacillus* strains that we have tested, (2) strains that produce proline, (3) strains that produce ectoine, (4) strains that synthesize both ectoine and hydroxyectoine, and (5) strains that produce both proline and ectoine (N. Pica, A. Kuhlmann and E. Bremer, unpubl. results). None of the *Bacillus* species investigated was capable of synthesizing glycine betaine de novo. We note that in particular the halophilic and halotolerant *Bacillus* spp. are ectoine and hydroxyectoine producers.

To analyze osmoregulatory ectoine production in the genus *Bacillus* in greater detail, we chose *Bacillus pasteurii* (recently reclassified as *Sporosarcina pasteurii*) as a representative of ectoine-synthesizing *Bacillus* spp. Growth of *B. pasteurii* in minimal media of different salinities revealed an essential linear relationship between ectoine content of the cells and the salinity of the growth medium, demonstrating that *B. pasteurii* sensitively adjusts its ectoine pool to the degree of the prevalent osmotic stress. The ectoine biosynthetic genes (*ectABC*) of *B. pasteurii* were cloned, and it was demonstrated that they are coordinately expressed as an operon from a unique and osmoregulated promoter (Kuhlmann and Bremer 2002). Hence ectoine accumulation in *B. pasteurii* depends primarily on increased *ectABC* transcription under hyperosmotic growth conditions (Kuhlmann and Bremer 2002). A comparison of the EctABC proteins from *B. pasteurii* with those of *Halomonas elongata* (Göller et al. 1998), *Chromohalobacter salexigens* (Canovas et al. 1998) and *Marinococcus halophilus* (Louis and Galinski 1997) revealed that these enzymes are evolutionarily closely related. This finding demonstrates that the ectoine biosynthetic pathway is well conserved

between the Gram-negative (*H. elongata*, *C. salexigens*) and Gram-positive (*M. halophilus*, *B. pasteurii*) branches of the bacterial world. Tracer studies, NMR spectroscopy and enzymatic analysis previously revealed both the identity of the precursor (L-aspartate-β-semialdehyde) for ectoine production and the three enzymatic steps involved in the formation of this compatible solute (Peters et al. 1990; Ono et al. 1999). This pathway also operates in *B. pasteurii* (Fig. 7.2).

7.6.3 Osmoregulatory Synthesis of Glycine Betaine from Choline

Glycine betaine (Fig. 7.1) is one of the most potent and widely used compatible solutes found in nature (Le Rudulier et al. 1984). Several routes for the synthesis of this trimethylammonium compound exist in microorganisms. The extreme halophiles *Actinopolyspora halophila* and *Ectothiorhodospira halochloris* produce glycine betaine de novo through a three-step series of methylation reactions of the amino acid glycine with sarcosin and dimethylglycine as intermediates and *S*-adenosyl methionine as methyl donor (Nyyssola et al. 2000). *B. subtilis* cannot synthesize glycine betaine de novo and instead produces it via two sequential oxidation reactions of the precursor choline (Boch et al. 1994, 1996). Since *B. subtilis* is unable to synthesize choline, it must actively acquire this precursor from exogenous sources through transport processes involving the highly substrate-specific ABC-transporter OpuB and a closely related ABC-transporter (OpuC) with a broad substrate specificity (Fig. 7.3; Kappes et al. 1999). Subsequent to choline import via OpuB and OpuC, glycine betaine synthesis proceeds via a two-step oxidation process involving a soluble type-III alcohol dehydrogenase (GbsB) and a highly salt-tolerant glycine betaine aldehyde dehydrogenase (GbsA; Fig. 7.2; Boch et al. 1996, 1997). The genes for these enzymes are encoded by the *gbsAB* operon.

Glycine betaine production depends on the availability of choline in the environment. The presence of exogenous choline stimulates the expression of the loci encoding the systems for its high-affinity uptake (OpuB) and its enzymatic oxidation (GbsAB) to glycine betaine (G. Nau-Wagner and E. Bremer, unpubl. results). High osmolality does not directly stimulate *gbsAB* transcription to any significant degree (Boch et al. 1996), whereas expression of the *opuB* operon is subjected to both induction by choline and osmotic control. The GbsR repressor, a novel type of choline-sensing protein (G. Nau-Wagner and E. Bremer, unpubl. results), mediates the induction of the *opuB* and *gbsAB* operons in response to exogenous choline levels. Consequently, GbsR coordinates the choline-to-glycine betaine synthetic pathway by simultaneously controlling the genes for the high-affinity choline-uptake system OpuB (Fig. 7.3) and those encoding the glycine betaine synthetic enzymes GbsAB (Fig. 7.2). The OpuC system is also involved in the high-affinity uptake of

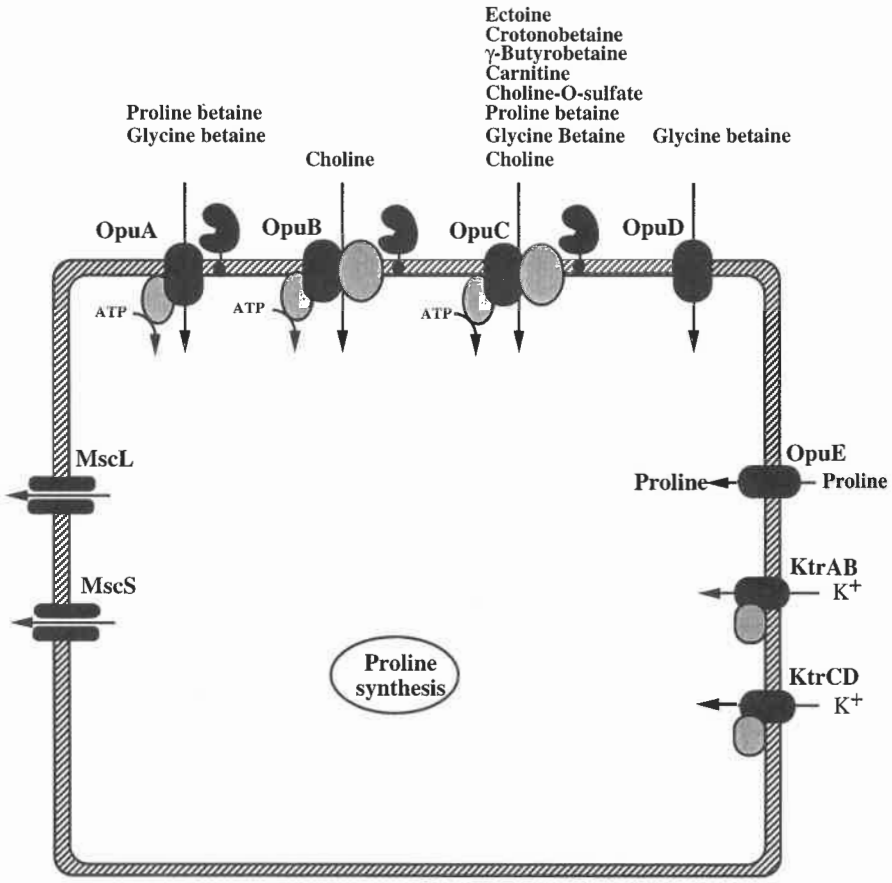


Fig. 7.3. Systems for the uptake and expulsion of compatible solutes in *B. subtilis*

choline (Fig. 7.3; Kappes et al. 1999), but the expression of the *opuC* operon is not under the control of the GbsR repressor (G. Nau-Wagner and E. Bremer, unpubl. results). This observation can readily be put in a physiological context: OpuC transports a wide variety of compatible solutes (Fig. 7.3) and it would be counterproductive for the *B. subtilis* cell to control the synthesis of OpuC solely on the basis of the availability of choline in the environment.

7.7 Acquisition of Preformed Compatible Solutes by *B. subtilis* from Environmental Resources

The varied habitats of *B. subtilis* (soil, roots, rotting plant material, etc.) 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 effectively use these preformed compatible solutes and their accumulation allows it to grow over a wide range of osmotic conditions and to colonize habitats that are otherwise inhibitory for its proliferation. The compatible solutes employed by *B. subtilis* are all structurally related to the amino acid proline, the trimethylammonium compound glycine betaine and the tetrahydropyrimidine ectoine (Fig. 7.1); it does not use any sugars, such as trehalose, for osmoprotective purposes.

A detailed genetic and physiological analysis has demonstrated that *B. subtilis* can take up a wide variety of compatible solutes via multiple osmoprotectant transport systems (Opu) that have overlapping substrate specificity and energy coupling mechanisms (Fig. 7.3). Each of these transporters exhibits a high affinity for its various substrates with K_m values in the low micromolar range (Kempf and Bremer 1995; Kappes et al. 1996, 1999; von Blohn et al. 1997; Nau-Wagner et al. 1999), thereby permitting the efficient scavenging of osmoprotectants that are usually present in natural habitats in very low concentrations. The Opu transporters also possess substantial transport capacities (V_{max}), which allow the accumulation of compatible solutes to the exceedingly high intracellular levels that are required for the effective maintenance of turgor under hyperosmotic conditions. Whatmore et al. (1990) determined the intracellular pool of glycine betaine and found that in a basal minimal medium *B. subtilis* accumulates this compatible solute to a level of 175 mM. The glycine betaine pool rose within 2 h subsequent to a sudden osmotic upshock with 0.4 M NaCl to a level of approximately 700 mM. We determined the correlation between cellular glycine betaine content and the salinity of the growth medium in more detail in actively growing cells and found that there is a linear relationship between the intracellular glycine betaine concentration and the osmolality of the environment. Cellular glycine betaine content rose from a basal level of approximately 200 mM in non-osmotically stressed cultures to a level of 1.4 M when cells were grown in a minimal medium containing 1 M NaCl and 1 mM glycine betaine (S. Moses, E. P. Bakker and E. Bremer, unpubl. results).

The proline transporter OpuE (von Blohn et al. 1997) is a member of the sodium/symporter family (Fig. 7.3). The glycine betaine transporter OpuD (Kappes et al. 1996) 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-posi-

tive bacteria. Like the glycine betaine transporter BetP from *Corynebacterium glutamicum* (Rübenhagen et al. 2001), 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). Activation at the level of transport activity as exhibited by OpuD allows the cells to take advantage of pre-existing OpuD proteins and thus permits immediate import of compatible solutes when the cell is osmotically challenged. The expression of both the *opuE* and *opuD* genes is interwoven with the general, SigB-controlled stress regulon of *B. subtilis* (von Blohn et al. 1997; Spiegelhalter and Bremer 1998; Spiegelhalter and Bremer, unpubl. results). A sudden increase in salinity is among the environmental cues that trigger the transient expression of the entire SigB regulon (Price 2000; Hecker and Völker 2001). SigB activity is controlled through a complex signal transduction cascade that involves an anti-sigma factor and several regulatory proteins whose activity is controlled through phosphorylation and dephosphorylation events. However, the actual mechanism by which changes in environmental salinity are detected by the cell and fed into this signal transduction cascade that controls SigB activity is still elusive. Both the *opuE* (von Blohn et al. 1997; Spiegelhalter and Bremer 1998) and *opuD* genes (F. Spiegelhalter and E. Bremer, unpubl. results) are part of the SigB-controlled general stress regulon but each has an additional, independently controlled promoter that responds to increases in medium osmolality. Thus, at least two different pathways for the transduction of osmotic signals must operate in *B. subtilis*: one of which relies on the sensory and regulatory proteins that control SigB activity (Price 2000; Hecker and Völker 2001) and another which is dependent on an unidentified signal transduction cascade.

The OpuA, OpuB and OpuC transport systems (Fig. 7.3) are members of the ABC-family of transporters (Kempf and Bremer 1995; Kappes et al. 1999). 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; Kappes et al. 1999). The *opuB* and *opuC* operons are located near one another on the *B. subtilis* chromosome, and the high amino acid sequence identity of the encoded gene products strongly suggests that these two ABC-transport systems have evolved from a duplication event of a primordial gene cluster (Kappes et al. 1999). Despite the close relatedness of the OpuB and OpuC systems, these transporters exhibit a striking difference in substrate specificity for osmoprotectants. OpuB is highly specific for choline, the precursor for glycine betaine synthesis, whereas OpuC transports a broad range of compatible solutes, most of which are structurally related to glycine betaine (Fig. 7.3). Collectively, the Opu transporters can provide *B. subtilis* with at least eight preformed osmoprotectants and also with the precursor (choline) for the biosynthesis of glycine betaine (Kempf and Bremer 1995; Jebbar et al. 1997; von Blohn et al. 1997; Kappes and Bremer 1998; Nau-Wagner et al. 1999). The complex arsenal of osmoprotectant transporters operat-

ing in *B. subtilis* (Fig. 7.3) highlights the important role of compatible solute acquisition in this bacterium's adaptation to high salinity and high osmolality surroundings.

Unlike *opuB* expression, which is induced by choline and is under the negative control of the GbsR repressor, expression of the *opuA*, *opuC*, *opuD* and *opuE* loci is not enhanced when a substrate for the encoded transporter is present in the growth medium. However, transcription of the structural genes of 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 in the environment and communicates this information to the transcription apparatus of the cell is completely unknown.

7.8 Expulsion of Compatible Solutes: Protection Against Extreme Turgor

Rain, flooding and washout into freshwater sources exposes *B. subtilis* to rapid and sometimes 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 osmotic downshift equivalent to 0.3 M salt was calculated to increase turgor from a basal level of approximately 4 atm (0.4 MPa) to approximately 11 atm (1.1 MPa; Booth and Louis 1999). To a certain extent, elevated turgor can be accommodated by the elasticity of the murein sacculus (Höltje 1998; Wood 1999), but when this capacity is exceeded the cell must eliminate water-attracting osmolytes and ions to avoid bursting. It has become clear in recent years that 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). Such mechanosensitive channels have been detected both in Bacteria and Archaea and the physiological function of two such channel types, MscL and MscS (YggB, KefA), have been characterized in *E. coli*. Their simultaneous disruption (MscL, YggB) resulted in cell death following a severe osmotic downshock (Levina et al. 1999).

In *B. subtilis*, the presence of ion-conducting channels 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; Szabo et al. 1992). The MscL protein (Moe et al. 1998) 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 conduc-

tance of 3.6 nS (Moe et al. 1998). We disrupted the chromosomal copy of the *B. subtilis mscL* gene and found that this mutant strain can only partially withstand an osmotic downshock equivalent to 0.8 M salt (C. Boiangiu and E. Bremer, unpubl. results). Three YggB homologues (*ydhY*, *ykuT*, *yfkC*) that might function as MscS-type channels are also present in *B. subtilis* (Levina et al. 1999). However, when we disrupted these three *yggB*-related genes either individually or in combination, the resulting mutant strains did not exhibit any phenotype with respect to their survival subsequent to a severe osmotic downshock. In contrast, combination of a *mscL* mutation with a gene disruption in *ykuT* (MscS) yielded a *B. subtilis* strain that was extremely sensitive against osmotic downshifts (C. Boiangiu and E. Bremer, unpubl. results). Taken together, these data and observations strongly suggest that mechanosensitive channels of the MscL and MscS type (Fig. 7.3) play a pivotal role in managing the transition of *B. subtilis* from high- to low-osmolality environments.

7.9 Perspectives

A solid framework for understanding how *B. subtilis* and closely related species adapt to changing osmolality has now been laid (Fig. 7.3). Furthermore, the participation of the SigB-controlled general stress response network in osmoadaptation has been demonstrated (Völker et al. 1999). *B. subtilis* mutants lacking SigB are highly sensitive to sudden and growth-restricting upshocks with NaCl. This observation implies that osmotic stress resistance is conferred by members of the SigB regulon, but the proteins that participate in this particular stress response are unknown (Price 2000; Hecker and Völker 2001). The general and specific osmostress response systems are interwoven (Bremer and Krämer 2000) and both enable *B. subtilis* to cope with growth-restricting and growth-preventing osmotic and high-salinity stress. This is particularly important since the prime defensive strategy of *B. subtilis*, the formation of a highly desiccation-resistant endospore, is severely impaired by high salinity (Kunst and Rapoport 1995; Ruzal et al. 1998).

The cellular responses of *B. subtilis* to high osmolality and salinity are not limited to the adaptation systems described above because high salinity exerts pleiotropic effects on the physiology of *B. subtilis*. Mutants lacking the alternative transcription factor SigM are sensitive to high salt concentrations (Horsburgh and Moir 1999), but this may be an indirect phenotype related to the major cell wall defects exhibited by such mutants. Interestingly, a sudden osmotic upshift triggers the induction of the structural gene for the extracytoplasmic sigma factor SigW and the entire regulon controlled by this alternative transcription factor (Petersohn et al. 2001). Increases in salinity also affect the phospholipid composition of the cytoplasmic membrane (Lopez et

al. 2000), the properties of the cell wall (Lopez et al. 1998), and the synthesis of the cell-wall-associated protein WapA (Dartois et al. 1998). In addition, the production of several extracellular degradative enzymes is regulated in a DegS/DegU-dependent manner at high salinity (Kunst and Rapoport 1995). Furthermore, increases in medium salinity transiently induce the structural gene for the membrane-associated protease FtsH, and *ftsH* mutants can grow efficiently in media with NaCl concentrations greater than 0.2 M only if the compatible solutes proline or glycine betaine are added (Deuerling et al. 1995). One also observes increases in negative supercoiling of reporter plasmids when *B. subtilis* is subjected to salt stress (Krispin and Allmansberger 1995; Alice and Sanchez-Rivas 1997), and such changes in DNA topology might have profound influences on gene expression under high osmolality growth conditions. Furthermore, 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). However, this response is of limited use when the entire habitat of *B. subtilis* undergoes a change in salinity and osmolality. Furthermore, proteome analysis has demonstrated that the synthesis of the major structural component of the flagellum, Hag, is strongly repressed in cells grown at high salinity, implying that *B. subtilis* can no longer effectively swim in hypertonic media (Hoffmann et al. 2002). Proteome analysis of *B. subtilis* cells grown at high salinity unexpectedly revealed an overlap between the cellular responses and iron limitation. Growth in high-salinity minimal media triggered the expression of the genes encoding the iron chelator bacillibactin and of transport systems involved in the scavenging of iron from the environment (Hoffmann et al. 2002). The unexpected finding that high salinity stress has an iron limitation component might be of special ecophysiological importance for the growth of *B. subtilis* in natural settings, in which bioavailable iron is usually scarce.

The adaptation of the *B. subtilis* cell to salt stress and osmotic challenges apparently has many facets. We have now begun to employ the method of transcriptional profiling of salt-stressed *B. subtilis* cells on a genome-wide scale (L. Steil, T. Hoffmann, U. Völker and E. Bremer, unpubl. results). This global look at the *B. subtilis* transcriptome revealed that the expression of approximately 120 genes is induced upon salt stress whereas transcription of approximately 100 genes is simultaneously repressed. Hence approximately 5% of all *B. subtilis* genes are either positively or negatively affected by increases in the salinity of the growth medium. It will be a challenging task in the coming years to characterize the physiological function of the salt-induced and salt-repressed *B. subtilis* genes, to study the molecular mechanisms of their gene regulation in response to increased salinity, and to unravel possible regulatory networks that control and integrate the cellular responses of *B. subtilis* to hypertonic environments.

Acknowledgements. We greatly appreciate the help of V. Koogle in editing the manuscript. Financial support for studies on osmoregulation in our laboratory was provided by the Deutsche Forschungsgemeinschaft through SFB-395, the Graduiertenkolleg "Proteinfunktion auf atomarer Ebene", the European Union (contract IAC4-CT-2000-30041), the BMBF through the "Genomnetzwerk Göttingen" and the Fonds der Chemischen Industrie.

References

Alcayaga C, Venegas R, Carrasco A, Wolff D (1992) Ion channels from the *Bacillus subtilis* plasma membrane incorporated into planar lipid bilayers. FEBS Lett 311:246–250

Alice A, Sanchez-Rivas C (1997) DNA supercoiling and osmoresistance in *Bacillus subtilis* 168. Curr Microbiol 35:309–315

Arakawa T, Timasheff SN (1985) The stabilization of proteins by osmolytes. Biochem J 47:411–414

Belitsky BR, Brill J, Bremer E, Sonenshein AL (2001) Multiple genes for the last step of proline biosynthesis in *Bacillus subtilis*. J Bacteriol 183:4389–4392

Blount P, Moe PC (1999) Bacterial mechanosensitive channels: integrating physiology, structure and function. Trends Microbiol 7:420–424

Boch J, Kempf B, Bremer E (1994) Osmoregulation in *Bacillus subtilis*: synthesis of the osmoprotectant glycine betaine from exogenously provided choline. J Bacteriol 176:5364–5371

Boch J, Kempf B, Schmid R, Bremer E (1996) Synthesis of the osmoprotectant glycine betaine in *Bacillus subtilis*: characterization of the *gbsAB* genes. J Bacteriol 178:5121–5129

Boch J, Nau-Wagner G, Kneip S, Bremer E (1997) Glycine betaine aldehyde dehydrogenase from *Bacillus subtilis*: characterization of an enzyme required for the synthesis of the osmoprotectant glycine betaine. Arch Microbiol 168:282–289

Bolen DW, Baskakov IV (2001) The osmophobic effect: natural selection of a thermodynamic force in protein folding. J Mol Biol 310:955–963

Booth IR, Louis P (1999) Managing hypoosmotic stress: aquaporins and mechanosensitive channels in *Escherichia coli*. Curr Opin Microbiol 2:166–169

Bourot S, Sire O, Trautwetter A, Touze T, Wu F, Blanco C, Bernard T (2000) Glycine betaine-assisted protein folding in a *lysA* mutant of *Escherichia coli*. J Biol Chem 275:1050–1056

Bremer E, Krämer R (2000) Coping with osmotic challenges: osmoregulation through accumulation and release of compatible solutes in bacteria. In: Storz G, Hengge-Aronis R (eds) Bacterial stress responses. ASM, Washington, DC, pp 79–97

Brown AD (1976) Microbial water stress. Bacteriol Rev 40:803–846

Burg M, Kwon E, Kültz D (1997) Regulation of gene expression by hypertonicity. Annu Rev Physiol 59:437–455

Calamita G (2000) The *Escherichia coli* aquaporin-Z water channel. Mol Microbiol 37:254–262

Calamita G, Bishai WR, Preston GM, Guggino WB, Agre P (1995) Molecular cloning and characterization of AqpZ, a water channel from *Escherichia coli*. J Biol Chem 270:29063–29066

Caldas T, Demont-Caulet N, Ghazi A, Richarme G (1999) Thermoprotection by glycine betaine and choline. Microbiology 145:2543–2548

Canovas D, Vargas C, Calderon MI, Ventosa A, Nieto JJ (1998) Characterization of the genes for the biosynthesis of the compatible solute ectoine in the moderately halophilic bacterium *Halomonas elongata* DSM 3043. Syst Appl Microbiol 21:487–497

Canovas D, Borges N, Vargas C, Ventosa A, Nieto JJ, Santos H (1999) Role of N-γ-acetyldiaminobutyrate as an enzyme stabilizer and an intermediate in the biosynthesis of hydroxyectoine. Appl Environ Microbiol 65:3774–3779

Canovas D, Fletcher SA, Hayashi M, Csonka LN (2001) Role of trehalose in growth at high temperature of *Salmonella enterica* serovar typhimurium. J Bacteriol 183:3365–3371

Csonka LN, Epstein W (1996) Osmoregulation. In: Neidhard FC, Curtiss R III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE (eds) *Escherichia coli* and *Salmonella*. Cellular and molecular biology. ASM, Washington, DC, pp 1210–1223

Da Costa MS, Santos H, Galinski EA (1998) An overview of the role and diversity of compatible solutes in *Bacteria* and *Archaea*. Adv Biochem Eng Biotechnol 61:117–153

Dartois V, Débarbouillé M, Kunst F, Rapoport G (1998) Characterization of a novel member of the DegS/DegU regulon affected by salt stress in *Bacillus subtilis*. J Bacteriol 180:1855–1861

Delamarche C, Thomas D, Rollander J-P, Froger A, Gouranton J, Svelto M, Agre P, Calamita C (1999) Visualization of AqpZ-mediated water permeability in *Escherichia coli* by cryoelectron microscopy. J Bacteriol 181:4193–4197

Delaunay AJ, Verma DPS (1993) Proline biosynthesis and osmoregulation in plants. Plant J 4:215–223

Dennis PP, Shimmin LC (1997) Evolutionary divergence and salinity-mediated selection in halophilic archaea. Microbiol Mol Biol Rev 61:90–104

Deuerling E, Paeslack B, Schumann W (1995) The *ftsH* gene of *Bacillus subtilis* is transiently induced after osmotic and temperature upshift. J Bacteriol 177:4105–4112

Durell SR, Guy HR (1999) Structural models of the KtrB, TrkH, and Trk1,2 symporters based on the structure of the KcsA K^+ channel. Biophys J 77:789–807

Durell SR, Hao Y, Nakamura T, Bakker EP, Guy HR (1999) Evolutionary relationship between K^+ channels and symporters. Biophys J 77:775–788

Galinski EA, Trüper HG (1994) Microbial behaviour in salt-stressed ecosystems. FEMS Microbiol Rev 15:95–108

Göller K, Ofer A, Galinski EA (1998) Construction and characterization of an NaCl-sensitive mutant of *Halomonas elongata* impaired in ectoine biosynthesis. FEMS Microbiol Lett 161:293–300

Hecker M, Völker U (2001) General stress response of *Bacillus subtilis* and other bacteria. Adv Microbial Physiol 44:35–91

Hoffmann T, Schutz A, Brosius M, Völker A, Völker U, Bremer E (2002) High-salinity-induced iron limitation in *Bacillus subtilis*. J Bacteriol 184:718–727

Hohmann S (1997) Shaping up: the response of yeast to osmotic stress. In: Hohmann S, Mager WH (eds) Yeast stress responses. Springer, Berlin Heidelberg New York, pp 101–145

Holtmann G, Bakker EP, Uozumi N, Bremer E (2003) KtrAB and KtrCD: two K^+ uptake systems in *Bacillus subtilis* and their role in adaptation to hypertonicity. J Bacteriol 185:1289–1298

Höltje JV (1998) Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. Microbiol Mol Biol Rev 62:181–203

Horsburgh MJ, Moir A (1999) σ^M, an ECF RNA polymerase sigma factor of *Bacillus subtilis* 168, is essential for growth and survival in high concentrations of salt. Mol Microbiol 32:41–50

Jebbar M, von Blohn C, Bremer E (1997) Ectoine functions as an osmoprotectant in *Bacillus subtilis* and is accumulated via the ABC-transport system OpuC. FEMS Microbiol Lett 154:325–330

Kappes R, Bremer E (1998) Response of *Bacillus subtilis* to high osmolarity: uptake of carnitine, crotonobetaine and γ-butyrobetaine via the ABC transport system OpuC. Microbiology 144:83–90

Kappes RM, Kempf B, Bremer E (1996) Three transport systems for the osmoprotectant glycine betaine operate in *Bacillus subtilis*: characterization of OpuD. J Bacteriol 178:5071–5079

Kappes RM, Kempf B, Kneip S, Boch J, Gade J, Meier-Wagner J, Bremer E (1999) Two evolutionarily closely related ABC-transporters mediate the uptake of choline for synthesis of the osmoprotectant glycine betaine in *Bacillus subtilis*. Mol Microbiol 32:203–216

Kempf B, Bremer E (1995) OpuA, an osmotically regulated binding protein-dependent transport system for the osmoprotectant glycine betaine in *Bacillus subtilis*. J Biol Chem 270:16701–16713

Kempf B, Bremer E (1998) Uptake and synthesis of compatible solutes as microbial stress responses to high osmolality environments. Arch Microbiol 170:319–330

Kempf B, Gade J, Bremer E (1997) Lipoprotein from the osmoregulated ABC transport system OpuA of *Bacillus subtilis*: purification of the glycine betaine binding protein and characterization of a functional lipidless mutant. J Bacteriol 179:6213–6220

Krispin O, Allmansberger R (1995) Changes in DNA supertwist as a response of *Bacillus subtilis* towards different kinds of stress. FEMS Microbiol Lett 134:129–135

Kuhlmann AU, Bremer E (2002) Osmotically regulated synthesis of the compatible solute ectoine in *Bacillus pasteurii* and related *Bacillus* spp. Appl Environ Microbiol 68:772–783

Kunst F, Rapoport G (1995) Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. J Bacteriol 177:2403–2407

Le Rudulier D, Strøm AR, Dandekar AM, Smith LT, Valentine RC (1984) Molecular biology of osmoregulation. Science 224:1064–1068

Levina N, Totemeyer S, Stokes NR, Louis P, Jones MA, Booth IR (1999) Protection of *Escherichia coli* cells against extreme turgor by activation of MscS and MscL mechanosensitive channels: identification of genes required for MscS activity. EMBO J 18:1730–1737

Lippert K, Galinski AA (1992) Enzyme stabilization by ectoine-type compatible solutes: protection against heating, freezing and drying. Appl Microbiol Biotechnol 37:61–65

Lopez CS, Heras H, Ruzal SM, Sanchez-Rivas C, Rivas EA (1998) Variations of the envelope composition of *Bacillus subtilis* during growth in hyperosmotic medium. Curr Microbiol 36:55–61

Lopez CS, Heras H, Garda H, Ruzal S, Sanchez-Rivas C, Rivas E (2000) Biochemical and biophysical studies of *Bacillus subtilis* envelopes under hyperosmotic stress. Int J Food Microbiol 55:137–142

Louis P, Galinski EA (1997) Characterization of genes for the biosynthesis of the compatible solute ectoine from *Marinococcus halophilus* and osmoregulated expression in *Escherichia coli*. Microbiology 143:1141–1149

Martin DD, Ciulla RA, Roberts MF (1999) Osmoadaptation in archaea. Appl Environ Microbiol 65:1815–1825

Martins L, Huber R, Huber H, Stetter K, Da Costa M, Santos H (1997) Organic solutes in hyperthermophilic *Archaea*. Appl Environ Microbiol 63:896–902

McNeil SD, Nuccio ML, Hanson AD (1999) Betaines and related osmoprotectants. Targets for metabolic engineering of stress resistance. Plant Physiol 120:945–950

Measures JC (1975) Role of amino acids in osmoregulation of non-halophilic bacteria. Nature 257:398–400

Mendum ML, Smith LT (2002) Characterization of glycine betaine porter I from *Listeria monocytogenes* and its roles in salt and chill tolerance. Appl Environ Microbiol 68:813–819

Miller KJ, Wood JM (1996) Osmoadaptation by rhizosphere bacteria. Annu Rev Microbiol 50:101–136

Moe PC, Blount P, Kung C (1998) Functional and structural conservation in the mechanosensitive channel MscL implicates elements crucial for mechanosensation. Mol Microbiol 28:583–592

Nakamura T, Yuda R, Unemoto T, Bakker EP (1998) KtrAB, a new type of bacterial K(+)-uptake system from *Vibrio alginolyticus*. J Bacteriol 180:3491–3494

Nau-Wagner G, Boch J, Le Good JA, Bremer E (1999) High-affinity transport of choline-*O*-sulfate and its use as a compatible solute in *Bacillus subtilis*. Appl Environ Microbiol 65:560–568

Nyyssola A, Kerovuo J, Kaukinen P, von Weymarn N, Reinikainen T (2000) Extreme halophiles synthesize betaine from glycine by methylation. J Biol Chem 275:22196–22201

Ono H, Sawada, K, Khunjakr N, Tao T, Yamamoto M, Hiramoto M, Shinmyo A, Takano M, Murooka Y (1999) Characterization of biosynthetic enzymes for ectoine as a compatible solute in a moderately halophilic eubacterium, *Halomonas elongata*. J Bacteriol 181:91–99

Peters P, Galinski EA, Trüper HG (1990) The biosynthesis of ectoine. FEMS Microbiol Lett 71:157–162

Petersohn A, Brigulla M, Haas S, Hoheisel JD, Völker U, Hecker M (2001) Global analysis of the general stress response of *Bacillus subtilis*. J Bacteriol 183:5617–5631

Price CW (2000) Protective function and regulation of the general stress response in *Bacillus subtilis* and related Gram-positive bacteria. In: Storz G, Hengge-Aronis R (eds) Bacterial stress responses. ASM, Washington, DC, pp 179–197

Roberts MF (2000) Osmoadaptation and osmoregulation in archaea. Front Biosci 5:796–812

Roosild TP, Miller S, Booth IR, Choe S (2002) A mechanism of regulating transmembrane potassium flux through a ligand-mediated informational switch. Cell 109:781–791

Rübenhagen R, Morbach S, Kramer R (2001) The osmoreactive betaine carrier BetP from *Corynebacterium glutamicum* is a sensor for cytoplasmic K^+. EMBO J 20:5412–5420

Ruzal SM, López C, Rivas E, Sánchez-Rivas C (1998) Osmotic strength blocks sporulation at stage II by impeding activation of early sigma factors in *Bacillus subtilis*. Curr Microbiol 36:75–79

Spiegelhalter F, Bremer E (1998) Osmoregulation of the *opuE* proline transport gene from *Bacillus subtilis*: contributions of the sigma A- and sigma B-dependent stress responsive promoters. Mol Microbiol 29:285–296

Szabo I, Petronilli V, Zoratti M (1992) A patch-clamp study of *Bacillus subtilis*. Biochim Biophys Acta 1112:29–38

Tholema N, Bakker EP, Suzuki A, Nakamura T (1999) Change to alanine of one out of four selectivity filter glycines in KtrB causes a two orders of magnitude decrease in the affinities for both K^+ and Na^+ of the Na^+ dependent K^+ uptake system KtrAB from *Vibrio alginolyticus*. FEBS Lett 450:217–220

Ventosa A, Nieto JJ, Oren A (1998) Biology of moderately halophilic aerobic bacteria. Microbiol Mol Biol Rev 62:504–544

Völker U, Maul B, Hecker M (1999) Expression of the σ^B-dependent general stress regulon confers multiple stress resistance in *Bacillus subtilis*. J Bacteriol 181:3942–3948

Von Blohn C, Kempf B, Kappes RM, Bremer E (1997) Osmostress response in *Bacillus subtilis*: characterization of a proline uptake system (OpuE) regulated by high osmolarity and the alternative transcription factor sigma B. Mol Microbiol 25:175–187

Welsh DT (2000) Ecological significance of compatible solute accumulation by microorganisms: from single cells to global climate. FEMS Microbiol Rev 24:263–290

Whatmore AM, Reed RH (1990) Determination of turgor pressure in *Bacillus subtilis*: a possible role for K^+ in turgor regulation. J Gen Microbiol 136:2521–2526

Whatmore AM, Chudek JA, Reed RH (1990) The effects of osmotic upshock on the intracellular solute pools of *Bacillus subtilis*. J Gen Microbiol 136:2527–2535
Wong LS, Johnson MS, Sandberg LB, Taylor BL (1995) Amino acid efflux in response to chemotactic and osmotic signals in *Bacillus subtilis*. J Bacteriol 177:4342–4349
Wood JM (1999) Osmosensing by bacteria: signals and membrane-based sensors. Microbiol Mol Biol Rev 63:230–262