Stress responses of *Bacillus subtilis* to high osmolarity environments: Uptake and synthesis of osmoprotectants

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A decrease in the water content of the soil imposes a considerable stress on the soil-living bacterium *Bacillus subtilis*: water exits from the cells, resulting in decreased turgor and cessation of growth. Under these adverse circumstances, *B. subtilis* actively modulates the osmolarity of its cytoplasm to maintain turgor within acceptable boundaries. A rapid uptake of potassium ions via turgor-responsive transport systems is the primary stress response to a sudden increase in the external osmolarity. This is followed by the massive accumulation of the so-called compatible solutes, i.e., organic osmolytes that are highly congruous with cellular functions and hence can be accumulated by bacterial cells up to molar concentrations. Initially, the compatible solute proline is accumulated via *de novo* synthesis, but *B. subtilis* can also acquire proline from the environment by an osmoregulated transport system, OpuE. The preferred compatible solute of *B. subtilis* is the potent osmoprotectant glycine betaine. This trimethylammonium compound can be taken up by the cell through three high-affinity transport systems: the multicomponent ABC transporters OpuA and OpuC, and the single-component transporter OpuD. The OpuC systems also mediates the accumulation of a variety of naturally occurring betaines, each of which can confer a considerable degree of osmotic tolerance. In addition to the uptake of glycine betaine from the environment, *B. subtilis* can also synthesize this osmoprotectant but it requires exogenously provided choline as its precursor. Two evolutionarily closely related ABC transport systems, OpuB and OpuC, mediate the uptake of choline which is then converted by the GbsA and GbsB enzymes in a two-step oxidation process into glycine betaine. Our data show that the intracellular accumulation of osmoprotectants is of central importance for the cellular defence of *B. subtilis* against high osmolarity stress.

1. Introduction

The osmotic strength of the environment is an important physical parameter that influences the ability of microorganisms to proliferate and successfully compete for a given habitat. Bacteria maintain an osmotic pressure in the cytoplasm that is higher than that in the surrounding environment, resulting in an outward directed tension, the turgor (figure 1). In Gram-negative bacteria, turgor has been estimated at 0·3–0·5 MPa (Csonka 1989), whereas Gram-positive bacteria, such as *Bacillus subtilis*, maintain a much higher turgor, estimated at approximately 1·9 MPa (Whatmore and Reed 1990). Since the cell envelope of microorganisms is permeable to water, fluctuations in the environmental osmolarity trigger the flux of water across the cytoplasmic membrane along the osmotic gradient. To avoid lysis under low-osmolarity or dehydration under high-osmolarity growth conditions, bacteria must possess active mechanisms to efficiently adjust the osmolarity of their cytoplasm.

Analyses of the cellular responses of bacteria to osmotic stress have been focused primarily on high-osmolarity environments (Csonka 1989; Csonka and Hanson 1991; Galinski and Trüper 1994; Lucht and Bremer 1994; Csonka and Epstein 1996; Miller and Wood 1996). Two basic schemes of adaptation to these environments have

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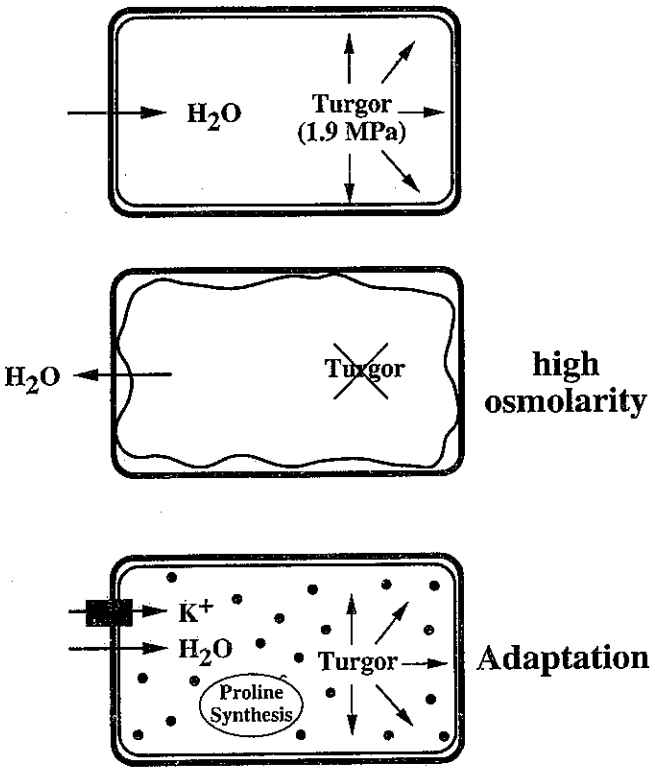

Figure 1. Initial adaptation of *B. subtilis* to high osmolarity.

been identified: (i) The accumulation of very high intracellular concentrations of ions. This strategy is followed by many halophilic and halotolerant microorganisms whose entire physiology has been adapted to a permanent life in a high-saline environment. (ii) The intracellular accumulation of compatible solutes, i.e., organic osmolytes that are highly congruous with the structure and function of the cell. This approach is of general importance; not only is it widely employed in the prokaryotic world by (bacteria and archaea), but it is also used as a stress reaction in yeast, plant, animal, and even human cells (Rhodes and Hanson 1993; Blomberg 1997; Burg *et al* 1997).

2. Characteristics of compatible solutes

Osmoprotectants are defined as exogenous organic solutes that enhance bacterial growth in media of high osmolarity. These substances may themselves be compatible solutes or they may be precursor molecules that can be enzymatically converted to these compounds (Galinski and Trüper 1994; Miller and Wood 1996). The spectrum of compatible solutes used by microorganisms comprise a limited number of compounds: amino acids and amino acid derivatives (e.g., proline, glutamate, β-alanine, ectoine), sugars and polyols (e.g., trehalose, glycerol, glycosylglycerol), quaternary amines and their sulphonium analogues (e.g., glycine betaine, proline betaine, dimethylsulphoniopropionate), sulphate esters (e.g., choline-O-sulphate), *N*-acetylated diamino acids (e.g., *N*δ-acetylornithine, *N*ε-acetyllysine), and small peptides (e.g., *N*-acetylglutaminylglutamine amide) (Csonka 1989; Galinski and Trüper 1994). In general, compatible solutes are highly soluble molecules and do not carry a net charge at physiological pH. In contrast to inorganic salts, they can reach high intracellular concentrations without disturbing vital functions such as DNA-replication, DNA-protein interactions, and cellular metabolism.

Compatible solutes serve a dual function in osmoregulating cells. Because they can accumulate up to molar concentrations, they make a major contribution to the osmotic balance of the cytoplasm (Galinski and Trüper 1994; Miller and Wood 1996). They also serve as stabilizers of enzymes and cell components against the denaturing effects of high ionic strength and hence can be described as chemical chaperones. This protective property of compatible solutes appears to stem from their preferential exclusion from the hydration shell of macromolecules (Potts 1994; Yancey 1994). In this overview, we focus on the physiological and molecular stress responses of *B. subtilis* to high osmolarity environments and report on the characteristics of the systems for the synthesis and uptake of osmoprotectants and compatible solutes in this soil bacterium.

3. *B. subtilis* as a model system for studies on osmoregulation

B. subtilis frequently occupies the upper layers of the soil, where desiccation often causes high salinity and high osmolarity (Miller and Wood 1996; Potts 1994). To survive and grow in this ecological niche, *B. subtilis* must be able to adapt to high osmolarity stress. It exhibits a behavioural response to brief increases in osmolarity, whereby it swims away from the area of high osmolarity (Wong *et al* 1995). It also responds genetically with the induction of a large general stress regulon (Antelmann *et al* 1997; Hecker *et al* 1996). The members of this stress regulon are induced not only by salt but also by a number of growth-limiting conditions and stationary phase signals; these stress proteins provide cross-protection for a variety of environmental challenges and are thought to prepare non-growing cells for a diverse range of stress conditions (Hecker *et al* 1996). Expression of many members of the general stress regulon is under the control of the alternative transcription factor sigma B (σ^B), whose activity is determined by a complex network of regulatory proteins (Hecker *et al* 1996; Yang *et al* 1996). Although the addition of salt transiently triggers the enhanced transcription of many genes of the σ^B regulon, *sigB* mutants are not at a survival disadvantage

compared with the wild-type when exposed to osmotic shock or extreme desiccation under laboratory conditions (Boylan *et al* 1993). Hence, σ^B is clearly not the master regulator for the osmostress response in proliferating *B. subtilis* cells. Under high osmolarity growth conditions, *B. subtilis* incites specific and highly co-ordinated genetic and physiological adaptation reactions to limit the loss of water from the cell and thus maintain turgor at a level suitable for growth (figure 1). At the core of this stress response is the intracellular accumulation of osmoprotectants and compatible solutes (figure 2) through synthesis and uptake from the environment.

4. The initial stress response to high osmolarity

4.1 *Uptake of K^+*

Potassium is abundant in nature and is the prevalent cellular cation in the cytoplasm of bacteria. Studies with the Gram-negative eubacteria *Escherichia coli* and *Salmonella typhimurium* have shown that K^+ plays a primary role in the initial stress response of these organisms (Csonka and Epstein 1996). Reed and co-workers (Whatmore *et al* 1990; Whatmore and Reed 1990) have carefully analysed the influx of K^+ into *B. subtilis* subsequent to sudden osmotic upshifts. A moderate osmotic upshock with 0·4 M NaCl results in a decrease in turgor (figure 1) and causes an increase in the cellular K^+ level from a basal value of approximately 315 mM to 650 mM within 1 h (Whatmore *et al* 1990). Uptake of K^+ under these conditions is apparently mediated by turgor-responsive transport systems, but the molecular details of these transporters have not yet been elucidated. The influx of K^+ is essential for the recovery of turgor after upshock (figure 1) and the subsequent resumption of growth (Whatmore *et al* 1990). Hence K^+ serves a crucial role in the initial cellular stress response of *B. subtilis* to sudden increases in the environmental osmolarity.

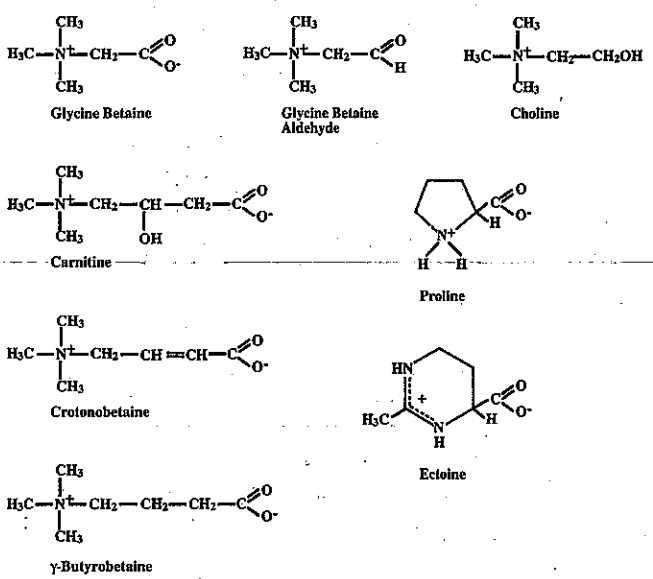

Figure 2. Structure of compatible solutes employed by *B. subtilis*.

4.2 *Synthesis of the compatible solute proline*

Because high intracellular concentrations of K^+ are deleterious for the bacterial cell, the massive accumulation of K^+ is an inadequate strategy for coping with prolonged high osmolarity. In *E. coli* and *S. typhimurium* (Csonka and Epstein 1996), the initial rapid accumulation of K^+ is followed by a long-term accumulation of compatible solutes such as trehalose via *de novo* synthesis and glycine betaine through uptake from the environment. Natural abundance ^{13}C-NMR spectroscopy studies revealed that in the absence of exogenous osmoprotectants, *B. subtilis* initiates a massive accumulation of proline (figure 1) by *de novo* synthesis (Whatmore *et al* 1990). Galinski and Trüper (1994) surveyed various *Bacillus* species and found that in addition to proline, many produce the cyclic amino acid derivative ectoine (figure 2) as their major organic osmolyte. Among the surveyed species, *B. subtilis* represents a minority of proline producers that are unable to synthesize ectoine.

When a *B. subtilis* culture grown in defined medium was subjected to a moderate osmotic upshock with 0·4 M NaCl the intracellular proline level increased from 16 mM to approximately 700 mM within 7 h after the osmotic challenge. The physiological importance of this remarkable increase is emphasized by the finding that proline represents 79% of the total free amino acid pool of the cell under these growth conditions, whereas the concentration of its biosynthetic precursor, glutamate, is only moderately increased from 103 mM to 167 mM (Whatmore *et al* 1990). Because proline production is dependent on the prior accumulation of K^+, this ion might serve as a signal for increased proline synthesis (Whatmore *et al* 1990). We found a direct correlation between the osmotic strength of the environment and the amount of intracellular proline. Addition of 1·4 M NaCl to the minimal medium resulted in the production of approximately 1·5 M proline by *B. subtilis* (unpublished results), emphasizing the very importance of *de novo* proline biosynthesis for the osmoadaptation process.

In a variety of bacteria, proline biosynthesis for anabolic purposes proceeds from glutamate and its regulation is frequently exerted through feed-back inhibition of the *proB*-encoded γ-glutamyl kinase, the first enzyme of the proline biosynthetic pathway (Leisinger 1996). Since relatively low proline concentrations result in strong inhibition of γ-glutamyl kinase activity, salt-stressed

B. subtilis cells require an adjustment of the biosynthetic pathway to permit production of proline even when high concentrations of this substance are present in the cell. Single amino acid substitutions in the ProB protein in several Gram-negative bacteria can greatly reduce the allosteric feed-back inhibition by proline (Leisinger 1996), resulting in proline overproduction and enhanced osmotolerance (Le Rudulier *et al* 1982; Dandekar and Uratsu 1988). One can thus readily envision that subtle modifications in the properties of the biosynthetic enzymes in *B. subtilis* might result in a proline biosynthetic pathway refractory to high intracellular levels of proline in osmotically stressed cells. Alternatively, *B. subtilis* might use two distinct sets of proline biosynthetic genes to meet the different demands of the cell for this amino acid under low- and high-osmolarity growth conditions. Since proline can serve as efficient sole carbon and nitrogen source in *B. subtilis*, additional cellular control mechanisms are required to ensure that proline degradation is curtailed under high-osmolarity growth conditions.

5. Uptake of osmoprotectants from the environment

5.1 *OpuE: an osmotically regulated proline transport system*

After exposure to osmotic stress, many plants accumulate large quantities of proline through *de novo* synthesis from glutamate (Peng *et al* 1996). Proline thus eventually reaches soil bacteria such as *B. subtilis* through root exudates and decaying plant material. Since exogenously provided proline is used by a wide spectrum of Gram-negative and Gram-positive bacteria as an osmoprotectant (Csonka 1989; Lucht and Bremer 1994), it is not surprising that *B. subtilis* can also use this amino acid for osmoprotective purposes. Transport assays with radiolabelled proline revealed that *B. subtilis* possesses an osmotically stimulated uptake activity for this amino acid (von Blohn *et al* 1997).

To facilitate the molecular analysis of transport systems for osmoprotectants in *B. subtilis*, we developed an *in vivo* complementation strategy that uses an *E. coli* mutant unable either to synthesize glycine betaine or to acquire osmoprotectants from the medium via transport systems (Kempf and Bremer 1995). Because this strain, MKH13, cannot grow in high-osmolarity minimal media even in the presence of exogenous osmoprotectants (Haardt *et al* 1995), a plasmid must be provided that encodes systems for osmoprotectant uptake or production. Using MKH13, we were able to select and clone complementing genes for glycine betaine synthesis, glycine betaine uptake, and proline transport from *B. subtilis* (Kempf and Bremer 1995; Boch *et al* 1996; Kappes *et al* 1996; von Blohn *et al* 1997). This strategy proved to be successful for the cloning of osmoprotectant uptake systems from the plant pathogen *Erwinia chrysanthemi* and the soil bacterium *Corynebacterium glutamicum* as well (Gouesbet *et al* 1996; Peter *et al* 1996).

The *opuE* (osmoprotectant uptake) structural gene encodes a proline transport system (figure 3) that operates under high-osmolarity growth conditions (von Blohn *et al* 1997). It consists of a single component (OpuE) and transports proline with high affinity. OpuE is a member of the sodium/solute symporter family, comprising proteins from both prokaryotes and eukaryotes that obligatorily couple substrate uptake to Na^+ symport. An elevation of the osmolarity of the medium by either ionic or non-ionic osmolytes results in a strong increase in the OpuE-mediated proline uptake. Disruption of the *opuE* gene yields a *B. subtilis* strain that is entirely deficient in osmoregulated proline transport activity and is no longer protected by exogenously provided proline (von Blohn *et al* 1997). Surprisingly, the *B. subtilis* OpuE protein displays highest similarity to the PutP proline permeases, which are used in *E. coli*, *S. typhimurium* and *Staphylococcus aureus* for the acquisition of proline as a carbon and nitrogen source, but not for osmoprotective purposes. PutP activity of *E. coli* and *S. typhimurium* is reduced when the cells are grown under high-osmolarity conditions (Wood 1988). In striking contrast, OpuE activity is strongly enhanced in hypertonic media, highlighting the distinct physiological functions of the PutP and OpuE proline transporters.

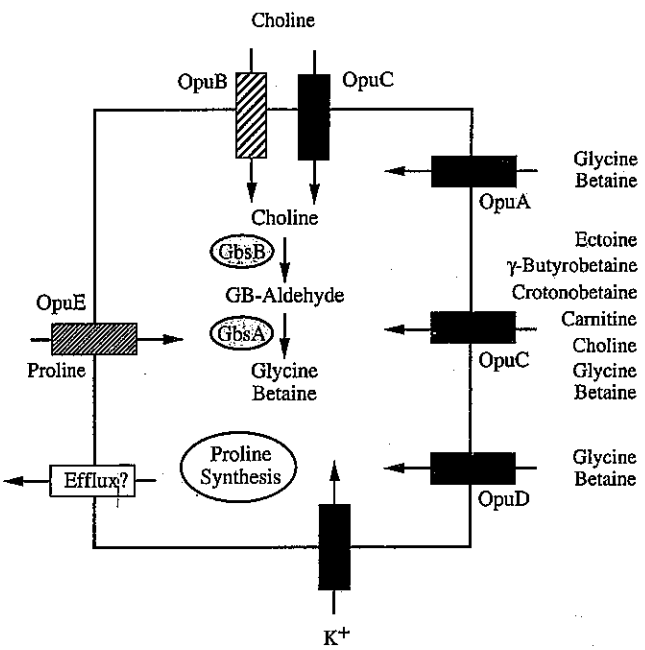

Figure 3. Uptake and synthesis of osmoprotectants in *B. subtilis*.

The OpuE-mediated transport of proline is strongly enhanced after an osmotic upshock. This increase in proline uptake is entirely dependent on *de novo* protein synthesis, and hence the activity of the OpuE protein is not stimulated by elevated osmolarity. Mapping of the initiation sites for the *opuE* transcripts by high-resolution primer extension reactions revealed the presence of two distinct *opuE* mRNA species whose amount is regulated in response to the osmolarity of the environment (von Blohn *et al* 1997). Mutant analysis demonstrated the presence of two promoters, *opuE*-P1 and *opuE*-P2, that are recognized by the house-keeping sigma factor of *B. subtilis*, σ^A, and the alternative transcription factor, σ^B, respectively. The σ^B-dependent control of *opuE*-P2 activity links the OpuE transporter to the general stress regulon of *B. subtilis*. Although the addition of salt triggers enhanced transcription of many genes of the σ^B regulon (Hecker *et al* 1996), no clear function in osmoadaptation had been identified for any of its members. *opuE* is thus the first example of a σ^B-responsive gene with a demonstrated physiological role in the osmoadaptation of *B. subtilis*. The activity of the *opuE*-P1 promoter remains fully osmoregulated in a *sigB* mutant (von Blohn *et al* 1997). It is apparent that two independent signal transduction pathways operate in *B. subtilis* to osmotically control the level of *opuE* expression. The *opuE* gene might thus serve as a useful model for unravelling the mechanism of osmosensing in *B. subtilis* and the processing of this information into a genetic signal that governs gene expression in response to a changing environment.

5.2 *Three glycine betaine transport systems operate in B. subtilis*

Like many other Gram-negative and Gram-positive bacteria, *B. subtilis* uses the trimethylammonium compound glycine betaine (figure 2) as a metabolically inert and highly effective compatible solute (Boch *et al* 1994). Its osmoprotective capacity for osmotically stressed *B. subtilis* cells greatly exceeds that of exogenously provided proline (von Blohn *et al* 1997). In *B. subtilis* grown in minimal medium with 1 mM glycine betaine, the intracellular level of this osmoprotectant increased after a moderate upshock with 0·4 M NaCl from approximately 175 mM to approximately 700 mM (Whatmore *et al* 1990). Since the addition of 0·4 M salt to a basal minimal medium does not strongly affect the growth of *B. subtilis* (Boch *et al* 1996), the intracellular concentration of glycine betaine is likely to well surpass 700 mM under high-salinity growth conditions. This high-level accumulation of glycine betaine confers a considerable degree of osmotic tolerance and permits the growth of *B. subtilis* in habitats that are otherwise strongly inhibitory for its proliferation (Boch *et al* 1994). Glycine betaine is widely found in nature. It is employed as an osmoprotectant by plants (Rhodes and Hanson 1993) and is released from decomposing plant material and root exudates into the habitat of *B. subtilis*. Its concentration is likely to vary considerably in the upper layers of the soil, and *B. subtilis* thus requires effective mechanisms for its uptake from the environment. Through gene complementation and mutagenesis experiments, we identified three highly effective glycine betaine transport systems in *B. subtilis*: OpuA, OpuC, and OpuD (figure 3) (Kempf and Bremer 1995; Kappes *et al* 1996). Each of these is under osmotic control and actively participates in the stress reaction of *B. subtilis* to a high-osmolarity environment. Osmoprotection by glycine betaine is completely abolished only in a mutant that lacks all three uptake systems (Kappes *et al* 1996). None of these glycine betaine transporters contribute to proline uptake (von Blohn *et al* 1997).

Molecular and biochemical characterization of the OpuA, OpuC, and OpuD systems revealed that the OpuA (OpuAA, OpuAB, OpuAC) and OpuC (OpuCA, OpuCB, OpuCC, OpuCD) transporters are multicomponent systems belonging to the ABC-type superfamily, whereas OpuD consists of a single component. The latter is an integral membrane protein and represents a new type of glycine betaine transport system. An OpuD-related glycine betaine transporter, BetP, has recently been characterized in the soil bacterium *C. glutamicum* (Peter *et al* 1996). OpuD and BetP, together with the choline transporter BetT and the carnitine transporter CaiT from *E. coli*, form a small family of structurally and functionally related uptake systems for various trimethylammonium compounds (Kappes *et al* 1996). The OpuA and OpuC systems are related to the binding-protein-dependent glycine betaine transporter ProU from *E. coli* and *S. typhimurium* (Lucht and Bremer 1994; Csonka and Epstein 1996; Gowrishankar and Manna 1996). The ProU system is characterized by a high-affinity ($K_D = 1{\cdot}4\ \mu M$) substrate-binding protein (ProX), that can freely diffuse in the periplasm. As a Gram-positive bacterium, *B. subtilis* lacks this compartment and the substrate binding proteins (OpuAC and OpuCC) of the OpuA and OpuC systems are anchored in the cytoplasmic membrane via a lipid modification at the amino-terminal cysteine residue to prevent their loss into the surrounding medium (Kempf *et al* 1997). By constructing a full set of *B. subtilis* mutant strains expressing or lacking particular glycine betaine uptake systems, we were able to assess the kinetic parameters and individual contributions made by the OpuA, OpuC, and OpuD transporters to osmoprotectant accumulation (Kempf and Bremer 1995; Kappes *et al* 1996). OpuA is clearly the dominating glycine betaine transport system of *B. subtilis*.

During the analysis of subtilin production in *B. subtilis*,

Lin and Hansen (1995) found a chimeric *proU* operon encoding a multi-component transport system for glycine betaine. This hybrid ProU system was artificially constructed by transforming a non-subtilin producing strain of *B. subtilis* with chromosomal DNA of a subtilin producer (Lin and Hansen 1995). The hybrid transporter is related to but not identical with the OpuC system characterized in our laboratory (Kappes *et al* 1996, 1998).

5.3 *Substrate specificity of the OpuA, OpuC and OpuD transporters*

A wide range of naturally occurring trimethylammonium and sulphonium compounds are known to function as osmoprotectants (Csonka and Epstein 1996; Galinski and Trüper 1994). We have begun to characterize the osmoprotective effects of such compounds for *B. subtilis* and discovered that in particular the OpuC system functions in the acquisition of a variaty of osmoprotectants from the environment. So far, we have carried out a detailed physiological and kinetic analysis of the uptake of the carnitine, crotonobetaine, γ-butyrobetaine and ectoine (Jebbar *et al* 1997; Kappes and Bremer 1998). The trimethylammonium compounds carnitine, crotonobetaine, and γ-butyrobetaine are ubiquitous in nature (Kleber 1997) and are structurally related to glycine betaine (figure 2). Kinetic and growth experiments revealed that OpuC recognizes each of these compounds with the same high affinity ($K_m = 5$ μM) as glycine betaine (Kappes and Bremer 1998). The cyclic amino acid derivative ectoine is employed by a variety of bacilli as an endogenously synthesized compatible solute (Galinski and Trüper 1994). We found that exogenously provided ectoine functioned as an osmoprotectant for the non-producer *B. subtilis*, and we have identified OpuC as its sole uptake route (Jebbar *et al* 1997). Osmoprotection of *B. subtilis* by ectoine proved to be inefficient, which can be attributed to the low affinity of the OpuC system ($K_i = 1{\cdot}56$ mM) for this compound (Jebbar *et al* 1997). These studies show the value of a genetically well characterized set of mutant strains with defined defects in osmoprotectant uptake systems and revealed that the ABC transporter OpuC plays a crucial role in supplying *B. subtilis* with a spectrum of osmoprotectants (figure 3). None of the osmoprotectants tested thus far (with the exception of proline) is used as sole carbon or nitrogen source by *B. subtilis*; therefore, these substances are accumulated by osmoregulating cells to serve as metabolically inert stress compounds.

6. The osmoregulatory choline glycine betaine synthesis pathway

In response to a water deficit, many bacterial and plant species can synthesize glycine betaine from the precursor choline with glycine betaine aldehyde (figure 2) as the intermediate (Rhodes and Hanson 1993; Galinski and Trüper 1994; Csonka and Epstein 1996). *B. subtilis* is no exception (Boch *et al* 1994). Data reported in the literature demonstrate that choline oxidation can be accomplished by three different enzymatic systems. In the Gram-positive bacterium *Arthrobacter globiformis* and *A. pascens*, and the fungus *Cylindrocarpon didymum* a bifunctional, soluble choline oxidase mediates glycine betaine production (Rozwadowski *et al* 1991). Higher plants employ a soluble choline monooxygenase to produce glycine betaine aldehyde and a glycine betaine aldehyde dehydrogenase to oxidize this intermediate into glycine betaine (Rathinasabapathi *et al* 1997). In the Gram-negative bacteria *E. coli* and *Sinorhizobium meliloti* a glycine betaine aldehyde dehydrogenase is also found; however, in these organisms it acts in conjunction with a membrane-bound FAD-containing choline dehydrogenase, which can oxidize both choline and glycine betaine aldehyde to glycine betaine at the same rate (Lamark *et al* 1991; Pocard *et al* 1997).

Our characterization of the glycine betaine synthesis pathway from *B. subtilis* revealed that this bacterium employs a novel combination of enzymes (Boch *et al* 1996). A soluble NAD-dependent type-III alcohol dehydrogenase (GbsB, glycine betaine synthesis) converts choline into glycine betaine aldehyde, and a soluble glycine betaine aldehyde dehydrogenase (GbsA) oxidizes the aldehyde into glycine betaine. The GbsA enzyme shows striking sequence identity (between 37 and 45%) to the glycine betaine aldehyde dehydrogenases known to be involved in osmoadaptation in plants and other microorganisms. However, use of a NAD-dependent type-III alcohol dehydrogenase in the biosynthesis of glycine betaine has not been reported before. The *B. subtilis gbsA* and *gbsB* genes are likely to form an operon whose transcription is enhanced by the presence of choline in the growth medium (Boch *et al* 1996). This observation hints to the presence of a regulatory protein that mediates the genetic control of *gbsAB* expression in response to the availability of choline.

In osmoregulating *B. subtilis* cells, the glycine betaine-synthesizing enzymes are initially faced with substantial amounts of K^+ and proline, and GbsA must function in the presence of molar concentrations of glycine betaine. To better understand the functioning of these enzymes, we have begun to analyse them biochemically. We have recently reported the characterization of the purified GbsA (Boch *et al* 1997). Although *B. subtilis* is not a halotolerant bacterium, the GbsA enzyme proved to be highly resistant to salt and retained 88% of its initial enzymatic activity in the presence of 2·5 M KCl. The enzyme was stimulated by proline, and its activity was only moderately inhibited by molar concentrations of glycine betaine. These features ensure that *B. subtilis* can produce high levels of the compatible

solute glycine betaine under conditions of high-osmolarity stress.

Uptake of the glycine betaine precursor choline is stimulated by the osmolarity of the environment (Boch *et al* 1994), and our recent molecular analysis of choline transport in *B. subtilis* has established the role of two multicomponent ABC-type transporters in choline uptake (Kappes *et al* 1998). One of these transporters corresponds to the OpuC uptake system for glycine betaine, carnitine, crotonobetaine and γ-butyrobetaine (figure 3), whereas the second transporter, OpuB, represents a new uptake system (figure 3). Both OpuC and OpuB exhibit a high affinity for choline and are evolutionarily closely related. The structural genes for the *opuC* and *opuB* operons are transcribed in the same direction, are located only a few kb apart on the *B. subtilis* chromosome and their gene products exhibit sequence identities of over 65% (Kappes R M, Kempf B, Kneip S, Boch J, Gade J and Bremer E, in preparation). These findings suggest that the *opuB* and *opuC* loci originate from a gene duplication event with a subsequent evolution in the substrate specificity of the OpuB and OpuC transporters. As detailed above, OpuC exhibits a wide substrate specificity, whereas the transport activity of the OpuB system appears to be restricted to choline and glycine betaine aldehyde (figure 3) (Kappes *et al* 1998). The unusual close structural relatedness of these transporters and their different spectra of osmoprotectant uptake pose intriguing questions with respect to the molecular basis of their distinct substrate specificity.

7. Efflux of osmoprotectants

It is apparent that growth of *B. subtilis* in high-osmolarity environments will lead to the massive intracellular accumulation of compatible solutes either via direct uptake from the environment or through synthesis. In addition, the cell will also contain substantial amounts of K^+ (figure 3). However, a soil bacterium is likely to experience frequent hypo osmotic shocks caused by rain and flooding. Such conditions will lead to a rapid and massive influx of water into the cell, and the bacteria must be able to quickly reduce their intracellular solute pool to avoid cell lysis. Decreases in osmotic pressure are known to cause the efflux of K^+, glycine betaine, proline, choline, and trehalose from *E. coli* and *S. typhimurium* cells (Csonka and Epstein 1996). In the Gram-positive bacteria *Lactobacillus plantarum* and *C. glutamicum*, stretch-activated channels have been detected that preferentially mediate the rapid efflux of compatible solutes subsequent to osmotic downshock (Glaasker *et al* 1996; Ruffert *et al* 1997). These rapid effluxes are probably mediated in both organisms by mechano-sensitive channels and proceed in *L. plantarum* by an additional carrier-like efflux system (Glaasker *et al* 1996). Detailed physiological studies on the release of compatible solutes in hypo-osmotically stressed cells have not yet been performed with *B. subtilis*, but stretch-activated ion channels are present in its cytoplasmic membrane and might well constitute part of the cellular protection mechanism against osmotic down shocks (Zoratti *et al* 1990).

8. Summary and outlook

Figure 3 provides a cartoon of the currently identified systems in *B. subtilis* for the uptake and synthesis of osmoprotectants and compatible solutes. The massive intracellular accumulation of these organic osmolytes is an integral part of the adaptation reaction of *B. subtilis* to high-osmolarity stress and allows this soil bacterium to proliferate in habitats which are otherwise strongly inhibitory for its growth. The genes encoding these systems for the uptake and synthesis of osmoprotectants thus provide a framework for the future characterization of the molecular and cellular stress responses to high osmolarity. In particular, this will allow us to experimentally address the signal transduction pathway(s) that allow *B. subtilis* to sense changes in the environmental osmolarity and to identify the molecular mechanisms converting this information into a cellular stress response. Part of this complex network are the mechanisms that allow the extrusion of compatible solutes and ions from the cells upon hypo osmotic shock. The complexity and number of systems already identified for synthesis and uptake of osmoprotectants make it apparent that *B. subtilis* must be able to integrate them into a homeostatic network of stress responses in order to survive and grow in an environment subjected to rapid changes in osmolarity.

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