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MINI-REVIEW

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Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments

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Abstract All microorganisms possess a positive turgor, and maintenance of this outward-directed pressure is essential since it is generally considered as the driving force for cell expansion. Exposure of microorganisms to high-osmolality environments triggers rapid fluxes of cell water along the osmotic gradient out of the cell, thus causing a reduction in turgor and dehydration of the cytoplasm. To counteract the outflow of water, microorganisms increase their intracellular solute pool by amassing large amounts of organic osmolytes, the so-called compatible solutes. These osmoprotectants are highly congruous with the physiology of the cell and comprise a limited number of substances including the disaccharide trehalose, the amino acid proline, and the trimethylammonium compound glycine betaine. The intracellular amassing of compatible solutes as an adaptive strategy to high-osmolality environments is evolutionarily well-conserved in Bacteria, Archaea, and Eukarya. Furthermore, the nature of the osmolytes that are accumulated during water stress is maintained across the kingdoms, reflecting fundamental constraints on the kind of solutes that are compatible with macromolecular and cellular functions. Generally, compatible solutes can be amassed by microorganisms through uptake and synthesis. Here we summarise the molecular mechanisms of compatible solute accumulation in *Escherichia coli* and *Bacillus subtilis*, model organisms for the gram-negative and gram-positive branches of bacteria.

Key words Osmoregulation · Stress protectants · Trehalose · Glycine betaine · K^+ uptake · ABC transporters · Efflux · Gene regulation · *Escherichia coli* · *Bacillus subtilis*

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Introduction

All microorganisms must cope with changes in water availability in their milieu since the concentration of solutes within the cell is higher than that in the environment. Sustaining such a difference on opposite sides of the semipermeable cytoplasmic membrane is essential since a positive turgor is considered as the driving force for cell expansion. Although turgor is quite difficult to quantify, values of $3–10 \times 10^5$ Pa (3–10 bar) for gram-negative bacteria and approximately 20×10^5 Pa (20 bar) for gram-positive microorganisms have been estimated (Whatmore and Reed 1990; Csonka and Epstein 1996). The elastic murein sacculus allows the bacteria to withstand these remarkable pressures, which in the case of the gram-positive soil bacterium *Bacillus subtilis* (Whatmore and Reed 1990) is ten times the pressure present in a standard car tire. Changes in the external osmolality trigger water fluxes along the osmotic gradient and thus cause either swelling and bursting of the cell in hypotonic environments or plasmolysis and dehydration under hypertonic conditions. Microorganisms actively respond to variations in the osmolality of their habitat (Galinski and Trüper 1994; Csonka and Epstein 1996; Miller and Wood 1996). This stress reaction, generally referred to as osmoregulation or osmoadaptation, is aimed at maintaining turgor and volume within boundaries acceptable for normal cellular physiology (Record et al. 1998a,b). Rapid fluxes of water accompany these osmoregulatory responses. Until the discovery of dedicated water channels (aquaporins) in eukaryotic cells, water permeation across lipid bilayers was considered to be an entirely passive diffusion process. However, the recent discovery of aquaporins (e.g. AqpZ) in microorganisms raises the possibility that these specific water channels participate in turgor control (Calamita et al. 1995).

Microorganisms do not possess active transport mechanisms for water; hence, turgor is adjusted by controlling the pool of osmotically active solutes in the cytoplasm. Two basic schemes of adaptation to high osmolality have been identified.

1. The accumulation of very high intracellular concentrations of ions, a strategy followed frequently by extreme halophilic archaea and by halotolerant bacteria whose entire physiology has been adapted to a high-saline environment (Galinski and Trüper 1994; Ventosa et al. 1998); this adaptive strategy, however, is not considered in detail in this review.

2. The intracellular amassing of osmotically active compounds that are highly congruous with cellular functions, the so-called compatible solutes (Brown 1976). These organic solutes are accumulated by many microorganisms through synthesis or through uptake from the environment to counteract the outflow of water under hypertonic growth conditions (Galinski and Trüper 1994; Csonka and Epstein 1996; Miller and Wood 1996). Osmoprotectants are operationally defined as exogenously provided organic solutes that enhance bacterial growth in media of high osmolality. These substances may themselves be compatible solutes, or they may act as precursor molecules that can be enzymatically converted into these compounds. The intracellular amassing of compatible solutes is not restricted to the prokaryotic world but is also widely used as an adaptive strategy in fungal, plant, animal, and even human cells to offset the deleterious effects of high osmolality and high ionic strength (Rhodes and Hanson 1993; Burg et al. 1997; Hohmann 1997). Furthermore, the nature of the osmolytes that are accumulated during water stress is evolutionarily well-conserved across the kingdoms, reflecting fundamental constraints on the kind of solutes that are compatible with macromolecular and cellular functions (Le Rudulier et al. 1984; Yancey 1994).

Compatible solutes: characteristics and function

A variety of bacteria (Galinski and Trüper 1994; Ventosa et al. 1998) and archaea (Martins et al. 1997) have been examined for compatible solute production by means of HPLC and NMR methods. The spectrum of compatible solutes used by microorganisms comprises only a limited number of compounds: sugars (e.g. trehalose), polyols (e.g. glycerol and glucosylglycerol), free amino acids (e.g. proline and glutamate), derivatives thereof (e.g. proline betaine and ectoine), quaternary amines and their sulfonium analogues (e.g. glycine betaine, carnitine and dimethylsulfoniopropionate), sulfate esters (e.g. choline-*O*-sulfate), and *N*-acetylated diamino acids and small peptides (e.g. *N*δ-acetylornithine and *N*-acetylglutaminylglutamine amide). In general, compatible solutes (Fig. 1) 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 cellular functions such as DNA replication, DNA-protein interactions, and the cellular metabolic machinery (Strøm and Kaasen 1993; Yancey 1994; Record et al. 1998a,b). Many microorganisms accumulate compatible solutes as metabolically inert stress compounds, and in those instances where these substances can be metabolised (Pichereau et al. 1998), sensitively balanced regulatory mechanisms ensure their high-level accumulation under hypertonic conditions. A spectrum of compatible solutes is usually used by a given micoorganism for osmoregulatory purposes, and the composition of the solute pool can vary in response to growth phase and growth medium (Galinski and Trüper 1994). The accumulation of compatible solutes not only allows the cells to withstand a given osmolality but also expands the ability of microorganisms to colonise ecological niches that are otherwise strongly inhibitory for their proliferation. Depending on the type, compatible solutes can also protect microorganisms against stresses other than dehydration. An example is the increased cold tolerance conferred by the accumulation of glycine betaine in the food-borne pathogen *Listeria monocytogenes* (Ko et al. 1994).

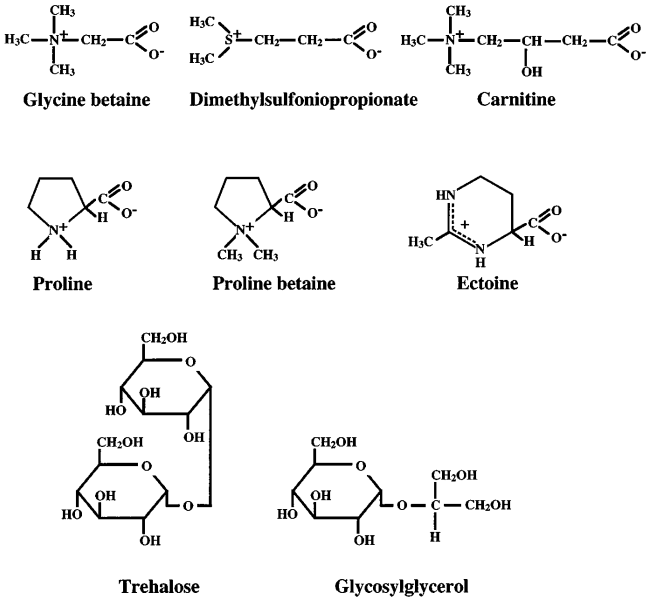

Fig. 1 Structures of selected osmoprotectants

Compatible solutes serve a dual function in osmoregulating cells. Because microorganisms frequently accumulate them up to molar concentrations, compatible solutes lower the cytosolic osmotic potential and hence make major contributions to the restoration and maintenance of turgor. The free cytoplasmic volume (unbound water, in contrast to water bound by macromolecules) is a key determinant for cell growth (Cayley et al. 1992). Compatible solutes such as glycine betaine and proline increase the cytoplasmic volume and free water content of the cells at high osmolality, and their accumulation thus permits continued cell proliferation under unfavourable conditions (Record et al. 1998a). Compatible solutes also serve as stabilisers of proteins and cell components against the denaturing effects of high ionic strength. This protective property is not fully understood, but according to the preferential exclusion model, osmoprotectants are kept away from the immediate vicinity of proteins, resulting in a preferential hydration of protein surfaces. This solvent distribution leads to a situation in which the disruption of wa-

ter structure in the hydration shell of proteins by local or global unfolding of the polypeptide chain is energetically unfavourable, and hence the native conformations of proteins are stabilised (Yancey 1994).

The intracellular amassing of compatible solutes is at the core of the osmostress response in many microorganisms. Here we concentrate on the molecular mechanisms of compatible solute accumulation in *Escherichia coli* and *B. subtilis*, model organisms for gram-negative and gram-positive bacteria. Studies on osmoadaptation in these two bacteria are not inheritantly more important or interesting than those focusing on other microorganisms. However, *E. coli* and *B. subtilis* are amenable to sophisticated genetic and molecular approaches, and these studies have consequently provided detailed insights into the systems for uptake and synthesis of compatible solutes.

Initial phase of osmoadaptation in *E. coli*: accumulation of K^+ glutamate

Bacteria that are subjected to a sudden increase in osmolality usually respond with an adaptation reaction that is characterised by two distinct phases. Studies with the enteric gram-negative bacteria *E. coli* and *Salmonella typhimurium* revealed that initially large amounts of K^+ are rapidly taken up from the environment via turgor-responsive transport systems (Fig. 2). Concomitantly, within 1 min of the osmotic upshock, glutamate synthesis is increased to provide counterions for the strong increase in positive charges. Glutamate synthesis is dependent on the prior uptake of K^+, and glutamate is required to maintain the steady-state K^+ pool (McLaggan et al. 1994; Yan et al. 1996). High intracellular concentrations of K^+, however, have negative effects on protein function and DNA-protein interactions in nonhalophilic bacteria; thus, the massive accumulation of K^+ is an inadequate strategy for coping with prolonged high osmolality. Instead, the initial increase in cellular K^+ content is followed by the accumulation of compatible solutes, which allows the cell to discharge large amounts of K^+ through specific (Kef) and nonspecific efflux systems (Fig. 2; Csonka and Epstein 1996; Stumpe et al. 1996).

Three different uptake systems for K^+ operate in *E. coli*: Kup, Trk and Kdp (Csonka and Epstein 1996; Stumpe et al. 1996). Low-level K^+ uptake via the minor transporter Kup is constitutive and is not influenced by medium osmolality. In contrast, K^+ uptake via the multicomponent Trk and Kdp systems is increased upon osmotic shock, and cellular K^+ levels are determined (at least in part) by the osmolality of the growth medium. Changes in turgor contribute to the regulation in the activity of both the Trk and the Kdp transporters, and the level of turgor is thought to control expression of the *kdpFABC* operon via the KdpD-KdpE regulatory module. This two-component system has attracted considerable attention since a regulatory model has been proposed that directly links osmotically mediated changes in turgor or envelope tension to the genetic control of *kdpFABC* expression (Csonka and Epstein 1996; Jung et al. 1997). Signal perception (e.g. a drop in turgor) induces autophosphorylation at a histidine residue of the KdpD kinase and is thought to proceed via four transmembrane segments found in the middle of the protein. The phosphorylated KdpD then rapidly transfers the phosphoryl group to an aspartate residue in the response regulator KdpE, which in turn activates expression of the *kdpFABC* operon. Transcription of *kdpFABC* is only transiently induced following osmotic upshifts, a regulatory pattern that is consistent with the observation that turgor is restored (at least partially) by an increase in the level of K^+.

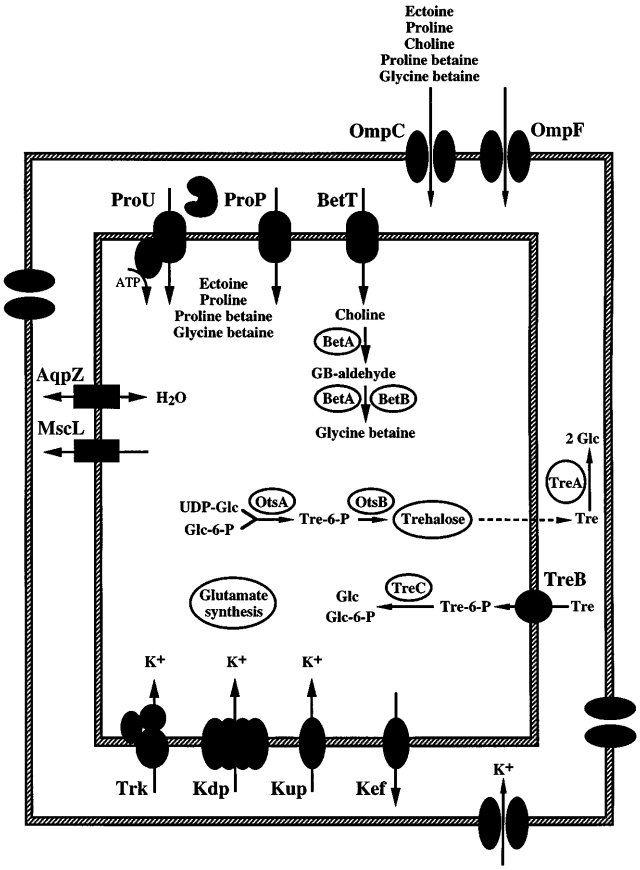

Fig. 2 Osmostress response systems of *Escherichia coli* (*GB* glycine betaine, *Glc* glucose, *Glc-6-P* glucose-6-phosphate, *Tre* trehalose, *Tre-6-P* trehalose-6-phosphate, *UDP-Glc* uridine disphosphate-glucose)

The temporal sequence of events during the initial phase in osmoadaptation, and the finding that many osmotic responses and the resumption of growth following a hyperosmotic shock are dependent on the prior accumulation of K^+ have led to the proposal that K^+/glutamate acts as a second messenger to trigger and coordinate subsequent osmotic responses (Epstein 1986; Higgins et al. 1987). This concept is quite attractive, but nevertheless it remains to be directly demonstrated whether the size of and temporary changes in the cellular K^+ glutamate pool can serve as an internal signal for the onset of the second phase of osmoadaptation: the accumulation of compatible solutes.

Endogenous osmoprotection by synthesis of trehalose

The disaccharide trehalose (Fig. 1) is an important stress compound in both prokaryotic and eukaryotic organisms. *E. coli* and *S. typhimurium* accumulate it via de novo synthesis as their predominant endogenous compatible solute in minimal medium and in the absence of exogenous compatible solutes such as glycine betaine and proline (Dinnbier et al. 1988; Strøm and Kaasen 1993). For osmoadaptation of growing *E. coli* cells, the uptake of proline and glycine betaine thus has priority over the synthesis of trehalose, which in turn is preferred over K^+ and glutamate as osmoprotectants (Dinnbier et al. 1988). Two enzymes encoded by the *otsBA* (osmoregulated trehalose synthesis) operon determine this osmoregulatory trehalose synthesis. OtsA, the trehalose-6-phosphate synthase, catalyses the enzymatic condensation of the precursors glucose-6-phosphate and UDP-glucose, and free trehalose is then generated from this intermediate by the *otsB*-encoded trehalose-6-phosphate phosphatase (Fig. 2). The accumulation of trehalose provides osmoprotection sufficient to withstand osmotic stress generated by adding 0.5 M NaCl to a minimal medium (Strøm and Kaasen 1993).

Osmotic stress induces the expression of the *otsBA* operon, which is entirely dependent on σ^S (RpoS), an alternative transcription factor of *E. coli* controlling or contributing to gene expression under various stress conditions (e.g. osmotic stress) and in stationary phase (Hengge-Aronis 1996; Conter et al. 1997). Hence, elevated levels of trehalose are also found in stationary-phase cells not subjected to high osmolality, reflecting the function of this sugar as a general stress protectant (Strøm and Kaasen 1993). The K^+-glutamate accumulated in osmotically stressed cells (Record et al. 1998a,b) appears to play a specific role for the trehalose synthesis pathway since the activity of the *otsA*-encoded trehalose-6-phosphate synthetase is strongly stimulated by K^+-glutamate and salts of other monovalent cations (Strøm and Kaasen 1993). Remarkably, only endogenously synthesised trehalose has an osmoprotective function because exogenously provided trehalose is degraded to two glucose molecules by an osmotically induced periplasmic trehalase (TreA) and because there is only low-level synthesis of a trehalose-specific, phosphoenolpyruvate:sugar phosphotransferase (PTS)-dependent transport system (enzyme IITre; TreB) (Fig. 2) in high osmolality-grown cells. The presence of TreA in the periplasm allows exogenously provided trehalose to be used as a carbon source under high osmolality growth conditions and permits extruded trehalose to be recaptured as glucose molecules via the glucose-specific PTS system (Fig. 2) in high osmolarity-grown cells. As in the case of *otsBA* operon, expression of *treA* is osmotically controlled and is partially dependent on RpoS. Under low-osmolality growth conditions, trehalose can also be acquired via TreB as trehalose-6-phosphate, which is then enzymatically hydrolysed by TreC to glucose and glucose-6-phosphate for further metabolism (Fig. 2). The dual use of trehalose as a compatible solute and as a carbon source requires that the corresponding biosynthetic and degradative pathways be strictly controlled and linked to the osmolality of the environment to avoid a futile cycle. In contrast to the *otsBA* and *treA* loci, trehalose mediates induction of *treBC*, an effect conferred by the TreR regulatory protein with trehalose-6-phosphate being the internal inducer. This inducer is effectively hydrolysed under high osmolality growth conditions by the *otsB*-encoded trehalose-6-phosphate phosphatase, thus preventing induction of the catabolic *treBC* operon under conditions under which trehalose is accumulated by the cell as a stress compound (Horlacher et al. 1996).

Characteristics of transporters for osmoprotectants

In addition to accumulating compatible solutes by endogenous synthesis, both Bacteria and Archaea have developed uptake systems for osmoprotectants. These compounds are released into ecosystems by primary microbial producers upon dilution stress, by decaying plant and animal cells, and by mammals in the form of excretion fluids (e.g. urine) (Galinski and Trüper 1994; Ventosa et al. 1998). Transporters for osmoprotectants have evolved to meet the special demands imposed by their physiological tasks. They must function most effectively at high osmolality and at high ionic strength, conditions that often inhibit transporters for the uptake of nutrients. Their level of activity is frequently osmotically controlled, and the expression of the genes encoding osmoprotectant uptake systems is often induced in high osmolality media (Csonka and Epstein 1996). In natural ecosystems, the supply of compatible solutes is likely to be low and varying. Therefore, osmoprotectant transporters usually exhibit high affinity for their substrates with K_m values in the micromolar range, and their capacity is geared to permit high-level compatible solute accumulation. The kinetic parameters for the uptake of a particular osmoprotectant greatly determine its effectiveness. Microorganisms frequently possess several transport systems for osmoprotectants. A number of these transporters exhibit a broad substrate specificity so that the cells can take advantage of a spectrum of compatible solutes that might be present in their habitat. The widespread ability of microorganisms to accumulate betaines might be exploited for the delivery of structurally related compounds with antibacterial activity (Peddie et al. 1998). Potential complications might arise, however, through the presence of betaine transporters in human kidney cells (Burg et al. 1997).

Accumulation of compatible solutes from exogenous sources generally strongly decreases – at least over a certain range of osmolarities – the synthesis of the indigenously produced osmoprotectants (Dinnbier et al. 1988; Whatmore et al. 1990). This observation implies a finely tuned cellular control over the pool of compatible solutes achieved by the bacteria via uptake, synthesis and excretion. The sensitivity of this regulatory network is illustrated by the finding that the presence of osmoprotectants in the growth medium frequently reduces the transcription

of osmotically induced genes encoding components for uptake and synthesis of osmoprotectants. Furthermore, for a number of uptake systems, intracellularly accumulated osmoprotectants can inhibit the transport of exogenously provided compatible solutes until the cell is subjected to increased osmolality, suggesting that alterations in membrane structure are transmitted to an allosteric binding site of the uptake system (Verheul et al. 1997).

Features of the osmoregulated ProP and ProU transport systems

An exogenous supply of osmoprotectants in micromolar to 1 mM concentrations confers a considerable degree of osmotic tolerance, allowing growth of *E. coli* and *S. typhimurium* in hyperosmotic media containing up to 1 M NaCl. Two transporters are primarily responsible for the uptake of osmoprotectants across the cytoplasmic membrane of these enteric bacteria: ProP and ProU (Fig. 2). These transporters were originally identified as osmotically stimulated uptake systems for proline, but subsequent studies established their pivotal role in the uptake of a wide spectrum of osmoprotectants (Table 1), of which glycine betaine and proline betaine (Fig. 1) are transported with high affinity (Csonka and Epstein 1996). Permeation of osmoprotectants across the *E. coli* and *S. typhimurium* outer membrane is accomplished by passive diffusion through the nonspecific porins OmpC and OmpF (Fig. 2). Expression of the *ompC* and *ompF* structural genes is regulated in a reciprocal fashion by medium osmolality such that OmpC synthesis predominates in hypertonic media and OmpF production occurs primarily under hypotonic conditions (Pratt et al. 1996). The level of *ompC* and *ompF* transcription is responsive to medium osmolarity and a variety of other environmental parameters. Osmoregulation of these genes is mediated by the cytoplasmic membrane-embedded sensor kinase EnvZ and by the response regulator OmpR, a classical example of the two-component regulatory module. In addition, fine-tuning of the OmpC and OmpF porin levels involves the antisense RNA MicF, which acts as a negative regulator of *ompF* expression at the post-transcriptional stage (Pratt et al. 1996).

ProP is a single-component transport system driven by cation symport. It mediates transport of a wide variety of osmoprotectants structurally related to proline and glycine betaine (Table 1) and also serves as an entry route for ectoine (Fig. 1). ProP-mediated import of compatible solutes is greatly enhanced in high osmolality media as a result of increased *proP* expression and stimulated transport activity. Transcription of *proP* is directed from two promoters, P1 and P2, both of which are activated by osmotic upshifts. The activity of *proP*-P1 is normally repressed by the cAMP-CRP complex, which binds to a site overlapping the –35 region. Activity of *proP*-P2 is dependent on RpoS and the nucleoid-associated protein Fis (Mellies et al. 1995; Xu and Johnson 1997). Thus, *proP* is a member of a growing class of RpoS-dependent genes that respond to both stationary-phase and high-osmolality signals (Hengge-Aronis 1996; Conter et al. 1997).

Activity of the ProP protein is enhanced by hypertonicity both in vivo and in vitro (Table 1), suggesting that the ProP transporter also functions as a sensor for osmot-

Table 1 Properties of osmoprotectant transport systems (*BB* γ-butyrobetaine, *Car* carnitine, *CB* crotonobetaine, *Cho* choline, *GB* glycine betaine, *DMG* dimethylglycine, *DMSA* dimethylsulfonioacetate, *DMSP* dimethylsulfoniopropionate, *Ect* ectoine, *HB* homobetaine, *PB* proline betaine, *PIP* L-pipecolate, and *nd* not determined)

Organism	System	Type of transporter[a]	Components	Osmotic stimulation of osmoprotectant transport by		Substrates
				Gene expression	Transport activity	
Escherichia coli	ProP	Secondary transporter	ProP	+	+	GB, PB, Pro, Car, Ect, PIP, DMP, DMG, DMSP, HB, BB
Escherichia coli	ProU	ABC transporter	ProV, ProW, ProX	+	+	GB, PB, Pro, Car, DMP, HB, BB, Cho
Escherichia coli	BetT	Secondary transporter	BetT	+	+	Cho
Corynebacterium glutamicum	BetP	Secondary transporter	BetP		+	GB
Bacillus subtilis	OpuA	ABC transporter	OpuAA, OpuAB, OpuAC	+	nd	GB, PB, DMSA, DMSP
Bacillus subtilis	OpuB	ABC transporter	OpuBA, OpuBB, OpuBC, OpuBD	+	nd	Cho
Bacillus subtilis	OpuC	ABC transporter	OpuCA, OpuCB, OpuCC, OpuCD	+	nd	GB, PB, Car, DMSA, DMSP, Cho, BB, CB, Ect, Choline-*O*-sulfate
Bacillus subtilis	OpuD	Secondary transporter	OpuD	+	+	GB, DMSA, DMSP
Bacillus subtilis	OpuE	Secondary transporter	OpuE	+	–	Pro
Erwinia chrysanthemi	OusA	Secondary transporter	OusA	+	–	GB, Pro, Ect, PIP

[a] ABC transporter, ATP binding cassette transporter

ically instigated changes in the physicochemical parameters of the cytoplasmic membrane. ProP possesses a carboxyterminal extension with a high propensity for forming an α-helical coiled coil; this unusual structural feature is thought to participate in the osmoregulation of ProP transport activity (Culham et al. 1993). Control of transporter activity is a critical aspect in the physiology of compatible solute acquisition by microorganisms, and this poorly understood property of these transport systems deserves greater attention in future investigations. Recent molecular studies have demonstrated the importance of the highly charged N-terminal and C-terminal extensions in controlling the activity of BetP, a high-affinity glycine betaine uptake system from *Corynebacterium glutamicum* (Peter et al. 1998). The BetP carrier is completely inactive under isoosmotic conditions, but osmotic stress triggers a strong increase in transport activity within seconds. Deletion derivatives of BetP with truncated N-terminal or C-terminal extensions exhibit altered or completely abolished responses to osmotic stress, and these alterations severely impair the ability of *C. glutamicum* to adapt to hyperosmotic conditions (Peter et al. 1998). Such genetic approaches are likely to make important contributions to our understanding of the molecular basis for the sensing of osmo-stress (mechano-stress) at the level of membrane and protein structure and for the processing of this information to result in a transport system with altered kinetic properties.

ProU, the second osmoprotectant transporter of *E. coli*, is a binding protein-dependent transport system and a member of the ABC superfamily of transporters (Table 1). The ProU system consists of a membrane-associated ATPase (ProV), the integral membrane protein ProW, and the periplasmic substrate-binding protein ProX (Fig. 2). ProU exhibits a broad substrate specificity for compatible solutes but displays a clear preference for certain compounds, most notably glycine betaine and proline betaine (Gouesbet et al. 1994; Haardt et al. 1995). These two compatible solutes are bound with high affinity (K_D = 1.4 μM and 5.2 μM, respectively) to ProX, whereas no binding could be demonstrated for a variety of osmoprotectants (e.g. proline, L-carnitine, γ-butyrobetaine and dimethylsulfoniopropionate) transported by the ProU system. However, presence of the substrate-binding protein is required for the successful uptake of these compounds (Gouesbet et al. 1994; Haardt et al. 1995). It is currently thought that the periplasmic binding protein recognises, binds and delivers the substrate to the inner-membrane components of the ABC transport system, and this sequence of events appears to be a prerequisite for successful transport. The apparent absence of binding of a variety of substrates to ProX raises intriguing questions about the molecular mechanism of substrate recognition and translocation and about the role of ProX in the ProU-mediated osmoprotectant uptake.

The large induction ratio of *proU* (≥ 100-fold) in response to increases in medium osmolality created a strong impetus for genetic studies aimed at understanding the molecular mechanisms of signal perception and osmotic gene regulation (Higgins et al. 1987; Lucht and Bremer 1994; Csonka and Epstein 1996; Gowrishankar and Manna 1996). Sudden osmotic upshocks result in a rapid increase in *proU* transcription to a level proportionally linked to the osmolality of the growth media. This kind of gene regulation reflects the physiological function of ProU since it permits a finely tuned adjustment in the number of ProU transporters to the degree of osmotic stress to ensure adequate levels of osmoprotectant acquisition. Consequently, *proU* expression is kept at elevated levels for as long as the osmotic stimulus persists. This regulatory pattern clearly distinguishes the osmoregulation of *proU* from that of the only transiently induced *kdp* operon, indicating that fundamentally different parameters are sensed to regulate the Kdp and ProU systems. Despite intensive efforts in a number of laboratories, no classical regulatory protein that specifically controls *proU* transcription has been identified. However, genetic searches for such proteins have yielded mutants with alterations in DNA-binding proteins (e.g. TopA, GyrAB, IHF, HU and H-NS) that have pleiotrophic effects on gene expression and DNA superstructure. The recovery of such mutants and the finding that cells grown at high osmolality show an increase in DNA supercoiling of reporter plasmids have led to a model in which changes in DNA supercoiling are responsible for the osmotic control of *proU* expression (Higgins et al. 1990). It is difficult, however, to envision how alterations in DNA supercoiling alone could bring about the finely interwoven correlation between the strength of *proU* transcription and the osmolality of the growth medium. It is also unclear which features make the *proU* control region so uniquely responsive to changes in DNA superstructure. Both in vivo and in vitro studies have suggested a direct effect of K^+-glutamate on proU transcription, but the beneficial effect of K^+ glutamate might be indirect and reflect a non-specific stimulation on the transcription apparatus of the cell (Csonka and Epstein 1996). Genetic studies have identified an important transcriptional regulatory element within *proV*, the first gene of the *proVWX* operon (*proU*), whose deletion results in a partial derepression of *proU* transcription at low osmolality. This silencer element is a target for the nucleoid-associated DNA-binding protein H-NS, which forms an extended nucleo-protein complex around the *proU* promoter at low osmolality and dissociates by an unknown mechanism from the silencer region and the promoter at high osmolality. Expression of *proU* remains osmotically controlled in mutants with lesions in *hns*, and thus the global regulatory protein H-NS functions as a modulator rather than as a regulator of *proU* expression. A model that takes into account the role of nucleoid-associated DNA-binding proteins (H-NS, HU, IHF), the intracellular accumulation of K^+-glutamate, and local changes in DNA supercoiling has been proposed and envisions the operation of multiple mechanisms in the osmotic control of *proU* (Gowrishankar and Manna 1996). Despite intensive efforts in the past by several laboratories, many questions remain with respect to the sensitive adjustment of *proU* transcription to the osmolality of the environment. Chief among them

are: what is the molecular mechanism of osmosensing and which physiological and physical parameters are actually sensed by cells subjected to sudden osmotic increases or prolonged growth in high osmolality environments.

Biosynthesis of glycine betaine: the Bet pathway

Of the above-mentioned amino acids, trimethylammonium, and sulfonium compounds (Fig. 1), only glycine betaine can be synthesised as an osmoprotectant by *E. coli*. A limited number of bacteria can produce this substance de novo via stepwise methylation of glycine (Galinski and Trüper 1994). However, *E. coli* does not belong to this group of bacteria. Rather, it synthesises glycine betaine by a two-step oxidation of the precursor choline with glycine betaine aldehyde as the intermediate (Fig. 2; Lamark et al. 1991). This biosynthetic pathway is widespread in nature in both prokaryotes and eukaryotes (Rhodes and Hanson 1993; Galinski and Trüper 1994), but different enzymatic systems can be employed to accomplish glycine betaine synthesis. *E. coli* utilises a membrane-bound, FAD-containing choline dehydrogenase (BetA) that oxidises choline to glycine betaine aldehyde, which is then further oxidised to glycine betaine at approximately the same rate. A second, highly substrate-specific enzyme (BetB) converts glycine betaine aldehyde into glycine betaine (Fig. 2); this dehydrogenase is evolutionarily highly conserved in bacteria and plants. Import of the precursor choline is mediated at low external choline concentration by the high-affinity (K_m = 8 μM) and proton-motive-force-driven BetT transporter (Fig. 2). At a high substrate supply, choline is additionally taken up by the cells with low affinity (K_m = 1.5 mM) via the ABC transport system ProU (Table 1). The Bet system responds to several environmental stimuli: osmolality, oxygen content of the medium, and the presence of choline (Lamark et al. 1996). Induction of the *betT* and *betIBA* genes by choline is achieved through the BetI repressor. Oxygen control is conferred through the ArcA-ArcB two-component system, a sensor-regulator pair controlling many oxygen-regulated genes of *E. coli*. Genetic control by oxygen tension is of physiological relevance since the functioning of the BetA enzyme is dependent on the supply of oxygen.

Cellular reactions of *B. subtilis* to salt stress

There has been a long-standing focus on osmoadaptation in gram-negative bacteria, but only in recent years has the osmostress response of gram-positive bacteria attracted wider attention. In particular, the pathogens *Staphylococcus aureus* and *L. monocytogenes*, the lactic acid bacterium *Lactobacillus plantarum*, and the soil microorganisms *B. subtilis* and *C. glutamicum* are studied by physiological and molecular approaches. Gene cloning, mutant analysis, and transport studies have led to the identification of the systems for uptake and synthesis of osmoprotectants in *B. subtilis* and *C. glutamicum*. Our laboratory is focusing on the adaptation reaction of *B. subtilis* to high-osmolality environments, and we summarise below those findings pertinent for its osmostress response.

B. subtilis is a facultative anaerobic, endospore-forming, rod-shaped bacterium that is widespread in nature. It is a ubiquitous inhabitant of the upper layers of the soil, where frequent fluctuations in the availability of water often cause severe alterations in the osmolality of this habitat (Miller and Wood 1996). It is also exposed to washout into both freshwater and marine environments, thus imposing considerable strains on the water balance of the cell. Sudden osmotic changes trigger a behavioural response (osmotaxis) such that the *B. subtilis* cells are repelled by both high and low osmolality (Wong et al. 1995). Like many bacteria in the soil, *B. subtilis* is mostly found in a nongrowing state due to nutrient limitations and a variety of stress conditions. Under severe starvation, *B. subtilis* can escape unfavourable circumstances by forming highly desiccation-resistant endospores, a cellular differentiation invariably accompanied by the death of the mother cell and thus the temporary sacrifice of the ecological niche. Intuitively, one would expect that high-osmolality would trigger sporulation in *B. subtilis* as an escape reaction from desiccation. However, these growth conditions actually strongly inhibit spore formation, probably by impeding the activation of early sigma factors that control the onset of sporulation (Ruzal et al. 1998).

As a less extreme alternative to sporulation, a general stress regulon of *B. subtilis* is thought to provide cells in transition from a growing to a nongrowing state with a multifaceted stress resistance (Hecker et al. 1996; Hecker and Völker 1998). The master regulator of this large stress response network with more than 50 members is the alternative transcription factor sigmaB (σ^B). A variety of environmental insults and growth-limiting conditions including high salinity (Bernhardt et al. 1997) result in the transient induction of the σ^B regulon. The fact that enhanced transcription of the genes of the σ^B regulon can be triggered by the addition of salt has fostered speculations about its function in the cell's adaptation to high osmolality and desiccation. However, a *sigB* deletion mutant is not at a survival disadvantage when exposed to osmotic shock or desiccation under laboratory conditions (Boylan et al. 1993). Efforts to elucidate the function of the σ^B-controlled genes in the osmostress response of *B. subtilis* might be complicated by the presence of multiple and differently regulated promoters in many genes of the σ^B regulon (Hecker et al. 1996). Furthermore, it might be rather difficult to simulate in the laboratory those conditions that appropriately mimic the growth and environmental circumstances faced by *B. subtilis* in its natural ecosystems (Hecker and Völker 1998). The expression of several genes controlled by the two-component DegS-DegU regulatory system has been shown to be affected either positively or negatively by salt stress. Since the DegS-DegU-instigated regulatory effects are salt-specific rather than osmotic (Dartois et al. 1998), the use of this sensor and regulator module as the central switch for the genetic control of the osmostress response of *B. subtilis* is unlikely.

Endogenous response of *B. subtilis* to high-osmolality: K^+ uptake and synthesis of proline

The key physiological role of compatible solute accumulation as an adaptive response of *B. subtilis* to high osmolality environments has been firmly established (Whatmore et al. 1990; Boch et al. 1994). *B. subtilis* responds to a sudden increase in the external osmolality by an initial rapid uptake of K^+ followed by the accumulation of large amounts of the compatible solute proline through de novo synthesis (Whatmore et al. 1990; Whatmore and Reed 1990). Influx of K^+ is essential for the recovery of turgor, increased proline biosynthesis, and the resumption of growth subsequent to an osmotic challenge. The nature of the counter-ion for K^+ in *B. subtilis* is unclear, since, in contrast to *E. coli*, glutamate levels increased only slightly after osmotic upshock. The transporters mediating K^+ uptake are apparently controlled by turgor, but the systems involved in K^+ acquisition in osmotically stressed cells have not been identified at the molecular level.

Proline plays a particularly important role in the osmostress response because it is accumulated by osmoregulating *B. subtilis* cells by both de novo synthesis and uptake from the environment. Unlike *E. coli*, which uses the disaccharide trehalose as the endogenously synthesised compatible solute (Strøm and Kaasen 1993), *B. subtilis* employs the amino acid proline for this purpose (Whatmore et al. 1990). The intracellular concentration of this osmoprotectant is raised from a basal level of 16 mM to well over 1 M under truly hypertonic growth conditions. Proline biosynthesis in many bacteria is frequently regulated through feedback inhibition of the first biosynthetic enzyme (γ-glutamyl kinase). Hence, salt-stressed *B. subtilis* cells require an adjustment of the proline biosynthetic pathway to sustain high-level proline production under hypertonicity. This could be accomplished either by producing enzymes that are refractory to feedback inhibition or by using two distinct sets of proline biosynthetic genes to meet the different demands for this amino acid under low- and high-osmolality growth conditions. Since proline can serve as the sole carbon and nitrogen source in *B. subtilis*, additional cellular control mechanisms are required to avoid a futile cycle in cells grown at high osmolality. The molecular and enzymatic mechanisms leading to the remarkable rise in proline content in *B. subtilis* at high osmolality are unclear, but it is known that both activation of pre-existing proline biosynthetic enzymes and their increased synthesis are involved (Whatmore et al. 1990).

Osmoregulated proline uptake in *B. subtilis* is mediated by a dedicated transporter

Proline is produced in large amounts by plants as an osmoprotectant, and it will eventually reach soil bacteria through root exudates and decaying plant material. *B. subtilis* uses a high-affinity and substrate-specific (Table 1) transport system, OpuE (osmoprotectant uptake), to scavenge proline efficiently for osmoprotective purposes (Fig. 3). Uptake of proline allows *B. subtilis* to grow in high-osmolality environments that are otherwise strongly inhibitory for its proliferation, and disruption of the *opuE* structural gene yields a *B. subtilis* mutant that is no longer protected by exogenously provided proline (Von Blohn et al. 1997). The OpuE transporter consists of a single component (Fig. 3) and is a member of the sodium/solute symporter family (SSF). Expression of *opuE* is strongly increased by either non-membrane-permeable ionic or nonionic osmolytes, but not by proline (von Blohn et al. 1997; Spiegelhalter and Bremer 1998). Surprisingly, OpuE displays high similarity to the proline-inducible PutP permeases, which are used in *E. coli* and *S. typhimurium* for the acquisition of proline as a carbon and nitrogen source, but not for osmoprotective purposes. Thus, it is apparent that structurally closely related proline transporters have been tailored in the course of evolution for different physiological functions by endowing their structural genes with special regulatory patterns. It is noteworthy that *B. subtilis* employs a dedicated osmoregulated proline transport system, whereas in *E. coli* and *S. typhimurium* proline uptake is mediated by ProP and ProU (Fig. 2; Csonka and Epstein 1996).

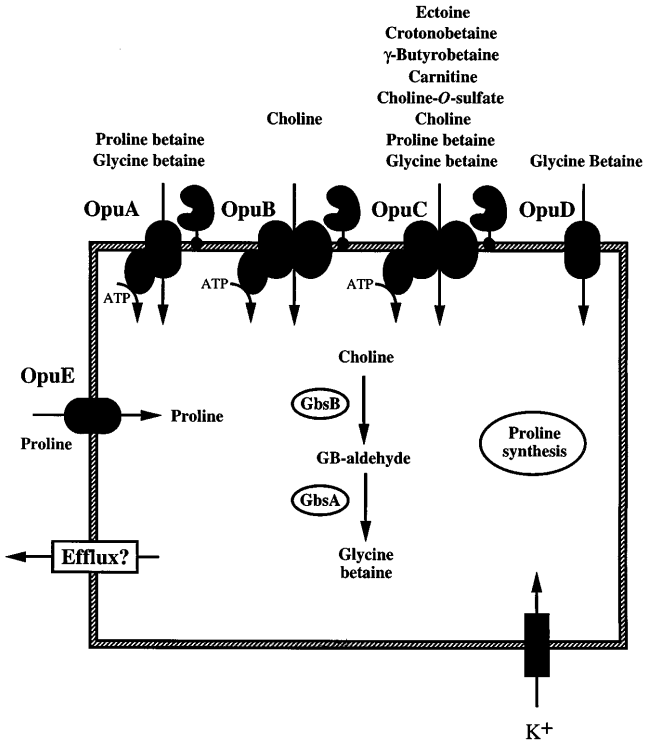

Fig. 3 Osmostress response systems of *Bacillus subtilis*

Expression of *opuE* is initiated from two closely spaced, osmoregulated promoters that are recognised by different σ-factors: *opuE* P-1, which is recognised by the vegetative σ^A, and *opuE* P-2, which is dependent on the stress-induced σ^B (von Blohn et al. 1997; Spiegelhalter and Bremer 1998). Therefore, *opuE* is the first member of the σ^B regulon with a clearly defined physiological func-

tion in the *B. subtilis* osmostress response. The σ^A and σ^B stress-responsive promoters have different roles in the osmotic control of this gene (Spiegelhalter and Bremer 1998). An osmotic upshock causes a rapid, but transient increase in *opuE* P-2 (σ^B) activity, whereas in cells subjected to long-term osmotic stress, regulation of *opuE* expression depends entirely on the σ^A-dependent *opuE* P-1 promoter. Its activity is proportionally linked to the environmental osmolality and is kept at elevated levels for as long as the osmotic stimulus persists. The independence of the osmoregulation of the σ^A-dependent *opuE* P-1 promoter from the σ^B regulon implies that at least two different signal transduction pathways operate in *B. subtilis* to communicate osmotic changes in the environment to the transcription apparatus of the cell (Spiegelhalter and Bremer 1998). Although the mechanisms of signal reception are unknown for both groups of genes, members of the signal transduction cascade have been identified for the σ^B-dependent pathway. Release of σ^B from inhibition by its anti-sigma factor RsbW following salt stress involves reactivation of the antagonist protein RsbV by dephosphorylation. The activity of the phosphatase RsbU is controlled by additional regulatory proteins such as RsbS, RsbT and RsbR (Akbar et al. 1997), each of which is encoded by the *sigB* operon (Hecker et al. 1996).

Uptake of glycine betaine and structurally related osmoprotectants in *B. subtilis*

B. subtilis employs three highly effective transporters (Table 1) to scavenge glycine betaine from the environment up to cellular levels that surpass 1 M in osmotically stressed cultures (Moses, S, von Blohn, C, Bakker, E, Bremer, E, unpublished work). These are the ABC transport systems OpuA and OpuC, and the secondary transporter OpuD (Fig. 3; Kempf and Bremer 1995; Kappes et al. 1996). The OpuA and OpuC transporters are related to each other and to the binding-protein-dependent glycine betaine uptake system ProU from *E. coli* and *S. typhimurium* (Fig. 2). Instead of a freely diffusable, periplasmic substrate-binding protein (ProX), both the OpuA and the OpuC systems employ extracellular substrate-binding proteins that are tethered via an amino-terminal lipid tail to the cytoplasmic membrane (Fig. 3; Kempf et al. 1997). The third uptake system, OpuD, consists of a single component (Fig. 3), and together with the glycine betaine transporter (BetP) from *C. glutamicum* and the choline (BetT) and carnitine (CaiT) transporters from *E. coli*, it forms a small family of uptake systems for trimethylammonium compounds (Kappes et al. 1996; Peter et al. 1996).

Each of the OpuA, OpuC and OpuD systems is under osmotic control (Table 1), and only in mutants lacking all three transporters is exogenously provided glycine betaine no longer osmoprotective. Thus, *B. subtilis* goes to considerable expense to acquire this osmoprotectant. Why are there so many uptake systems for glycine betaine? OpuA, OpuC and OpuD exhibit similar high affinities for glycine betaine (K_m = 2–13 μM), but their transport capacities [*V*max = 60–280 nmol min^{-1} (mg protein)$^{-1}$] differ substantially, as is reflected in the different contributions of these transporters to osmoprotection (Kappes et al. 1996). The OpuC system (Fig. 3) is particularly remarkable since it displays a broad substrate specificity (Table 1) combined with a high affinity for a variety of trimethylammonium and sulfonium compounds (Kappes and Bremer 1998). It also serves as a low-affinity system for ectoine (Jebbar et al. 1997), a compatible solute synthesised by a variety of microorganisms (but not by *B. subtilis*) as their dominant endogenous osmoprotectant (Galinski and Trüper 1994; Ventosa et al. 1998). Although closely related in structure to proline, the effective osmoprotectant proline betaine is efficiently taken up by the cells via the OpuA and OpuC transporters (Kempf, B, Jade, J, Bremer, E, unpublished work), but not through the proline transport system OpuE (Fig. 3). The OpuA, OpuC and OpuD osmoprotectant transporters (Table 1) also play a major role in the acquisition of 2-dimethylsulfonioacetate (DMSA), the sulfonium analogue of glycine betaine, and of 3-dimethylsulfoniopropionate (DMSP), a dominant compatible solute in marine algae (Nau-Wagner, G, Jebbar, M, Bremer, E, unpublished work). The different characteristics of the OpuA, OpuC, OpuD and OpuE transport systems with respect to substrate specificity (Table 1), affinity, and transport capacity permit a flexible adaptation of *B. subtilis* to high osmolality ecological niches with a different spectrum of compatible solutes. With the exception of proline, the osmoprotectants employed by *B. subtilis* are not used for anabolic purposes and are therefore accumulated as metabolically inert osmostress compounds.

An important difference exists between gram-negative and gram-positive bacteria with respect to the accumulation of compatible solutes in osmotically nonstressed cells: gram-negative bacteria do not amass these compounds unless they are subjected to hyperosmotic conditions, whereas gram-positive microorganisms tend to accumulate compatible solutes in standard rich and minimal laboratory media (Whatmore and Reed 1990; Boch et al. 1994; Glaasker et al. 1996; Peddie et al. 1998). This is likely to reflect the differences in turgor between gram-negative and gram-positive bacteria, requiring an adjustment in the regulation of osmoprotectant uptake and synthesis systems in these microorganisms. Compatible solutes might be accumulated by non-stressed gram-positive cells to assist in maintaining high turgor in preference to ionic osmolytes, which are deleterious at high concentrations.

Synthesis of glycine betaine in *B. subtilis*

Of the above-mentioned osmoprotectants, only proline can be accumulated by *B. subtilis* through de novo synthesis. However, glycine betaine can be synthesised when the precursor choline is provided (Boch et al. 1994). A novel combination of enzymes is employed to mediate the conversion of choline, which is not an osmoprotectant per se, into glycine betaine. A soluble, NAD-dependent type

III alcohol dehydrogenase (GbsB; glycine betaine synthesis) oxidises choline into glycine betaine aldehyde; this intermediate is then further oxidised by a soluble, highly salt-tolerant and substrate-specific glycine betaine aldehyde dehydrogenase (GbsA) to the osmoprotectant glycine betaine (Fig. 3; Boch et al. 1996). The structural genes *(gbsAB)* for these enzymes form an operon whose transcription is enhanced by the presence of choline in the growth medium, but not by high osmolarity. This pattern of *gbsAB* expression hints at the presence of a regulatory protein that controls transcription of this operon in response to the availability of choline. However, *B. subtilis* does not possess a polypeptide related to the choline-regulatory protein BetI from *E. coli*.

Molecular analysis has revealed that choline uptake in *B. subtilis* is mediated by two evolutionarily closely related ABC transport systems (Kappes, RM, Kempf, B, Kneip, S, Boch, J, Gade, J, Meier-Wagner, J, Bremer, E, unpublished work). One of these transporters is the above-described uptake system with the broad substrate specificity, OpuC, and the other system is OpuB, a novel transporter exhibiting high substrate specificity for choline (Fig. 3; Table 1). The structural genes for the OpuB and OpuC systems are only a few kilobases apart on the *B. subtilis* chromosome, and their gene products exhibit sequence identities of over 65% (Kappes, RM, Kempf, B, Kneip, S, Boch, J, Gade, J, Meier-Wagner, J, Bremer, E, unpublished work). These findings suggest that the *opuB* and *opuC* loci originate from a gene duplication event. Of the five known transport systems for osmoprotectant uptake in *B. subtilis*, three are multicomponent ABC transport systems (Fig. 3). Remarkably, the soil bacterium *C. glutamicum* appears to employ only secondary transport systems for osmoprotectant acquisition (Peter et al. 1996; R. Krämer, University of Cologne, Cologne, Germany, personal communication).

Efflux of osmoprotectants

The growth of microorganisms in high-osmolality environments leads to the massive intracellular accumulation of compatible solutes and ions. In their natural ecosystems, bacteria are likely to experience hypoosmotic shocks caused by rain, flooding, and washout into freshwater sources. Such conditions lead to a rapid and massive influx of water into the cell, requiring the bacteria to quickly reduce their intracellular solute pool in order to avoid cell lysis. For a variety of both gram-negative and gram-positive microorganisms, there is now increasing experimental evidence for the presence of osmoprotectant efflux systems that operate independently of their importers (Galinski and Trüper 1994; Csonka and Epstein 1996). Within seconds of hypotonic shocks, *L. plantarum*, *C. glutamicum* and *L. monocytogenes* exhibit a massive efflux of osmoprotectants, thus implicating stretch-activated channels in the fast release of compatible solutes from the cell (Glaasker et al. 1996; Ruffert et al. 1997; Verheul et al. 1997). Carrier-like systems also appear to contribute to the discharge of these compounds since the initial rapid efflux is followed in some microorganisms by a slow process with different kinetic and metabolic parameters (Strøm and Kaasen 1993; Glaasker et al. 1996). Remarkably, the osmoregulated channels in *C. glutamicum* mediate efflux of selected compatible solutes (Ruffert et al. 1997), suggesting a certain degree of substrate specificity. Opening of the mechanosensitive efflux channels is linked to the extent of osmotic downshock. Hence, a finely tuned and selective release of those compounds that are preferentially accumulated by the bacterial cells under hyperosmotic conditions is possible. The properties of these channels appear to be different from those operating in *E. coli*, which release more or less all low-molecular-mass compounds into the medium upon a harsh osmotic downshock (Berrier et al. 1992). However, under better-controlled conditions, *E. coli* releases only osmolytes after osmotic downshock (Schleyer et al. 1993) in a manner similar to that of *C. glutamicum* cells (Ruffert et al. 1997). Further physiological experiments and molecular approaches to identify the export systems for compatible solutes are urgently needed to provide greater insight into this important aspect of osmoregulating cells. In *L. plantarum*, the rapid decrease in osmolality not only results in the efflux of glycine betaine via channels and carriers but also inhibits its uptake, thus permitting the integration of glycine betaine fluxes into a homeostatic system. Analogous control mechanisms must also operate in hyperosmotically shocked cells to allow activation of glycine betaine uptake and inhibition of its efflux (Glaasker et al. 1996).

Bacterial systems for glycine betaine synthesis as resources for metabolic engineering in plants

In their seminal review on the molecular biology of osmoregulation, Le Rudulier et al. (1984) have noted striking parallels in the use of osmoprotectants between microorganisms and plants and have suggested that osmoprotective compounds might be exploited for the genetic enhancement of drought and salinity tolerance in crop plants. The concept that compatible solutes operate independently of a particular cell has been widely exploited by microbiologists for the cloning of genes that encode systems for uptake or synthesis of osmoprotectants by functional complementation of appropriately constructed *E. coli* mutants. More recently, this strategy has beeen employed to generate plants with an enhanced osmotolerance. Examples are the synthesis of glycine betaine by introducing the *E. coli betBA* genes into tobacco and the integration of the *Arthrobacter globiformis* choline oxidase *codA* gene into *Arabidopsis thaliana* (Holmstrøm et al. 1994; Hayashi et al. 1997). By genetically manipulating their glycine betaine content, it might be possible to generate desiccation-resistant varieties of commercially important crops such as rice, potatoes and tomatoes that are not natural glycine betaine accumulators. The genetic components of the microbial systems for compatible solute accumulation have thus become beneficial gene resources for agricultural

biotechnology and more generally for the metabolic engineering of stress tolerance in eukaryotic systems.

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