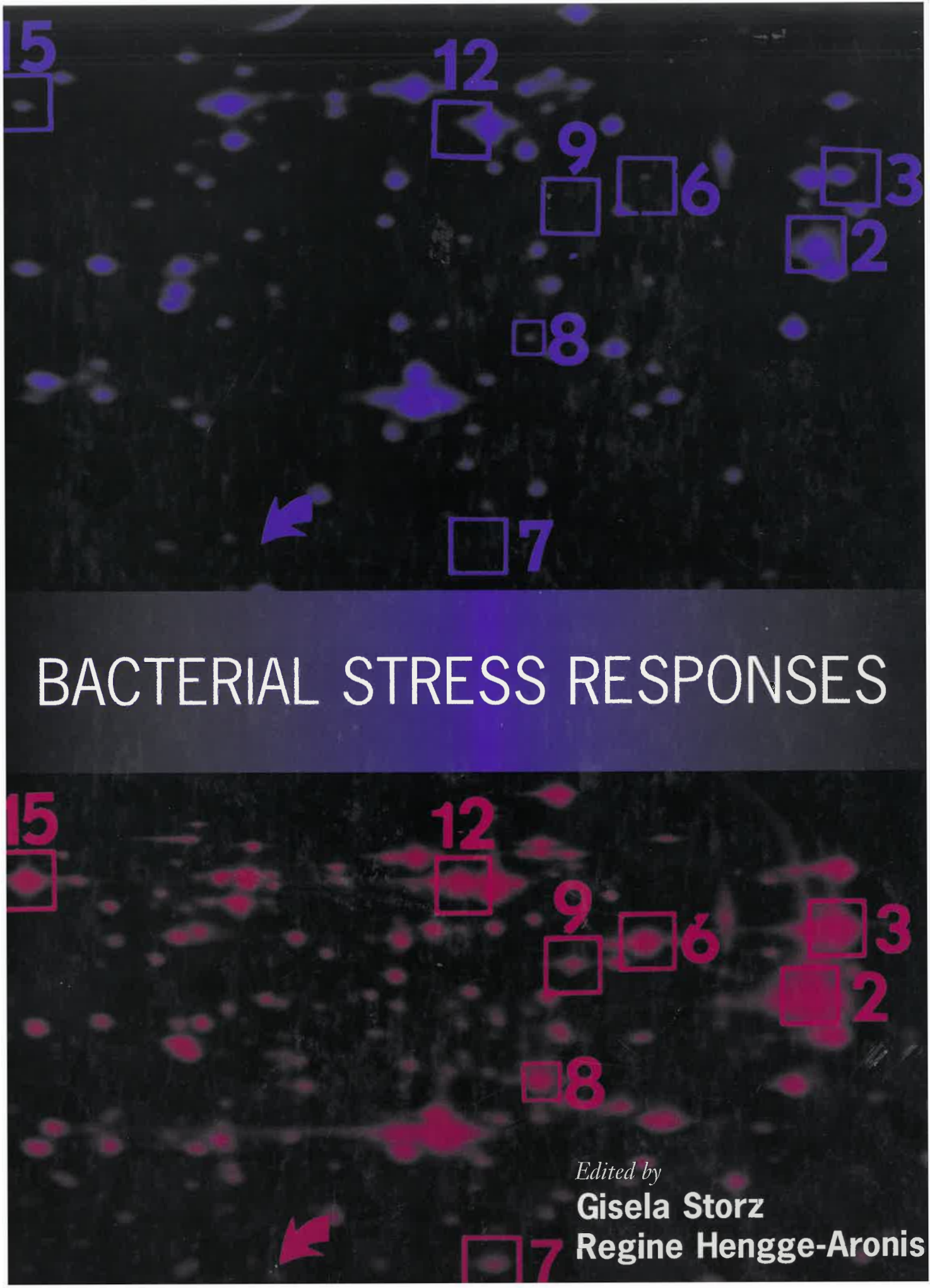

BACTERIAL STRESS RESPONSES

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Chapter 6

Coping with Osmotic Challenges: Osmoregulation through Accumulation and Release of Compatible Solutes in Bacteria

ERHARD BREMER AND REINHARD KRÄMER

Maintenance of turgor within physiologically acceptable boundaries is a key determinant for the growth of microorganisms. Bacteria that do not live permanently in hyperosmotic environments must adjust to fluctuations in the water content of their habitat. Osmotically instigated transmembrane water movements can cause either dehydration of the cytoplasm under hypertonic conditions or bursting of the cell in hypoosmotic environments. Microorganisms lack systems for active water transport and adjust cellular water content via osmosis by actively controlling the level of their intracellular solute pool. A limited group of organic osmolytes, the so-called compatible solutes, are used to balance environmental osmolality. These compounds can be accumulated to very high levels without disturbing the functioning of the cell and the most widely used are trehalose, proline, ectoine, and glycine betaine. Compatible solutes are amassed through synthesis or uptake from the environment, and their intracellular concentration is sensitively adjusted to the degree of osmotic stress. This process is controlled by an interplay between several finely tuned mechanisms that regulate either gene transcription or activity of the encoded enzymes and transporters used for compatible solute accumulation. The same compounds that protect the cell from the detrimental effects of hyperosmotic conditions become a threat to the cell's survival upon osmotic downshock. To reduce the driving force for water entry, the cell rapidly expels excess compatible solutes in a controlled manner through mechanosensitive channels. The amassing of organic osmolytes under hyperosmotic conditions and their expulsion when external osmolality drops allow the cell to control water content and turgor and thus sustain growth under unfavorable conditions.

Essentially all microorganisms other than intracellular parasites and symbionts face ever-changing environmental conditions. Their ability to thrive under often highly stressful circumstances depends on the sensing of environmental changes and responding to these challenges via highly integrated stress reactions. Two types of bacterial stress responses operate in microorganisms. General stress responses, which are frequently controlled through a single or a few master regulators, provide cross-protection against a wide variety of environmental insults regardless of the initial stimulus (54, 55) (see also chapters 11 and 12). While effective in ensuring the cell's survival, they may not be sufficient to allow growth when the cell is confronted with a particular stress condition for a prolonged period. In these circumstances, cells use specific stress responses that involve highly integrated networks of physiological and genetic adaptation mechanisms. There is also often a complex interplay between global regulators and cellular response systems directed against specific types of stress, thereby adding an additional level of sophistication to the cell's coordination of emergency and long-term stress reactions (57).

The availability of water is one of the most significant environmental parameters affecting the survival and growth of microorganisms (66, 121). Changes in the external osmolality immediately trigger fluxes of water along the osmotic gradient that could result either in swelling and bursting of the cell in hypotonic environments or in plasmolysis and dehydration under hypertonic conditions. The cell avoids these devastating alternatives by using active countermeasures to retain a suitable level of cytoplasmic water and turgor (30, 42, 76, 118, 149). Microorganisms control turgor by actively modulating

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the pool of osmotically active solutes in their cytoplasm, thereby allowing water content to be adjusted by osmosis. The physiological and molecular mechanisms through which this is accomplished are the focus of this chapter.

THE CELL AND THE SURROUNDING SOLVENT

When two compartments are separated by a semipermeable membrane, the solvent water flows from the compartment with high chemical potential of water to that with lower chemical potential. The osmotic pressure (Π) is defined as the hydrostatic pressure that, in an equilibrium state, balances the difference in chemical potential such that no movement of solvent occurs between the two compartments. The osmotic pressure (Π) of an aqueous solution is defined as $\Pi = (RT/V_w) \ln a_w$ and is thus directly related to the water activity (a_w, the mole fraction of water in a solution) and to V_w, the partial molal volume of water. Osmolality describes the osmotic pressure Π of a solution and is expressed in units of osmoles (moles of osmolyte) per kilogram of solvent. The term osmolarity, which is defined as the sum of the concentrations of osmotically active solutes (osmolytes) in solution, is a frequently used approximation for osmolality (149).

The total concentration of osmolytes within a cell is generally higher than in the environment, causing water to flow down its chemical potential into the cell. This influx increases the cellular volume, thereby pressing the cytoplasmic membrane toward the murein sacculus. The pressure difference across the inner membrane/cell wall complex is the turgor, $\Delta\Pi$ ($\Delta\Pi = \Pi_{in} - \Pi_{out}$). Turgor balances the difference in osmotic pressure between the cell interior and exterior and must be maintained through the growth cycle as the cell elongates (25). Turgor is considered essential for cell viability and critical for growth, and it is thought to provide the mechanical force for expansion of the cell wall (63, 80). Although turgor is quite difficult to quantify (149), values of 3 to 5 bars (0.3 to 0.5 MPa) for gram-negative bacteria and approximately 20 bars (2 MPa) for gram-positive organisms have been estimated (30, 66, 148). The much higher value in gram-positive bacteria is thought to reflect the large cytoplasmic solvent pool needed for expansion of the multilayered peptidoglycan.

WATER PERMEATION ACROSS THE CYTOPLASMIC MEMBRANE

Osmotically instigated water fluxes across the cytoplasmic membrane are accomplished by two distinct mechanisms. Simple diffusion of water across the lipid bilayer is usually sufficient to balance solute levels, but a much accelerated water transit is achieved by diffusion through water-selective channels. These so-called aquaporins are abundant in plant and animal cells that exhibit rapid transmembrane water movement (1, 102). They are also present in the lower eukaryote *Saccharomyces* (13) and have been detected in several bacterial species. The *Escherichia coli* aquaporin (AqpZ) (19) serves as a model for bacterial water channels and is a member of the ubiquitous major intrinsic protein family (MIP). Expression studies in *Xenopus* oocytes established the function of AqpZ as a water channel, and cryoelectron microscopy examinations have suggested that AqpZ mediates rapid and large water fluxes in both directions in response to sudden osmotic up- or downshifts (33). This indicates an important role for AqpZ in the cell's management of water flow. However, this channel is not essential (at least under laboratory conditions) since *aqpZ* deletion mutants exhibit only minor growth defects (20), and the physiological role of AqpZ in bacterial osmoregulation remains to be more fully explored.

MICROBIAL STRATEGIES FOR COPING WITH HYPEROSMOTIC ENVIRONMENTS

Microorganisms have developed two fundamentally different schemes for maintaining turgor under hyperosmotic conditions (42, 76, 109, 143). These are frequently referred to as the "salt-in" and "salt-out" strategies. The former has been adopted by microorganisms whose entire physiology has been adapted to a permanent life in high-osmolality surroundings; this group of bacteria accumulates large amounts of ions in their cytoplasm. Two phylogenetically unrelated groups of *Archaea* and *Bacteria*, the aerobic *Halobacteriales* and the *Haloanaerobiales*, colonize hypersaline environments, with salt concentrations ranging between 2 M and 5 M, as their preferred ecological niches (97, 109, 143). In their hypersaline habitats, K^+, Mg^{2+}, Na^+, and Ca^{2+} are generally the dominant cations, and Cl^-, SO_4^{2-}, and CO_3^{2-} are the major anions. These organisms selectively accumulate molar concentrations of K^+ and Cl^- in their cytoplasm and usually actively extrude the deleterious Na^+ ions (see chapter 8), resulting in a cytoplasmic ion composition that differs substantially from the surroundings. However, high concentrations of inorganic ions induce aggregation of macromolecules by enhancing hydrophobic interactions, and they restrict the availability of water for biochemical processes by salt ion hydration. These

effects necessitated evolutionary changes in essentially all proteins to adjust the entire cell physiology to the high ion content of the cytoplasm (17, 35, 38, 117). As a result, 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 true halophiles leads to cell disintegration at lower salt concentrations. Ultimately, the salt-in strategy employed by the *Halobacteriales* and *Haloanaerobiales* severely limits their habitats and precludes the colonization of environments with moderate osmolalities.

A more versatile and flexible osmostress response is provided by the salt-out strategy. It is used by bacteria that live either in environments of moderate salinity or in ecological niches that are only periodically subjected to conditions of low water activity. This group of microorganisms avoids high ionic conditions in their cytoplasm and instead amasses large amounts of specific organic osmolytes that are highly congruous with cellular functions (30, 42, 69, 76, 109, 125, 143). These so-called compatible solutes (Fig. 1) can be accumulated up to molar concentrations in the cytoplasm and thus contribute significantly to the maintenance of turgor under hyperosmotic conditions. Because the salt-out adaptation mechanism does not require evolutionary adjustment of proteins and cellular processes to high salt concentrations, this response to osmotic stress is prevalent not only in *Bacteria* and *Archaea* (28, 32, 42, 76, 109, 118) but also in fungal, plant, animal, and human cells (18, 61, 100).

CHARACTERISTICS OF COMPATIBLE SOLUTES

By definition, compatible solutes are compounds that can be accumulated to very high levels (several moles per liter) without disturbing cellular physiology (3, 16, 34, 135, 153). 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 (90, 154). The spectrum of compatible solutes identified comprises amino acids (e.g., glutamate, proline), amino acid derivatives (e.g., ectoine, proline betaine), small peptides (e.g., *N*-acetylglutaminylglutamine amide), methylamines (e.g., glycine betaines, carnitine) and their sulfonium analogs (e.g., dimethylsulfonium propionate), sulfate esters (e.g., choline-*O*-sulfate), polyols (e.g., glycerol, glycosylglycerol), and sugars (e.g., trehalose, sucrose). However, in the past few years, as more organisms have been examined, in particular those that thrive in extreme environments, a variety of novel organic osmolytes have been identified (32, 101). A given bacterium usually employs a selection of compatible solutes, and the composition of its compatible solute pool can vary in response to growth phase and growth conditions. Glycine betaine, ectoine, proline, and trehalose (Fig. 1) are probably the most widely used compatible solutes in the microbial world.

In general, compatible solutes are polar, highly soluble molecules, and they usually do not carry a net charge at physiological pH. Exceptions are negatively charged organic osmolytes (such as sulfotrehalose, diglycerolphosphate, and di-*myo*-1,1′-inositol phosphate) used by thermophilic *Archaea* (32, 101). K^+ is used as the counterion for these solutes; this ion is also accumulated transiently (often in combination with glutamate) by many nonhalophilic bacteria to initially offset the detrimental effects of an osmotic upshock (14, 30, 39, 152). The compatibility of organic osmolytes with cell functioning can be correlated with their effects on the behavior of water at interfaces and has been related to the so-called Hofmeister series (26, 153, 154). Compounds that break water structure and consequently destabilize proteins are called chaotropic, whereas those that increase wa-

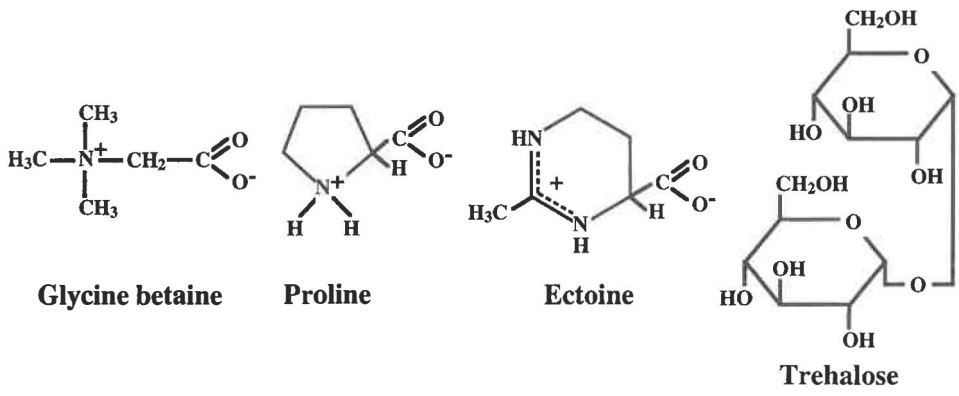

Figure 1. Structures of selected compatible solutes.

ter structure and stabilize proteins are termed kosmotropic. Compatible solutes are excluded from the immediate hydration shell of polypeptides, resulting in a preferential hydration of protein surfaces. The disruption of the ordered water structure around proteins by local or global unfolding becomes energetically unfavorable, and therefore the native conformations of proteins are stabilized (3, 123, 149, 154).

The accumulation of compatible solutes not only allows microbial cells to withstand a given osmolality, but it also extends their ability to colonize more saline ecological niches (42). Depending on the type, compatible solutes can also protect microorganisms against stresses other than dehydration. An example is the increased cold tolerance conferred on *Listeria monocytogenes* by the accumulation of glycine betaine from food sources (79). The beneficial effect of compatible solutes on membrane integrity and protein folding and stability has been demonstrated in in vitro experiments (129, 149, 153). These findings have heightened the biotechnological interest in these compounds as stabilizers in processes such as desiccation, freezing, and heating (23, 92). In addition, the common response of plant and microbial cells to high osmolality by producing compatible solutes (90, 100) has fostered interest in using bacterial systems for osmoprotectant synthesis as resources for the metabolic engineering of tolerance against high salinity and drought in commercially important crops (53, 62).

BIOSYNTHESIS OF COMPATIBLE SOLUTES: TREHALOSE, GLYCINE BETAINE, ECTOINE, AND PROLINE

Cell growth depends largely on the cell's ability to modulate its intracellular solvent pool in response to osmotic changes in the environment (125), and synthesis of compatible solutes plays an important role in this process. A considerable variety of compatible solutes can be produced by microorganisms (28, 32, 42, 101), but the biosynthetic pathways involved have been elucidated at the molecular level for only a few. Examples are the sugar trehalose, the trimethylammonium compound glycine betaine, the tetrahydropyrimidine ectoine, and the amino acid proline in *Bacteria* (Fig. 1).

The Disaccharide Trehalose

The sugar trehalose (Fig. 1) is an important stress compound and membrane-stabilizing agent in both prokaryotic and eukaryotic organisms (137). *E. coli* and *Salmonella enterica* serovar Typhimurium accumulate it via de novo synthesis as a compatible solute. Two enzymes encoded by the *otsAB* operon determine the osmoregulatory trehalose production (Fig. 2). OtsA, the trehalose-6-phosphate synthase, catalyzes the enzymatic condensation of the precursors glucose-6-phosphate and UDP-glucose; free trehalose is then generated from this intermediate by

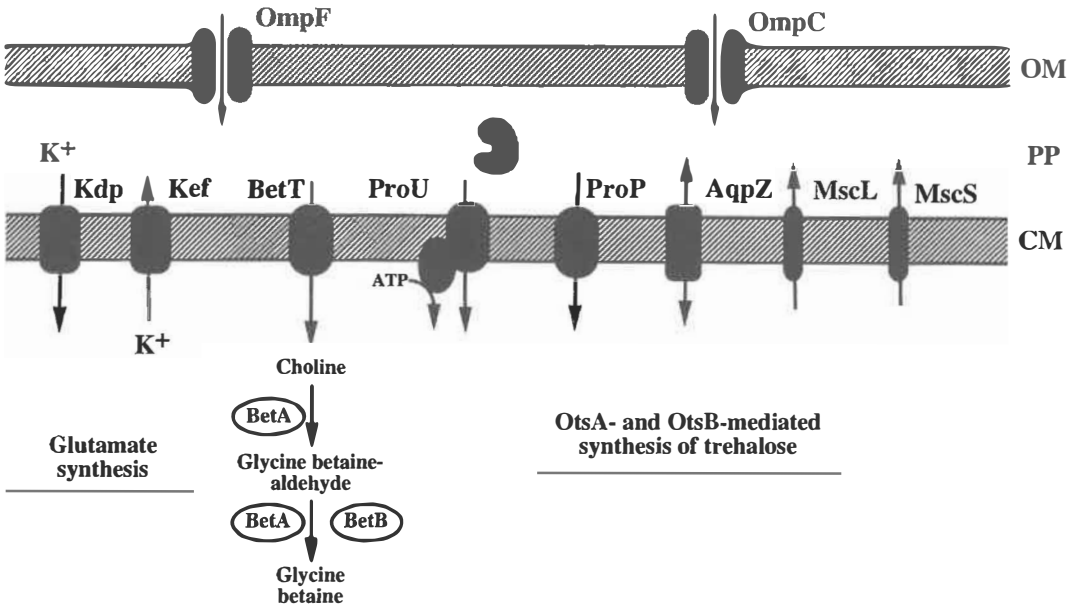

Figure 2. Osmostress response systems of *E. coli*.

rapid dephosphorylation via the o*tsB*-encoded trehalose-6-phosphate (Tre-6-P) phosphatase. Transcription of the *otsAB* operon is induced in osmotically stressed cells and is also enhanced when unstressed cells enter stationary phase (54, 71). This pattern of *otsAB* expression reflects its control by RpoS, an important alternative transcription factor controlling (or contributing to) gene expression of a large regulon of stationary-phase and stress-induced genes (56, 58) (see chapter 11). However, stationary-phase levels of trehalose are at least severalfold lower than the levels reached in response to osmotic stress (43). This finding indicates that stationary-phase cells that are highly osmotolerant (88, 98) are not dependent on a massive amount of trehalose to withstand a high degree of osmotic stress (43). Increased levels of trehalose are also accumulated under carbon-starvation conditions, pointing to the role of this nonreducing disaccharide as a general stress protectant (129, 137).

While trehalose serves as a stress protectant under hyperosmotic conditions, it also functions as an energy source at moderate osmolalities (64). The role that it plays is determined by the osmotic strength of the growth medium. Under conditions of low osmolality, extracellular trehalose is taken up by the cells via a specific phosphotransferase system (PTS) involving the $EIIBC^{Tre}$(TreB)/$EIIA^{Glc}$ complex, and it is released into the cytoplasm as Tre-6-P. A Tre-6-P hydrolase (TreC) cleaves this phosphorylated intermediate into glucose and glucose-6-phosphate for further metabolism. The genes (*treBC*) encoding the TreB transporter protein and the TreC enzyme are negatively controlled in response to the availability of trehalose in the growth medium by the TreR repressor protein, a member of the LacI family of prokaryotic DNA-binding proteins (52). Tre-6-P serves as the actual inducer. Since Tre-6-P is an intermediate in both the degradative and biosynthetic pathways, binding of the inducer to TreR must be sensitively controlled. The decision for one of the two pathways is governed by the intracellular ratio of Tre-6-P and trehalose, both of which can bind to TreR (52). While Tre-6-P prevents the binding of TreR to its operator, the TreR:trehalose complex retains DNA-binding capacity. As trehalose accumulates in osmotically stressed cells, this sugar competes (despite its lower affinity) with the inducer Tre-6-P for the available binding site on TreR and maintains this regulatory protein in a DNA-bound form that represses the expression of the catabolic *treBC* operon. A futile cycle of trehalose biosynthesis and degradation under hyperosmotic conditions is thus avoided.

Only de novo synthesized trehalose functions as a compatible solute in *E. coli* since this bacterium cannot take up free trehalose from the environment. Under high-osmolality growth conditions exogenously provided trehalose is hydrolyzed into two glucose molecules by a periplasmic trehalase (TreA) that are then transported into the cytoplasm via the glucose-specific PTS (50). Transcription of the *treA* structural gene is induced under hyperosmotic growth conditions and, like the *otsAB* genes, is regulated by RpoS (56, 137). The presence of TreA in the periplasm permits the cell to efficiently recapture exuded trehalose that was originally synthesized for osmoregulatory purposes and use it as a carbon source.

The Trimethylammonium Compound Glycine Betaine

The compound most widely used as an osmoprotectant by bacterial, plant, animal, and even human cells is glycine betaine (Fig. 1) (18, 30, 76, 100, 126). Two different routes for its production have been detected in bacteria. Some microorganisms have the ability to synthesize it de novo by a stepwise methylation of the amino acid glycine, with sarcosine and dimethylglycine as the intermediates and S-adenosyl methionine as the methyl donor (42). The second biosynthetic pathway, the enzymatic oxidation of choline to glycine betaine, is commonly used by both prokaryotic and eukaryotic cells, but the types of enzymes involved can vary.

The osmoregulatory choline to glycine betaine pathway has been characterized at the molecular level for the gram-negative and gram-positive model organisms, *E. coli* (85) and *B. subtilis* (12, 74). These bacteria cannot synthesize the precursor choline and acquire it from environmental sources such as decomposing plant and animal tissues. A single-component choline transporter (BetT) driven by the proton motive force (Table 1) is found in *E. coli* (Fig. 2), whereas two multicomponent, binding-protein-dependent ABC transporters (OpuB and OpuC) mediate high-affinity choline uptake in *B. subtilis* (Fig. 3). The first step in glycine betaine synthesis is performed by two different types of enzymes in *E. coli* and *B. subtilis*. *B. subtilis* uses a soluble, metal-containing, type III alcohol dehydrogenase (GbsB) (Fig. 3) to convert choline into glycine betaine aldehyde, whereas *E. coli* uses an FAD-containing, membrane-bound choline dehydrogenase (BetA) that can also oxidize glycine betaine aldehyde to glycine betaine at the same rate (Fig. 2). Both organisms possess evolutionarily well-conserved and highly salt-tolerant glycine betaine aldehyde dehydrogenases (BetB, GbsA) that convert the toxic intermediate into

Table 1. Uptake systems for compatible solutes

Organism	System	Type of mechanism	Substrate spectrum	Major substrate(s)[a]	Regulation at the level of:	
					Expression	Activity
E. coli	ProP	H^+ symport	Broad	GB, PB, Pro, Car, Ect, others	+	+
	ProU	ABC transporter[b]	Broad	GB, PB, Pro, others	+	+
	BetT	Secondary transport[c]	Narrow	Cho	+	+
B. subtilis	OpuA	ABC transporter	Medium	GB, PB, others	+	ND[d]
	OpuB	ABC transporter	Narrow	Cho	+	ND
	OpuC	ABC transporter	Broad	GB, PB, Cho, Car, others	+	ND
	OpuD	Na^+ symport	Narrow	GB, others	+	+
	OpuE	Na^+ symport	Narrow	Pro	+	
C. glutamicum	BetP	Na^+ symport	Narrow	GB	+	+
	EctP	Na^+ symport	Broad	GB, Pro, Ect		+
	ProP	H^+ symport	Medium	Pro, Ect	+	+

[a] Car, carnitine; Cho, choline; Ect, ectoine; GB, glycine betaine; PB, proline betaine; Pro, proline.
[b] ABC, ATP-binding cassette.
[c] The cotransported ion (Na^+ or H^+) is not known.
[d] ND, not determined.

the well-tolerated and metabolically inert glycine betaine (Fig. 2 and 3).

Glycine betaine production depends on the availability of choline. The presence of this precursor in the environment significantly affects the expression of genes encoding the systems for its uptake and enzymatic oxidation both in *E. coli* and *B. subtilis*. In *E. coli*, BetI, a member of the TetR family of bacterial regulators, serves as a specific choline-sensing repressor. It coordinates the transcription of the divergently oriented *betIBA* operon and the *betT* gene (127). Choline oxidation mediated by the choline dehydrogenase BetA (Fig. 2) can only occur in the presence of oxygen. Under anaerobic conditions, the DNA-binding protein ArcA represses both the *betT* and *betIBA* promoters (87). The *bet* genes are therefore under the control of the two-component Arc (aerobic respiration control) system, which consists of the membrane-bound histidine kinase ArcB and the cytoplasmic response regulator ArcA. Increases in medium osmolality stimulate *betI* and *betIBA* expression, but the way in which osmotic changes are sensed and transmitted to the *bet* gene cluster is unknown.

B. subtilis possesses no BetI-related protein. Instead, a different type of choline-sensing regulatory

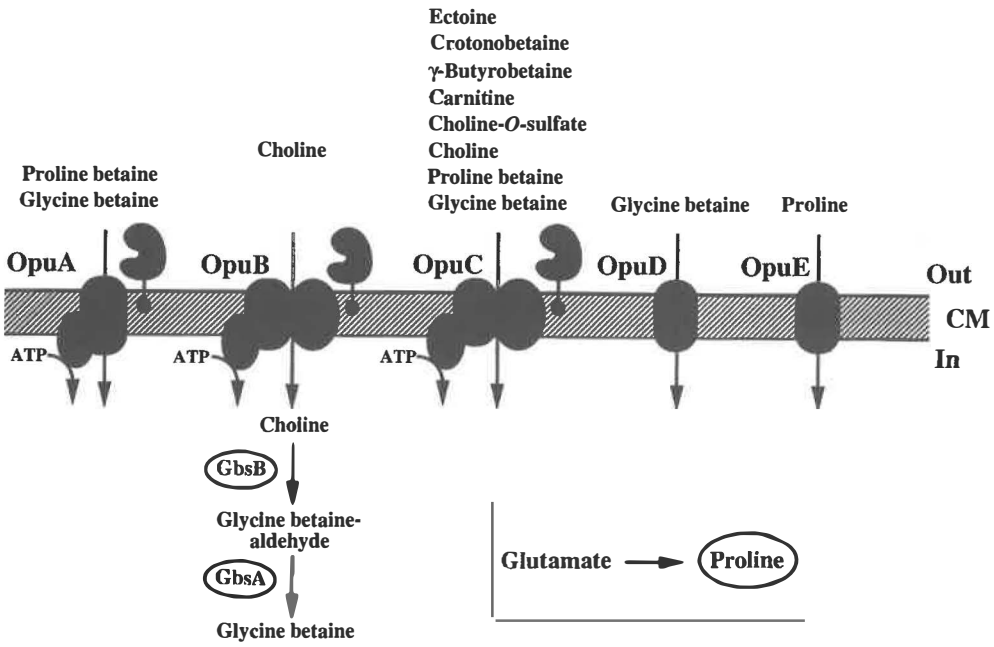

Figure 3. Osmostress response systems of *B. subtilis*.

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	OpuB	ABC transporter	Narrow	Cho	+	ND
	OpuC	ABC transporter	Broad	GB, PB, Cho, Car, others	+	ND
	OpuD	Na^+ symport	Narrow	GB, others	+	+
	OpuE	Na^+ symport	Narrow	Pro	+	
C. glutamicum	BetP	Na^+ symport	Narrow	GB	+	+
	EctP	Na^+ symport	Broad	GB, Pro, Ect		+
	ProP	H^+ symport	Medium	Pro, Ect	+	+

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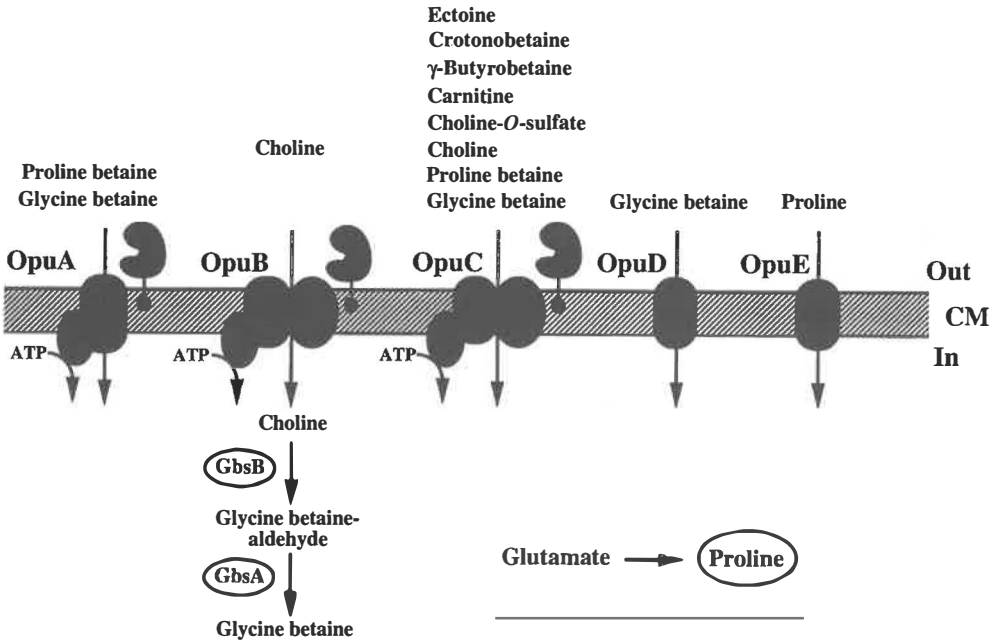

Figure 3. Osmostress response systems of *B. subtilis*.

protein (GbsR) is encoded by a gene that is transcribed divergently from the *gbsAB* glycine betaine biosynthetic gene cluster (G. Nau-Wagner, B. Kempf, J. Boch, and E. Bremer, unpublished data). GbsR negatively regulates both *gbsAB* and *opuB*, an operon encoding a high-affinity and highly substrate-specific choline transport system (Fig. 3). Expression of the *opuB* operon increases in response to elevations in medium osmolality (74), but like the choline-sensing BetI protein (87), the GbsR repressor is not involved in this regulatory process (Nau-Wagner et al., unpublished data). In contrast to the expression of the glycine betaine biosynthetic genes (*betBA*) in *E. coli*, *gbsAB* transcription is not greatly affected by osmotic stimuli.

The Tetrahydropyrimidine Ectoine

In addition to glycine betaine, ectoine (Fig. 1) is without doubt one of the most widely synthesized osmoprotectants in the microbial world (42). Many bacteria, including those that do not produce ectoine, such as *E. coli*, *B. subtilis*, and *Corynebacterium glutamicum*, have the ability to scavenge preformed ectoine from exogenous sources for osmoprotective purposes through transport processes (67, 68, 116). The pathway for ectoine biosynthesis was elucidated at the biochemical and molecular levels for two moderately halophilic eubacteria, the gram-positive *Marinococcus halophilus* (93) and the gram-negative *Halomonas elongata* (22). Disruption of the *ectABC* synthesis genes causes salt sensitivity in the resulting mutant strain (21). L-Aspartate-β-semialdehyde serves as the precursor for ectoine, and the consecutive action of three biosynthetic enzymes (EctBAC) is required for its production. Some microorganisms can modify ectoine by hydroxylation via a still uncharacterized pathway; in high-osmolality environments, these bacteria accumulate either a mixture of ectoine and hydroxyectoine or convert ectoine entirely into the hydroxylated derivative (23, 42).

The Amino Acid Proline

Proline plays a crucial role in osmotolerance in many bacteria. Often, it is taken up from environmental sources at times of osmotic stress (28, 29), and it is also synthesized in an osmotically controlled fashion by various bacteria, e.g., *Streptomyces* and *Bacillus* (147). Attesting to the effectiveness of this amino acid as an osmostress protectant, proline production is also widely used in plant cells as a defense against high salinity and drought (34). In bacteria, synthesis of proline for anabolic purposes usually proceeds from glutamate in three enzymatic steps involving γ-glutamyl kinase (ProB), glutamate-semialdehyde-dehydrogenase (ProA), and pyrroline-5-carboxylate-reductase (ProC). The activity of the first enzyme (ProB) of this pathway is frequently controlled by feedback inhibition through proline, thereby linking the production of this amino acid to the cell's biosynthetic requirements and preventing proline buildup. Substantially increased concentrations of proline are found in *proB* mutants of serovar Typhimurium and *E. coli*, where single amino acid substitutions cause a strong reduction in feedback inhibition of the γ-glutamyl kinase (27, 134). This confers enhanced osmotic stress tolerance in both species, although neither naturally synthesizes proline at elevated levels under hypersosmotic conditions.

While *E. coli* and serovar Typhimurium synthesize trehalose as their endogenous osmoprotectant (137), the soil bacterium *B. subtilis* increases proline production for this purpose. Upon a sudden osmotic upshock with 0.4 M NaCl, the intracellular proline concentration in *B. subtilis* is strongly increased via de novo synthesis from a basal level of 16 mM to approximately 500 mM, whereas the pool size of the other amino acids does not change (147). The intracellular proline content is proportionally linked to the osmolality of the growth medium over a considerable range of salt concentrations. A new proline biosynthetic pathway, which is different from that used for anabolic purposes, operates in high-osmolality stressed cells. It consists of two isoenzymes (ProH and ProJ) of the *proB*- and *proC*-encoded γ-glutamyl kinase and pyrroline-5-carboxylate-reductase, respectively, and also includes the *proA*-encoded glutamate-semialdehyde-dehydrogenase that functions in anabolic proline biosynthesis. Osmotic stress stimulates the expression of the operon encoding the ProHJ isoenzymes. Genetic disruption of the *proHJ* operon resulted in a strain that was proline prototrophic but no longer accumulated proline under high osmotic growth conditions; it exhibited a severe growth defect in hyperosmotic media (J. Brill, F. Spiegelhalter, and E. Bremer, unpublished data). This phenotype exemplifies the crucial role of compatible solute biosynthesis in the osmotic stress response of microorganisms.

TRANSPORT OF COMPATIBLE SOLUTES

In addition to accumulating compatible solutes by endogenous synthesis, a large variety of *Bacteria* and *Archaea* have developed the ability to acquire preformed osmoprotectants from exogenous sources. These compounds are released into ecosystems by primary microbial producers from osmotically down-

shocked cells; by decaying microbial, plant, and animal cells; by root exudates; and by mammals in their excretion fluids (e.g., urine).

Transporters for osmoprotectants (Table 1) have evolved to meet the special demands imposed by their physiological tasks. In natural ecosystems, the supply of osmoprotectants and their biosynthetic precursors is varying and generally very low, with concentrations usually in the nanomolar to micromolar range (78). Therefore, osmoprotectant transporters commonly exhibit very high affinity for their major substrates, and their capacity is geared to permit accumulation of compatible solutes to molar concentrations. In addition, they function effectively at high osmolality and at high ionic strength, conditions that frequently impair the performance of transporters for nutrients (128). To take advantage of the spectrum of osmoprotectants available in their habitat, microorganisms often possess several transport systems, some of which exhibit broad substrate specificity. Transporters for osmoprotectants have been most fully investigated at the molecular level in the gram-negative enteric bacteria *E. coli* and serovar Typhimurium and in the gram-positive soil bacteria *B. subtilis* and *C. glutamicum*.

Compatible Solute Uptake in the Gram-Negative Bacteria *E. coli* and Serovar Typhimurium

Two transport systems, ProP and ProU, are primarily responsible for the uptake of preformed compatible solutes in *E. coli* and serovar Typhimurium (Fig. 2). They were originally discovered as transporters for the uptake of proline under hyperosmotic growth conditions (hence their names), but subsequent studies revealed their broader substrate specificity (Table 1) and, in particular, their involvement in glycine betaine uptake (14, 30, 48, 77, 94).

ProP is a single-component system located in the cytoplasmic membrane (Table 1) and functions as an H^+ symporter for a wide variety of osmoprotectants structurally related to proline and glycine betaine (31, 96). ProP-mediated transport 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 (103). The activity of *proP*-P1 is normally repressed by cAMP-CRP complex, which binds to a site overlapping the −35 region. Activity of *proP*-P2 is dependent on the alternative sigma factor RpoS and the nucleoid-associated DNA-binding protein FIS (150, 151). Thus *proP* is a member of a growing class of RpoS-dependent genes that respond to both stationary-phase and high-osmolality signals (56). Activity of the ProP protein is enhanced by hypertonicity both in vivo and in vitro (Table 1), suggesting that upon hyperosmotic upshock the ProP transporter functions as a sensor, transducer, and responder (124). ProP possesses a carboxyterminal extension with a high propensity for forming an alpha-helical coiled coil; this domain is thought to be critical for the osmoregulation of ProP transport activity (31).

The second osmoprotectant uptake system, ProU, is a multicomponent system and a member of the ABC superfamily of transporters (Table 1) in which substrate translocation across the cell membrane is dependent on ATP hydrolysis (30, 48, 94). It consists of three proteins. The periplasmic substrate-binding protein ProX recognizes glycine betaine and proline betaine with high affinity (47, 51) and delivers them to the integral inner membrane component ProW. The ProW-mediated substrate translocation across the cytoplasmic membrane depends on the hydrolysis of ATP by the inner membrane-associated ATPase ProV. ProU also serves as low-affinity transporter for a considerable range of other compatible solutes, but curiously none of them seems to bind to ProX (47, 51). A sudden osmotic upshock results in a rapid induction of *proU* (*proVWX*) transcription (up to 100-fold) to a level proportionally linked to the osmolality of the growth medium. This pattern of gene expression reflects the physiological function of ProU since it permits the number of ProU transporters to be sensitively adjusted to the degree of osmotic stress. Consequently, *proU* expression is kept at elevated levels as long as the osmotic stimulus persists (30, 48, 59, 94). This regulatory pattern distinguishes osmoregulation of *proU* from that of the *kdp* operon, which is only transiently induced (39, 84) and controlled through the two-component regulatory system KdpD and KdpE (70, 139, 144). The Kdp transport system mediates K^+ uptake as an immediate response to osmotic upshock, loss of turgor, and limiting K^+ concentrations, but it does not serve in the long-term adaptation to hyperosmotic environments. This contrast in regulatory patterns indicates that fundamentally different parameters are sensed to increase *proU* and *kdp* transcription in response to osmotic stimuli. No classical regulatory protein that specifically controls *proU* transcription has been identified. Genetic searches for such proteins have yielded mutants with alterations in DNA-binding proteins (e.g., H-NS, TopA, GyrAB, IHF, HU) that have pleiotropic effects on gene expression and DNA superstructure. The recovery of these types of mutants and the finding that DNA supercoiling is increased in high-osmolality-grown cells have suggested a model in which changes in DNA superstructure directly con-

trol the strength of *proU* expression (60, 112). However, the correlation between the level of *proU* expression and medium osmolality seems too finely tuned to be explained by DNA supercoiling alone, and it is unclear which features might make the *proU* control region so uniquely responsive to changes in DNA superstructure (30). The nucleoid-associated DNA-binding protein H-NS (5) plays an important role in governing *proU* expression and is one of the proteins that binds to an important transcriptional regulatory element (the silencer) within *proV*, the first gene of the *proU* operon (95, 111, 112). H-NS functions as a modulator rather than a regulator of *proU* because osmoregulation is not abolished in strains with lesions in *hns* (60). In vitro and in vivo studies have suggested a direct effect of K^+ glutamate on *proU* transcription, but the beneficial effects of these solutes might be indirect and reflect a nonspecific stimulation on the transcription apparatus of the cell. A model has been proposed in which the role of nucleoid-associated DNA-binding proteins (H-NS, HU, IHF), the intracellular accumulation of K^+ glutamate, and local changes in DNA supercoiling all play a part in the genetic control of *proU* (48). Despite intensive efforts in a number of laboratories, a full understanding of the molecular mechanisms controlling osmoregulated *proU* expression has not been achieved and remains a formidable challenge.

In gram-negative bacteria, the first step in uptake of osmoprotectants involves their movement across the outer membrane permeability barrier. The OmpC and OmpF porins form water-filled open channels and allow the passage of a wide variety of low-molecular-mass compounds (107). The levels of OmpC and OmpF vary in response to different demands and stresses, in particular to changes in medium osmolality (122). Hyperosmotic conditions favor the synthesis of OmpC and reduce the production of OmpF, whereas hypoosmotic conditions reverse this pattern. Glycine betaine can diffuse through both porins, but OmpC has a greater role in osmoprotectant permeation simply because it is synthesized at a much higher rate than OmpF when medium osmolality is high (40).

A two-component regulatory system comprising a membrane-bound sensor kinase (EnvZ) and a cytoplasmic response regulator (OmpR) serves to detect changes in environmental osmolality and to set the level of OmpF and OmpC synthesis (65, 122). However, these proteins do not serve as a general osmoregulatory module for other osmoadaptive cellular responses. The EnvZ protein monitors environmental osmolality and transmits information across the cytoplasmic membrane to the soluble transcription factor OmpR through phosphorylation and dephosphorylation reactions. The level of OmpR phosphorylation is critical for its DNA interactions with both the *ompC* and *ompF* regulatory regions. High levels of phosphorylated OmpR activate *ompC* expression but repress *ompF* transcription; low levels of phosphorylated OmpR can activate *ompF* expression but fail to increase *ompC* transcription. Despite extensive analysis, the nature of the signal to which the EnvZ sensor protein responds is not known. However, medium osmolality is not likely to be a direct signal since *ompF* expression is considerably increased when the high-osmolality growth medium is supplemented with glycine betaine without changing its osmotic strength (7).

Compatible Solute Uptake in the Gram-Positive Bacteria *B. subtilis* and *C. glutamicum*

The identification of osmoprotectant transporters in *B. subtilis* (Fig. 3) was facilitated by the availability of *E. coli* mutants defective in all osmoprotectant uptake systems (51) that permitted the cloning of transporter genes through a functional complementation approach (73, 75, 146). Five transport systems for osmoprotectants have been found: two are secondary transporters (OpuD and OpuE), and three are members of the ABC superfamily (OpuA, OpuB, and OpuC [Table 1]) (76). The latter are related to the *E. coli* ProU system, but the location of the substrate-binding proteins differs. While ProX is freely diffusable in the periplasm, the ligand-binding proteins from OpuA, OpuB, and OpuC are extracellular and tethered to the cytoplasmic membrane via a lipid modification at their amino terminus (Fig. 3) (77). The OpuA, OpuC, and OpuD transporters mediate high-affinity glycine betaine uptake (Fig. 1). OpuA and OpuD exhibit a restricted substrate specificity, whereas OpuC can transport a wide variety of compatible solutes with high affinity (72, 106). The OpuB transporter is closely related to OpuC, and both systems probably evolved through a gene duplication event (74). However, OpuB differs significantly from OpuC in that it essentially recognizes only one substrate, choline (Fig. 3). OpuB is part of the choline to glycine betaine biosynthetic pathway, and its structural genes are regulated along with the genes for the biosynthetic enzymes (GbsA and GbsB) by the GbsR repressor in response to the availability of choline in the growth medium. Proline uptake in *E. coli* and serovar Typhimurium under highly osmotic conditions is mediated through the ProP and ProU systems with moderate affinity (30), but *B. subtilis* possesses a dedicated, high-affinity transporter (OpuE) for this compound (Fig. 3) (146).

OpuE is unrelated to the proline transporter ProP, but surprisingly it does resemble PutP, a permease used by *E. coli* and serovar Typhimurium to acquire proline for catabolic purposes (146). Unlike *putP*, *opuE* is not induced by proline in the growth medium; instead, its transcription is upregulated in response to increase in medium osmolality (136, 146). Transcription of the *opuE* structural gene is sensitively adjusted to the osmolality of the environment and mediated by two overlapping and independently controlled promoters. The *opuE*-P1 promoter is controlled by the housekeeping sigma factor SigA, and *opuE*-P2 is recognized by the alternative transcription factor SigB (136, 146), the master regulator for a large stress regulon in *B. subtilis* that protects the cells against various environmental insults (54, 55) (see chapter 12).

The dual regulation of *opuE* exemplifies the complexity and sophistication of the cellular response to a single stress factor by illustrating the connection between the uptake of a specific osmoprotectant and the onset of a general stress defense reaction (F. Spiegelhalter and E. Bremer, unpublished data). Osmotic upshock triggers the rapid induction of *opuE* transcription, with the level of *opuE* expression proportionally linked to medium osmolality. Transcription initiating from *opuE*-P1 is sensitively adjusted to the degree of osmotic stress and is kept at elevated levels as long as the osmotic stimulus persists. In contrast, the activity of the SigB-controlled *opuE*-P2 promoter rises only transiently after an osmotic upshock and does not significantly contribute to the level of *opuE* expression in cells subjected to long-term osmotic stress. Thus at least two different signal transduction pathways must operate in *B. subtilis* to communicate osmotic changes in the environment to the transcription apparatus of the cell (136). Essentially all SigB-controlled genes can be induced by salt stress (54, 55), but the SigB regulon cannot adequately protect growing cells against the negative effects of high osmolality. In fact, the *sigB* gene can be deleted without affecting the growth of osmotically stressed cultures. However, SigB is crucial in protecting the cells against osmotic upshock under growth-restricting conditions (145). The linkage of this general stress response system to a particular proline transporter (OpuE) clearly shows the importance of compatible solute uptake in osmotically threatened and even nongrowing cells.

C. glutamicum is another gram-positive soil bacterium whose osmoregulatory systems have been studied in detail (116). It is a member of the high G/C group of gram-positive bacteria and is not phylogenetically closely related to *B. subtilis*, which is a representative of the low G/C group of gram-positive microorganisms. *C. glutamicum* synthesizes several compatible solutes such as trehalose, proline, and glutamine. Biosynthesis of trehalose and proline is regulated by osmotic stress conditions, but the type and the amount of the accumulated compounds depend highly on the available carbon and energy sources. High internal concentrations of glutamate (up to 1 M) are also found; however, in contrast to the situation observed in *E. coli*, this amino acid is not directly involved in osmoregulation in *C. glutamicum* (R. Krämer, unpublished data). *C. glutamicum* achieves osmoprotection primarily by the uptake of external compatible solutes, in particular, glycine betaine, ectoine, and proline (41, 116). It is equipped with three osmoregulated transporters: BetP, a high-affinity system specific for glycine betaine; ProP, a medium-affinity system for proline and ectoine; and EctP, a low-affinity system for all three compounds. All three systems are effectively regulated at the level of activity, and the former two are also controlled at the level of gene expression (113, 114). Recently, a carboxy-terminal domain of BetP was found to be directly involved in osmosensing (115). The BetP and EctP transporters from *C. glutamicum* (113) are related to the glycine betaine uptake systems OpuD from *B. subtilis* (73) and BetL from *L. monocytogenes* (133) and to the choline transporter BetT from *E. coli* (85); they are all members of the BCCT (betaine-choline-charnitine transporters) family of secondary transporters. In contrast to *B. subtilis*, *C. glutamicum* exclusively uses secondary transport systems for osmoprotectant acquisition (Table 1), although it does possess ABC-type transporters (83). An additional proline transport system (PutP) is present in *C. glutamicum*, but it is used for anabolic purposes and its physiological function is unrelated to osmoprotection (113); such a transporter is also present in *B. subtilis* (S. Moses and E. Bremer, unpublished data). *C. glutamicum* responds to hypoosmotic stress through the controlled release of osmoprotectants via mechanosensitive channels (130, 131).

OSMOTIC REGULATION OF OSMOPROTECTANT TRANSPORTER ACTIVITY

The activity of many transporters that take up and mechanosensitive channels that release (see below) compatible solutes is governed by environmental osmotic conditions. This inherent feature allows the cell to respond to osmotic fluctuations very rapidly by enhancing or diminishing the performance of existent proteins. Osmotically activated solute uptake systems have been detected in many bacterial species.

They comprise members of different families of transporters and include the *E. coli* K^+ uptake systems Kdp (a P-type ATPase) and Trk, the ABC transporter ProU from *E. coli*, as well as several secondary systems (e.g., ProP and BetT from *E. coli*, OpuD from *B. subtilis*, BetP from *C. glutamicum*, QacT of *Lactobacillus plantarum*, and transporters from *L. monocytogenes*) (6, 40, 46, 49, 73, 114, 115, 124, 138, 139, 144). The degree of hyperosmotic stress that leads to optimal activation of the transport systems closely parallels the degree of osmotolerance of the respective bacterial species, which is low for *E. coli* (approximately 0.2 to 0.3 M NaCl for ProP and ProU) and higher for more salt-tolerant species like *L. monocytogenes*, *Staphylococcus aureus*, *C. glutamicum* (0.7 to 0.8 M NaCl) (79, 81, 115, 119), and *L. plantarum* (1.2 M NaCl) (45, 46). Detailed molecular and biochemical studies on osmotic activation have been initiated for the ProP and BetP transporters from *E. coli* and *C. glutamicum*, respectively, and for the sensor kinase KdpD, a regulatory protein for the Kdp K^+ transport system (70, 115, 124, 139, 144). These studies focus on correlating the structure and function of the involved membrane proteins and have identified regions that seem to serve as sensory domains; however, the mechanism for recognizing the signal and transforming it into an activity change has not yet been deciphered.

OSMOREGULATION: STRATEGIES AND EVENTS

Within a given organism the stress responses discussed above are coordinated into a particular sequence of reactions resulting in cellular adaptation to unfavorable osmotic growth conditions. The best illustration of this coordination is provided by the intensively studied enteric bacteria *E. coli* and serovar Typhimurium (14, 30, 76, 149). The primary event after an osmotic upshift is the efflux of water by diffusion across the lipid bilayer and through aquaporins. This leads to many physical and structural changes in the cell that presumably trigger the osmosensing process. The first response in *E. coli* is a massive uptake of K^+, which is mediated by the Trk and Kdp systems (Fig. 2). This event occurs within seconds after the osmotic shift and is accompanied by putrescine efflux (6, 39, 141). Within a few minutes, glutamate begins to accumulate and serves to balance charges within the cell (30, 36, 99, 141, 152). The accumulation occurs via an increase in biosynthesis and a decrease of glutamate utilization. Changes in turgor contribute to the regulation in activity of both the Trk and the Kdp transporters, and the level of turgor and the availability of K^+ appear to control expression of the *kdpFABC* operon via the KdpD-KdpE two-component regulatory module. An interesting regulatory model has been proposed that directly links osmotically mediated changes in turgor (or envelope tension) to the expression of the *kdp* operon (30, 39, 84). Signal perception induces autophosphorylation of the inner-membrane-embedded KdpD kinase; this phospor group is then transferred to the soluble response regulator KdpE (70), which then can activate *kdp* transcription. Transcription of the *kdp* operon is only transiently induced following an osmotic upshock, a regulatory pattern that is consistent with the observed restoration of turgor by an increase in the intracellular K^+ levels. K^+ availability also plays an important role in the genetic control of *kdp*: expression is transient when K^+ is freely available and permanent when K^+ is limiting. Furthermore, the threshold K^+ concentration for *kdp* expression is increased when the cells accumulate the osmoprotectants trehalose or glycine betaine (4). These findings challenge the validity of the turgor model, which predicts that the threshold concentration for K^+ will fall when accumulated compatible solutes contribute to turgor restoration and thereby lower the need for K^+ uptake via Kdp.

Because high cytoplasmic K^+ concentrations have negative effects on protein function and DNA-protein interactions, the massive accumulation of K^+ is an inadequate strategy for coping with prolonged osmotic stress. The initial increase in cellular K^+ content is followed by the accumulation of compatible solutes, allowing the cells to discharge large amounts of the initially acquired K^+ through specific and nonspecific efflux (Kef) systems (125, 141). The availability of compatible solutes in the environment determines whether synthesis or uptake will predominate in their cellular accumulation. A scarcity leads to the full expression of genes (*otsAB*) encoding enzymes for trehalose biosynthesis. Concomitantly, there is induction of genes coding for osmoprotectant transporters (ProP, ProU, and BetT) and enzymes for glycine betaine biosynthesis (BetBA), so that the cell also can take advantage of preformed osmoprotectants (or their biosynthetic precursors) that might be present in the environment. When exogenous osmoprotectants are abundant, uptake of these compounds is preferred over the endogenous synthesis of compatible solutes, and the expression of genes encoding the biosynthetic enzymes is repressed. Similarly, transcription of the osmoregulated genes encoding osmoprotectant transporters is reduced when the intracellular levels of compatible solutes are sufficient to counteract the osmotic stress (94). These observations imply a dedicated cellular and genetic

control over the intracellular pool of compatible solutes through which synthesis and uptake of osmoprotectants are integrated into a finely tuned cellular stress response system.

The temporal sequence of events during the initial phase in osmoadaptation and the finding that many subsequent osmotic responses (e.g., induction of the *proU* operon) are dependent on the prior accumulation of K^+ have led to the proposal that K^+ and its counterion glutamate act as a second messenger (30, 39, 49). This concept is attractive, but 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 in osmoadaptation—the accumulation of compatible solutes.

Osmosensing

Cellular responses to osmotic challenges are triggered by the cell's perception of a stress situation. In contrast to the response systems, the mechanisms for sensing osmotic fluctuations and the parameters that are actually sensed are poorly understood. In principle, changes in osmotic conditions may be detected directly as changes in extracellular water activity or indirectly as changes in the cell wall/plasma membrane structure or in the composition of the cytoplasm. Consequently, numerous parameters may be recognized by an osmosensor (29, 149). Some of these stimuli are related to properties of the cytoplasm and the periplasm, such as hydrostatic pressure, osmolality, ionic strength, concentration of particular signal molecules, and macromolecular crowding. Other potential stimuli are related to membrane-based osmosensors, such as lateral pressure within the membrane, bilayer curvature, density and structure of the phospholipid head groups, and thickness of the membrane. There is already good evidence that integral membrane proteins can function as sensors, transducers, and also responders to osmotic shifts (115, 124).

Osmosensing can be fully understood only by considering the mechanical and elastic properties of the cell wall and plasma membrane. The peptidoglycan sacculus of the cell wall is more flexible than previously believed (37, 142). It is able to swell and shrink to a certain extent in response to the influx and efflux of water, and these changes could indirectly affect intrinsic parameters of the cytoplasmic membrane. The mechanical properties of the cell wall differ greatly in gram-negative and gram-positive bacteria and are reflected in substantially different values for turgor. In contrast, the mechanical properties of the plasma membrane can be assumed to be similar in both groups of microorganisms, although the size of the cell must be taken into account. The strain at the surface of a spherical cell depends on its radius, i.e., larger cells experience a greater strain than smaller cells for a given stress (149). Further investigations are now needed to clarify the nature of the parameters that act as osmostress stimuli for the cell, the mechanisms by which these stimuli are recognized, and the pathway(s) by which the signal is transduced to further targets, such as regulatory proteins or the catalytic domains of transporters.

PROTECTION AGAINST EXTREME TURGOR: RELEASE OF COMPATIBLE SOLUTES

The stress response of bacterial cells to high-osmolality environments causes a massive intracellular buildup, often in molar concentrations, of compatible solutes. The same compounds that protect the cell from the detrimental effects of hyperosmotic conditions become a threat to the cell's survival in hypoosmotic habitats. In their natural ecosystems, bacteria are likely to experience osmotic downshocks caused by rain, flooding, and washout into freshwater sources (42, 108). Such conditions result in a rapid movement of water into the cell with a concomitant dramatic increase in turgor (15). To avoid bursting under this strain the cell must rapidly eliminate the accumulated organic osmolytes and ions (e.g., K^+) to reduce the driving force for water entry. This cannot be achieved simply by breaking down compatible solutes since this process is rather slow and many of these compounds are metabolically inert in various bacterial species. Instead, the organic osmolytes and other cellular solutes are rapidly expelled in a controlled manner. The extent of solute loss is related to the severity of the hypoosmotic shock, allowing the cell to sensitively adjust turgor to the altered osmolality of its habitat (44, 118, 131, 132).

E. coli cells grown at high osmolality release potassium glutamate, trehalose, and glycine betaine upon osmotic downshock (36, 132). Expulsion of more than one molar glycine betaine in less than 200 ms (the time resolution limit of the experiments) was observed (2). This massive solute efflux apparently occurs without severely damaging the cells because they can reaccumulate glycine betaine to preshock concentrations when the osmolality of the growth medium is raised to the previous level. Electrophysiological studies with the patch-clamp technique have revealed that the cytoplasmic membrane of both *Bacteria* and *Archaea* contains gated channels with various levels of conductances (10, 89). Since their discovery in bacteria, mechanosensitive channels have

been proposed to play a role in the sensing of and responding to osmotic changes and to serve as safety valves for the release of solutes when the environmental osmolality suddenly drops (9, 11, 110, 140). Recent genetic studies in *E. coli* have provided compelling support for this concept (15, 91).

Patch-clamp experiments have shown that *E. coli* contains at least three different species (MscL, MscM, and MscS) of efflux channels, with conductances ranging from 0.1 to 3 nS. These channels display little ion or solute specificity. Pioneering work by C. Kung and coworkers resulted in the isolation of the MscL protein, the identification of its structural gene (*mscL*), and the construction of mutants lacking MscL channel activity (140). Reconstitution of the MscL protein into artificial liposomes demonstrated that stretch forces in the lipid bilayer cause the channel to open, thereby establishing that mechanosensation is an inherent property of the MscL channel. Recently, Chang et al. succeeded in determining the crystal structure of the closed MscL channel from *Mycobacterium tuberculosis* at a resolution of 3.5 Å (24). The MscL protein has two transmembrane helices (TM1 and TM2) and a short periplasmic loop; both the N- and C-termini face the cytoplasm. The funnel-shaped channel is formed by a pentameric MscL configuration. All the transmembrane helices are slanted with respect to the plane of the membrane, and the five TM1 helices come together at their cytoplasmic ends, forming a gate that is held closed primarily by hydrophobic interactions between neighboring helices. It is thought that mechanical stretch pushes the cytoplasmic ends of the TM1 helices apart (9, 24) to form a large, nonspecific channel that can accommodate even the 12-kDa thioredoxin (2). The crystal structure of MscL (24) and mutant analysis of this integral membrane protein (11, 110) provide now a framework for understanding how a stretch in the lipid bilayer of the membrane can effect functional changes in a channel protein.

The search for the physiological function of MscL was originally inconclusive because a deletion mutant did not exhibit significant growth or survival phenotypes. The recent work of Levina et al. (91) not only characterized a new gene family (*yggB*) critical for MscS channel activity but also permitted new studies into the physiological role of MscL. Like the *mscL* deletion mutant, the *yggB* mutant did not have a severe growth phenotype. However, when both the *mscL* and *yggB* mutations were present in the same strain, these cells lost the ability to survive a severe osmotic downshock. Further physiological experiments with this double mutant revealed that the MscL and YggB channels open at a pressure change just below that which would cause cell disruption. These studies establish that the rapid release of solutes is necessary after osmotic downshock to prevent the excessive buildup of turgor and that the MscL and YggB mechanosensitive channels are crucial for managing hypoosmotic stress. As expected for proteins with such important physiological functions, database searches and physiological studies have revealed homologs of MscL and YggB in a wide variety of microorganisms (91, 104). In addition to YggB, the KefA channel has MscS-like properties, but it does not play any major role in managing the transition from high to low osmolality (91). Likewise, the physiological function of the MscM channel is unknown, and its structural gene has not been identified.

Clearly, mechanosensitive channels play a primary role in osmolyte expulsion as a stress response to a sudden reduction in osmolality, but they are not the only means for removing compatible solutes from the cell. For instance, the efflux of glycine betaine and proline from osmotically downshocked *L. plantarum* cells is characterized by two kinetically distinct pathways. One, which acts very rapidly, fits the pattern of mechanosensitive channels, and the other, which exhibits substantially slower kinetics, is consistent with a carrier system (44). Compatible solute efflux carriers also appear to be present in enteric bacteria (81, 86). It should be kept in mind that a number of solute efflux systems exist in bacteria that are unrelated to osmotic stress, e.g., those for amino acids in *C. glutamicum* (82).

CONTRIBUTION OF MULTIPLE STRESS RESPONSES TO CELL SURVIVAL IN HYPEROSMOTIC ENVIRONMENTS

In addition to modulating the intracellular solute, bacteria alter the expression of a sizeable number of genes that are not directly involved in osmoprotectant uptake or synthesis. Osmotic induction of these genes has been observed during searches with reporter gene fusions randomly inserted into the chromosome and in two-dimensional gel electrophoresis studies. In *E. coli*, many of these genes are under the control of the alternative transcription factor RpoS, and their osmotic induction is either abolished or strongly reduced in *rpoS* mutants (56). The σ^s subunit of the RNA polymerase is itself subjected to posttranscriptional osmotic regulation (8, 105), and the increase in the cellular RpoS content provides a link between hyperosmotically induced and stationary-phase-associated processes (56, 57) (see chapter 11). Although the physiological function of most of these osmotically induced genes in stress resistance has not been determined, the genetic regulation of

some has been studied in detail. These studies have revealed that RpoS-mediated gene expression is frequently governed by a complex interplay between global regulatory proteins such as H-NS, Lrp, CRP, IHF, and Fis, thus providing the cell with a finely tuned and interwoven stress response network (57). This network contributes significantly during stationary phase to the development of a broad stress resistance, including a high tolerance against osmotic challenges (88, 98). Increased osmotic sensitivity is observed in stationary-phase cells of *E. coli* lacking RpoS, but the cellular events involved in RpoS-mediated osmoadaptation are unknown. A similar phenomenon is observed in *B. subtilis* where a very large stress regulon that is controlled through the alternative sigma factor σ^B confers high stress resistance against a wide variety of environmental insults (54, 55) (see chapter 12). Mutants lacking σ^B are sensitive to a severe and growth-preventing osmotic upshock, and this osmosensitivity can be alleviated by glycine betaine (145). It is apparent that the general osmostress responses in both *E. coli* and *B. subtilis* add a level of osmoprotection that goes beyond compatible solute accumulation, and the underlying molecular and physiological mechanisms clearly deserve further exploration.

FUTURE PROSPECTS

The accumulation and release of compatible solutes have been clearly established as the principal physiological response of bacteria to hyper- and hypoosmotic challenges, and much has been learned about the systems for compatible solute synthesis and uptake. However, it is not yet possible to describe unambiguously in any bacterium the molecular events involved in perceiving osmotic changes and processing this information into a genetic signal that leads to altered gene expression and ultimately to physiological adaptation reactions. Considerable advances have been made in understanding these processes in yeast with respect to the synthesis of the compatible solute glycerol. Although two membrane-bound osmosensors (Sho1 and Sln1) and an elaborate signaling cascade involving MAP-kinases have been detected, the sensing process itself remains enigmatic (61, 120). This is also the case for the bacterial sensors EnvZ and KdpD, which control expression of the *ompF* and *ompC* porin genes and synthesis of the Kdp potassium transporter, respectively. There is genetic evidence that osmolality per se is not perceived by some osmoregulatory systems; the central problem that now must be urgently addressed is the precise nature of the stimulus that is actually sensed in osmotically stressed cells.

It is well established that the activity of many transporters and channels that take up or release compatible solutes is governed by environmental osmotic conditions. We do not understand sufficiently how these membrane-bound proteins sense osmotic up- or downshifts, nor do we understand the nature of the signal and its transformation into an activity change. The successful functional reconstitution of the purified channel protein MscL and the osmoprotectant transporters ProP and BetP and that of the sensor kinase KdpD into artificial membrane provides important tools to further our understanding of the osmotic influence on protein activity.

It is now possible to approach the osmostress response of microorganisms on a genomic and cellular scale by analyzing the transcriptome and proteome. These techniques not only allow the detection of new osmoregulated genes but also permit the exploration of connections among individual osmostress response systems. In addition, interactions between osmotic and other types of stress responses can be analyzed, allowing an integrated view of the complex relationship between the cell and its ever-changing environment.

Acknowledgments. We greatly appreciate the help of Vickie Koogle in editing the manuscript.

Work on osmoregulation in our laboratories was provided by the Deutsche Forschungsgemeinschaft and through the Fonds der Chemischen Industrie.

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