Adaptation to Changing Osmolarity

ERHARD BREMER

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OSMOTIC CHALLENGES

Microorganisms lack systems for active water transport; therefore, their cellular water content and turgor is governed by osmosis and is strongly affected by the osmolarity of the environment (14). For *Bacillus subtilis*, turgor has been estimated at 1.9 MPa (89). In its natural habitats, the upper layers of the soil (69, 91), *B. subtilis* experiences drastic changes in osmolality as a result of drought or rain, thereby threatening the cell with dehydration or rupture as water permeates across the cytoplasmic membrane along the osmotic gradient (59). The effective management of these osmotically instigated water fluxes is therefore of central importance for the survival and growth of microorganisms in habitats with changing osmolarities. In some microorganisms, dedicated water channels, the aquaporins, mediate accelerated water fluxes in both directions when the external osmolarity changes (22, 28). The *Escherichia coli* AqpZ protein is the prototype of bacterial aquaporins (20, 21, 71), but like many other gram-positive microorganisms, *B. subtilis* possesses no AqpZ-related protein (22). Thus, either *B. subtilis* contains no aquaporins or it possesses water channels of a yet unidentified type.

In many bacteria, mechanosensitive channels located in the cytoplasmic membrane act as safety valves against a detrimental buildup of turgor pressure, rapidly releasing compatible solutes and ions when turgor rises beyond a threshold value (6, 9, 14, 83). In their natural ecosystems, bacteria are likely to experience osmotic down-shocks caused by rain, flooding, and washout into freshwater sources. Two such mechanosensitive channels, MscL (1, 8, 82) and MscS (YggB) (52), have been characterized in some detail in *E. coli*, and their simultaneous disruption results in cell death following an osmotic down-shock (52). The molecular, biochemical, and physiological characteristics of the mechanosensitive channels in *B. subtilis* are just beginning to be explored. Heterologous expression of the evolutionarily well-conserved *B. subtilis mscL* (*ywpC*) gene in *E. coli* led to the formation of mechanosensitive channels with a conducting capacity of 3.6 nS (60). When a *mscL* mutant of *B. subtilis* was subjected to a severe osmotic down-shock, a substantial number of the cells did not survive this osmotic challenge (13). Three YggB (MscS) homologues (YhdY, YkuT, YfkC) are also encoded by the *B. subtilis* genome (52), and the functioning of these proteins as ion-conducting pores (Fig. 1) might account for some of the stretch-activated composite channels detected through electrophysiological approaches (2, 84).

The survival and growth of *B. subtilis* in osmotically changing habitats depends on highly integrated cellular adaptation reactions that are either part of the SigB-controlled general stress regulon (34, 87) (see chapter 26) or specific to osmotic stress (17, 44). The specific stress reactions of many *Bacillus* spp. comprise the synthesis and uptake of certain organic osmolytes, in particular proline, glycine betaine, and ectoine (Fig. 2), under hyperosmotic conditions and their expulsion under hypoosmotic circumstances (17).

When *B. subtilis* experiences a sudden osmotic up-shift and the resulting loss of cell water, it first responds by rapidly accumulating K^+ from environmental sources (89, 90). Glutamate is likely to function as the predominant counterion to K^+. Uptake of K^+ is mediated by the high-affinity KtrAB (YuaA, YubG) and the low-affinity KtrCD (YkqB, YkrM) transport systems (Fig. 1) (36). Despite the importance of K^+ in the initial osmostress reaction, high intracellular concentrations of this ion interfere with many important cellular functions. *B. subtilis* therefore accumulates large quantities of organic osmolytes, the so-called compatible solutes (90), which are more congruous with its physiology. The amassing of these compatible solutes through synthesis and uptake permits the reduction of the K^+ pool as the cell expands and proliferates under high osmolality conditions (89, 90).

SYNTHESIS OF PROLINE AS AN OSMOSTRESS PROTECTANT

When a *B. subtilis* culture growing in a chemically defined medium is subjected to a sudden but relatively mild osmotic upshift by the addition of 0.4 M NaCl, the cellular proline pool increases from a basal level of 16 mM to approximately 700 mM within 7 h (90). Proline levels substantially higher

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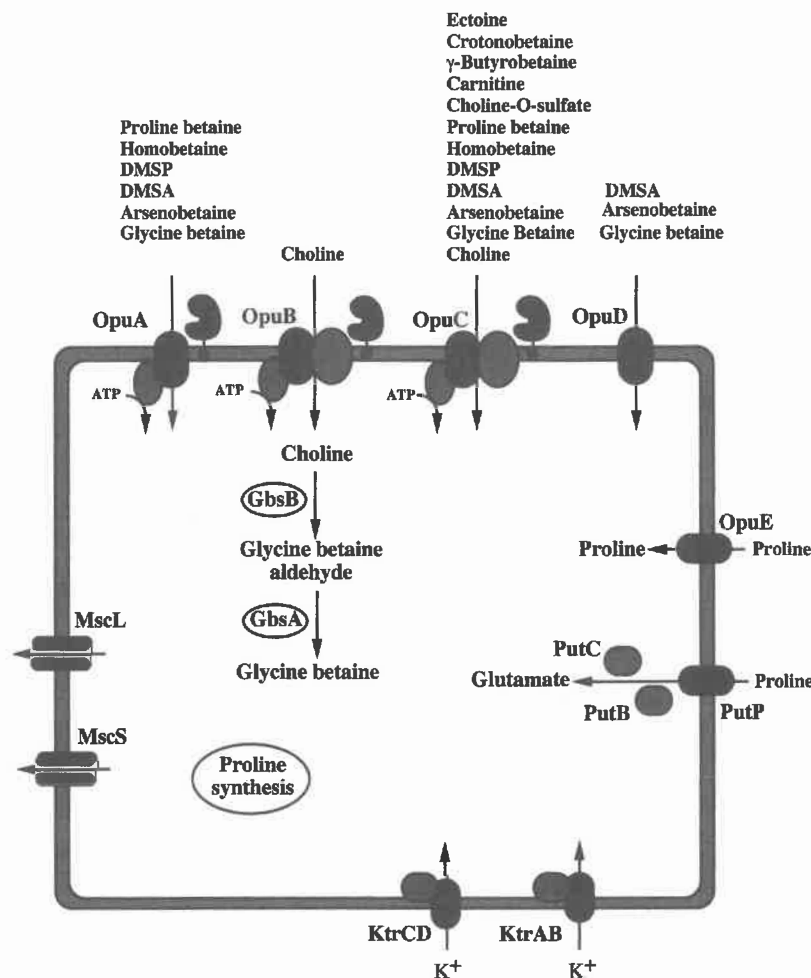

FIGURE 1 Systems for the uptake and expulsion of K^+ and compatible solutes in *B. subtilis*.

than 700 mM are therefore expected for truly osmotically challenged cells. The molecular details of this osmoregulatory proline synthesis have only recently become clear (18). The *proHJ* operon, encoding orthologs of the proline biosynthetic *proB* and *proI* (*yqjO*) genes (see chapter 16), is specifically needed for high-level proline synthesis under osmotic stress conditions. In contrast, the ProA protein is used for both anabolism and osmoprotection (Fig. 3). The *proHJ* operon is transcribed from a σ^A-type promoter; its level of expression increases upon osmotic up-shock and is linked in a linear fashion to the degree of osmotic stress (18). At the same time, transcription of the *proBA* operon is very moderately elevated, thereby ensuring that osmotically challenged *B. subtilis* cells possess increased levels of all enzymes required for osmoregulatory proline synthesis (18). The very high accumulation of proline raises two important regulatory issues. In *E. coli*, the initial, ATP-requiring step in the anabolic synthesis of proline is feedback inhibited by proline (51). The enormous quantities of proline produced under osmotic stress (90) strongly suggest that the ProJ enzyme (Fig. 3) is either regulated inefficiently by feedback control or not regulated at all.

Degradation of proline to glutamate in *B. subtilis* is induced when millimolar concentrations of proline are present in the growth medium (5) (see chapter 14). Therefore, the cell needs to tightly coordinate proline degradation and its osmoregulatory production to avoid the wasting of energy and precious resources through a futile cycle. Disruption of the *putBCP* (*ycgMNO*) operon abolishes the ability of *B. subtilis* to use an exogenous supply of proline as sole nitrogen or carbon source. The PutP protein serves as a high-affinity proline uptake system, and the PutBC proteins function in the sequential enzymatic degradation of proline (Fig. 1) (61). The *putBCP* operon is induced by the presence of low (10 to 500 μM) concentrations of proline in the growth medium. In contrast, the very large amounts of proline that are accumulated intracellularly via de novo synthesis under osmotic stress conditions do not result in strong induction of the *putBCP* operon (61). The genetic mechanism by which *B. subtilis* distinguishes between exogenously provided and endogenously synthesized proline is currently unknown.

COMPATIBLE SOLUTES: CHARACTERISTICS AND FUNCTION

Proline belongs to a restricted group of organic osmolytes, the so-called compatible solutes, that can be accumulated to exceedingly high intracellular levels (up to several moles per liter) without disturbing protein function and cellular physiology (4, 26, 33, 88, 93). Accumulation of compatible so-

lutes under high osmolarity conditions is not only common in the microbial world (*Archaea* and *Bacteria*) (17, 25, 26, 33, 58, 72) but is also characteristic of fungal, plant, animal, and even human cells (19, 35, 78). Twelve compounds are currently known to function as osmoprotectants and compatible solutes for *B. subtilis* (Fig. 1), and with the exception of ectoine, each of these compounds is structurally related to either proline or glycine betaine (Fig. 2). The exact biophysical mechanism(s) through which compatible solutes act is not completely understood, but their functioning is generally explained in terms of the preferential exclusion model (4). This model suggests that compatible solutes are excluded from the immediate hydration shell of proteins because of unfavorable interactions with the peptide backbone (70). This results in a nonhomogeneous distribution of these solutes within the cell water and a preferential hydration of protein surfaces. As a consequence of this thermodynamic disequilibrium, there is a driving force for the protein to occupy a smaller volume to reduce the amount of excluded water, thereby promoting the stabilization of the protein's native structure. In addition to their role in maintaining cellular water content and turgor, compatible solutes also protect proteins in vitro from denaturation caused by freezing, heating, desiccation, and high ionic conditions and have beneficial effects on membrane integrity, protein stability, and folding (26, 33, 54, 88). Recent data strongly suggest that compatible solutes also serve a protein stabilizing function in vivo (15, 23).

SYNTHESIS OF ECTOINE AND GLYCINE BETAINE FOR OSMOREGULATORY PURPOSES

In the genus *Bacillus*, the second most frequently synthesized compatible solute in response to high osmolality is ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) (Fig. 2) (48). As in the moderate halophile *Halomonas elongata* (24, 57), aspartate β-semialdehyde, an intermediate in the biosynthesis of lysine, threonine, methionine, and diaminopimelic acid (see chapter 16), serves as the precursor, and three enzymatic steps are required for its conversion to ectoine (67) (Fig. 3). The structural genes (*ectABC*) encoding the ectoine biosynthetic enzymes are clustered in the genome of *B. pasteurii*, and their transcription is induced by increases in medium osmolality (48). *B. subtilis* cannot produce this tetrahydropyrimidine, but, like many other bacterial species, it can take up ectoine for osmoprotective purposes, albeit with low affinity (38).

Glycine betaine (*N,N,N*-trimethyl glycine) (Fig. 2) is one of the most potent compatible solutes found in nature (25, 26, 33). A number of microorganisms, in particular halophilic phototrophic eubacteria (33) and some extreme halophiles (65), can synthesize it de novo by stepwise methylation of glycine. But many bacterial species (50), including *B. subtilis* (10, 11), produce glycine betaine from an exogenous supply of choline through two sequential oxidation reactions. *B. subtilis* uses a soluble, metal-contain-

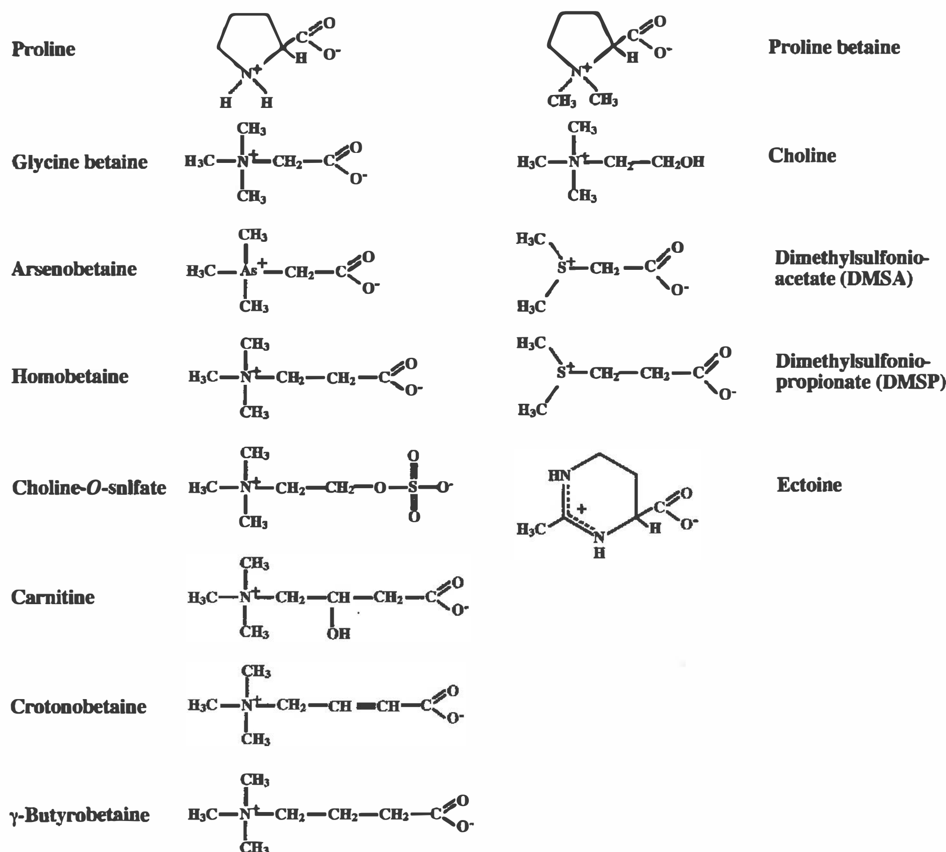

FIGURE 2 Chemical structures of osmoprotectants used by *B. subtilis*.

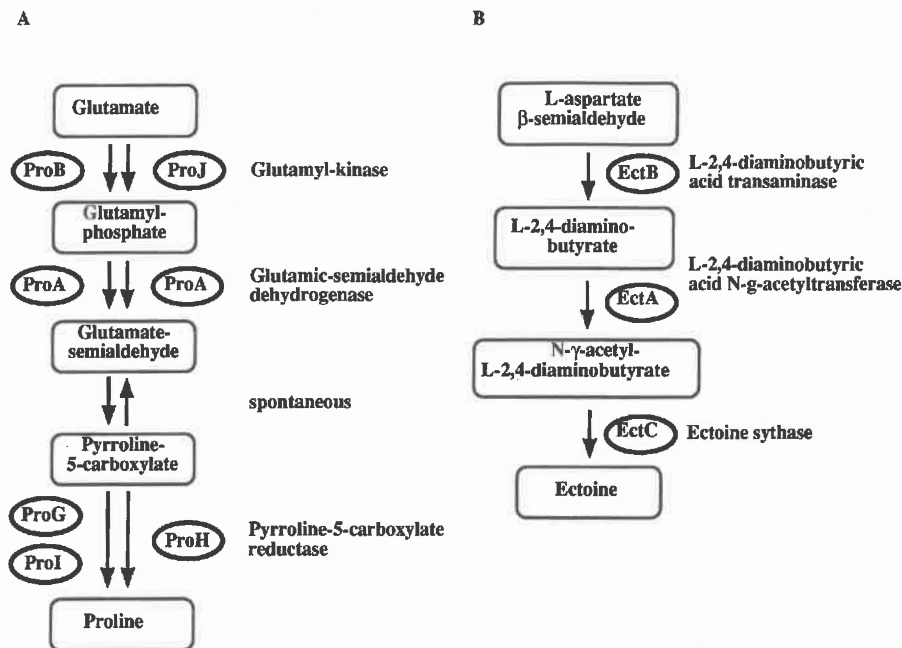

FIGURE 3 Biosynthesis pathways for proline and ectoine and glycine betaine in *Bacillus* sp. (A) Proline biosynthesis for anabolic and osmostress protective purposes in *B. subtilis*. (B) Osmoregulatory synthesis for the compatible solute ectoine in *Bacillus pasteurii*.

ing, type III alcohol dehydrogenase (GbsB) to convert choline to glycine betaine aldehyde and a highly salt-tolerant glycine betaine aldehyde dehydrogenase (GbsA) (12) to oxidize this toxic intermediate to the well-tolerated and metabolically inert glycine betaine (Fig. 1) (10, 11). The structural genes (*gbsAB*) encoding these enzymes form an operon. *B. subtilis* possesses two multicomponent, high-affinity transport systems (OpuB and OpuC) (Fig. 1) for the acquisition of choline from exogenous sources (41); both are members of the ABC superfamily of binding protein-dependent transport systems. OpuC functions as a broad-spectrum osmoprotectant uptake system (12 substrates) (38, 40, 45, 63), whereas OpuB transports only choline (Fig. 1). The high degree of sequence identity of the OpuB and OpuC systems and the close proximity of their structural genes in the *B. subtilis* genome argue that these two loci evolved through a gene duplication event (41).

GbsR, a repressor encoded by a gene upstream of *gbsAB*, coordinates the activities of the *opuB* (but not *opuC*) and *gbsAB* operons by sensing the intracellular concentration of choline (64). High osmolality increases the expression of the *opuB* and *opuC* operons but does not significantly stimulate the expression of the *gbsAB* genes. However, the increased intracellular levels of choline achieved via the osmotic control of *opuB* and *opuC* do indirectly stimulate transcription of the *gbsAB* operon in a GbsR-dependent manner (64). GbsR is a novel type of choline-sensing regulatory protein and is not related to the choline-sensing BetI repressor from *E. coli* (50, 73). GbsR-type proteins for the control of the choline to glycine betaine synthesis pathway may be widespread in gram-positive bacteria, since *gbsR*-related genes are present in *Staphylococcus carnosus* (74) and *Staphylococcus aureus* (29) in the vicinity of genes that are likely involved in glycine betaine synthesis.

ACQUISITION OF PREFORMED COMPATIBLE SOLUTES FROM ENVIRONMENTAL SOURCES

Within its varied habitats (69, 91), *B. subtilis* has access to a wide variety of compatible solutes that are released into ecosystems by primary producers (e.g., osmotically downshocked microbial cells, root exudates, and decaying bacterial and plant cells) (33, 88). *B. subtilis* imports these solutes via five transport systems (OpuA to OpuE) with overlapping substrate specificities (39, 41–43, 53, 87) (Fig. 1). Their high-level intracellular accumulation allows the growth of *B. subtilis* under conditions that are otherwise inhibitory for its proliferation (10, 38–41, 62, 63). Transcription of the structural genes for each of the *B. subtilis* Opu transporters is induced by an unknown mechanism when the cells are exposed to increased medium osmolality (41, 42, 80, 87). Osmoregulated compatible transporters related to the *B. subtilis* OpuA and OpuC systems have also been detected in the gram-positive bacteria *Listeria monocytogenes* (32, 46) and *Lactococcus lactis* (16, 66, 85).

HIGH SALINITY EXERTS PLEIOTROPIC EFFECTS ON THE PHYSIOLOGY OF *B. SUBTILIS*

The cellular responses to high salinity are not limited to the high-level accumulation of K^+ and compatible solutes. Mutants lacking the extracytoplasmatic function (ECF) sigma factor SigM are highly sensitive to high salt (37); however, this might be an indirect phenotype related to the major cell wall defects exhibited by such mutants. Increases in salinity affect the phospholipid composition of the cytoplasmic membrane (56), the properties of the cell wall (55), and the synthesis of the cell wall-associated protein WapA (27). In addition, the synthesis of several extracellular degradative enzymes is repressed in a DegS/DegU-dependent manner by

high salinity (49), and the structural genes of these regulatory proteins are upregulated in hypertonic media (75). Under such growth conditions, the structural gene for the membrane-associated protease FtsH is transiently induced, and *ftsH* mutants can only grow efficiently in media with NaCl concentrations greater than 0.2 M if the compatible solutes proline or glycine betaine are added (30, 31). One also observes increases in negative supercoiling of reporter plasmids when *B. subtilis* is subjected to salt stress (3, 47). Furthermore, sudden osmotic changes trigger a behavioral response (osmotaxis) such that the *B. subtilis* cells are repelled by both high and low osmolality (92).

OVERLAP BETWEEN THE SPECIFIC OSMOSTRESS REACTIONS AND THE SigB-CONTROLLED GENERAL STRESS RESPONSE NETWORK

B. subtilis possesses a very large general stress regulon whose expression depends on the alternative sigma factor SigB (σ^B) (see chapter 26), and a sudden increase in salinity triggers the transient expression of the entire σ^B regulon (34, 68). Proteome analysis has demonstrated that the proteins induced under these circumstances can be divided into two groups: the salt-specific stress proteins and the general stress proteins, which can be further subgrouped into SigB-dependent and SigB-independent classes (7). *B. subtilis* mutants lacking σ^B are highly sensitive to sudden and growth-restricting up-shocks with NaCl (86), perhaps because of the failure to synthesize the GsiB and YtxH proteins, both of which resemble plant desiccation proteins. The sensitivity of *sigB* mutants to severe increases in salinity can be counteracted by the uptake of glycine betaine, indicating that the onset of the *B. subtilis* general stress response and the accumulation of compatible solutes constitute two alternative routes to manage growth-restricting increases of NaCl (86).

The structural genes for both the glycine betaine transporter OpuD (81) and the proline uptake system OpuE (80, 87) (Fig. 1) are members of the σ^B regulon. Both genes are transcribed from two closely spaced and independently controlled promoters that respond to osmotic stress. One of these promoters is recognized by the housekeeping sigma factor σ^A, and the second promoter is recognized by σ^B. The σ^B-controlled promoters respond only transiently to a rapid osmotic up-shift. In contrast, the level of transcription initiating from the σ^A-controlled promoters remains elevated as long as the osmotic stimulus persists (80), suggesting that at least two different signal transduction pathways must operate in *B. subtilis* to communicate environmental osmotic changes to the transcription apparatus of the cell. The prime defensive strategy of *B. subtilis*, the formation of a highly desiccation-resistant endospore (79), is severely impaired by high salinity (49, 76, 77). Therefore, both the specific osmostress reactions (uptake of potassium and accumulation of compatible solutes) and the induction of the SigB-dependent general stress response are likely to play important physiological roles for the effective adaptation of *B. subtilis* to changing osmolarity in its natural habitats.

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