Chapter 15
Cellular Adjustments of *Bacillus subtilis* and Other Bacilli to Fluctuating Salinities

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15.1 The Osmotic Challenge

Bacilli are exposed, either suddenly or on a sustained basis, to changes in the osmotic conditions of the varied ecological niches that they can colonize. This can be vividly visualized by considering the main habitat of *Bacillus subtilis*, the upper layers of the soil (Earl et al. 2008). Rainfall and drying of the soil cause fluctuations in water availability and in the osmotic potential of this ecological niche. Such changes pose a considerable challenge to the microbial cell since they elicit water fluxes across the cytoplasmic membrane. These water fluxes are driven by the differential in the osmotic potential between the cell's interior and that of the surrounding microenvironment (Bremer and Krämer 2000). Water entry at low osmolarity can increase turgor to such an extent that the elastic and stress-bearing peptidoglycan sacculus can no longer cope with it and the cell will rupture. Water efflux at high osmolarity will trigger dehydration of the cytoplasm and the ensuing reduction or even collapse of turgor will cause growth arrest or even cell death. Turgor, an intracellular hydrostatic pressure considered to be essential for cell expansion and growth, is difficult to determine experimentally but has been estimated at 1.9 MPa (19.37 atm) for *B. subtilis* (Whatmore and Reed 1990), a pressure that is close to ten times the pressure present in a standard car tire.

To maintain turgor within physiologically acceptable boundaries, the *B. subtilis* cell needs to take active countermeasures to redirect the flow of water in or out of the cell when it faces decreases or increases in the external osmolarity. A considerable number of bacteria, including several Bacilli (but not *B. subtilis*), possess AqpZ-type aquaporins, water-selective channels embedded in the cytoplasmic membrane that can mediate accelerated water fluxes along osmotic gradients. However, the potential role of AqpZ-type water channels in the osmotic adjustment

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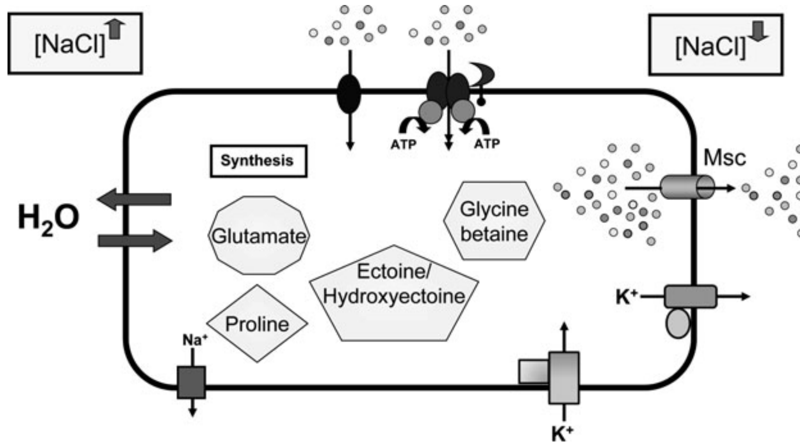

Fig. 15.1 The core of the osmostress response of Bacilli. Schematic overview of the initial and sustained cellular stress responses to high salinity through the uptake of K^+, synthesis and import of various compatible solutes and the active export of K^+ and Na^+ ions. Non-selective expulsion of ions and organic solutes occur in response to sudden osmotic downshifts via mechanosensitive channels (Msc)

processes of microorganisms has not been rigorously worked out (Tanghe et al. 2006). It is important to recall that no microorganism can actively move water across the cytoplasmic membrane in a directed fashion; in other words: through an energy-consuming transport process. Hence, microorganisms cannot counteract the environmentally imposed water fluxes by actively pumping water in or out of the cell. However, by carefully setting the differential in the osmotic potential of the cytoplasm and that of the environment, bacteria can actively determine the direction and magnitude of the water fluxes across their cytoplasmic membrane (Fig. 15.1) (Bremer and Krämer 2000).

15.2 Exposure to Hypo-osmolarity: Opening the Floodgates

A *Bacillus* cell living in the upper layers of dried-out soil will experience a sudden osmotic downshift when rain sets in. This creates an emergency situation, which the cell must deal with very rapidly. The stress-bearing peptidoglycan sacculus is elastic (Vollmer and Seligman 2010), but only to a certain degree. The rapid inflow of water following an osmotic downshift can drive up turgor almost instantaneously and to such a degree that the cell will burst. A calculation by Booth and Louis (1999) suggests that the turgor of *Escherichia coli*, which is estimated to lie in the range between 2 and 6 atm (Booth et al. 2007), will be driven up in a few milliseconds by about 10 atm when cells that were pre-adapted to high osmolarity media are suddenly transferred to low-osmolarity media (Booth and Louis 1999). Detailed genetic, electrophysiological, and structural studies have demonstrated

that microorganisms transiently open mechanosensitive channels embedded in their cytoplasmic membrane to rapidly jettison water-attracting solutes when they are suddenly exposed to hypo-osmotic surroundings (Booth et al. 2007; Kung et al. 2010). This reduces the osmotic potential of the cytoplasm quickly and thereby curbs water influx; consequently, the undue rise in turgor is counteracted and cell lysis is prevented.

Mechanosensitive channels remain in a closed state when turgor is properly balanced, but they can sense, via their protein–lipid interface, tension in the cytoplasmic membrane that is caused by increased turgor. Consequently, they are stretched opened to form huge holes through which both solvents and solutes can rapidly pass. Microorganisms typically possess several types of these channels (e.g., MscM, MscS, MscK, MscL) and their gating at different threshold values of membrane tension provides the cell with the tools for a graded response to osmotic down-shifts (Booth et al. 2007; Booth and Louis 1999; Kung et al. 2010). The opening of the channel of large conductance, MscL, is typically the measure of last resort, since MscL opens just below the turgor pressure threshold value at which the cells are about to lyse (Levina et al. 1999). The diameters of the *E. coli* MscS and MscL channels have been estimated to about 16 Å and 30 Å, respectively (Kung et al. 2010), and thereby exceed the diameters (about 12 Å) of the permanently open channels of porins (e.g., OmpC and OmpF) present in the outer membrane of Gram-negative bacteria (Nikaido 2003). Hence, the transient opening of mechanosensitive channels embedded in the cytoplasmic membrane will not only reduce turgor at a time when this is desperately needed, but also result in the loss of valuable metabolites and ions. However, the cell has to endure this negative side effect in order to survive. For obvious reasons, the timing and duration of the opening of such floodgates in the cytoplasmic membrane needs to be very carefully controlled by the microbial cell. The critical role of mechanosensitive channels for managing the transition from high to low salinities is manifest by the fact that mutants lacking these channels typically do not survive sudden osmotic downshifts (Levina et al. 1999; Hoffmann et al. 2008).

Electrophysiological studies with whole cells have shown that *B. subtilis* possesses mechanosensitive channels that can gate at different pressure set points (Szabo et al. 1992). Inspection of the *B. subtilis* genome sequence revealed one MscL-type and three MscS-type channel-forming proteins (YhdY, YfkC, YkuT) (Hoffmann et al. 2008; Wahome and Setlow 2008). Mutational analysis demonstrated that the simultaneous disruption of the structural genes for the MscS-type YhdY, YfkC, YkuT proteins does not cause a noticeable effect on cell survival when the corresponding *B. subtilis* triple mutant strain is subjected to a severe osmotic downshift. In contrast, 70% of the cells carrying an *mscL* gene disruption mutation do not survive such a downshift (Hoffmann et al. 2008), indicating that the channel-activity of MscL is key for the management of a rapid transition of *B. subtilis* from hyper- to hypo-osmotic surroundings (Hoffmann et al. 2008; Wahome and Setlow 2008). The inactivation of the MscS-type YkuT protein has no phenotype by itself, but the combination of a *ykuT* mutation with that of an *mscL* gene disruption potentiates the phenotype of the *mscL* mutant to such an extent that almost all cells

are killed upon a severe osmotic down-shock (Hoffmann et al. 2008). Indeed, Wahome and Setlow (2008) have shown that the overproduction of the YkuT protein in an *mscL* mutant background can rescue the osmotically down-shock-sensitive phenotype of a *B. subtilis mscL* mutant strain, thereby providing further evidence for a channel function of the MscS-related YkuT protein. It is currently unclear whether the MscS-related YhdY and YfkC proteins are actually channel-forming proteins and what the functions of such channels might be in the physiological context of the *B. subtilis* cell.

Interestingly, the *ykuT* gene is part of the SigB-controlled general stress regulon of *B. subtilis*, an emergency stress response system that provides environmentally or nutritionally challenged *B. subtilis* cells with a multifaceted and pre-emptive stress resistance (Hecker et al. 2007). This includes cellular resistance against the imposition of a severe and growth-restricting salt shock; such a salt shock elicits the transient induction of almost the entire SigB regulon (Höper et al. 2005, 2006). Consistent with the SigB control of the *ykuT* gene, genome-wide transcriptional profiling studies of *B. subtilis* cells subjected to an osmotic up-shift with 6% (wt/vol) NaCl revealed that *ykuT* expression is transiently up-regulated upon the hyperosmotic challenge. In contrast, the expression of the SigB-independent *mscL* gene was suppressed under these conditions (Hahne et al. 2010). Using GFP- and YFP-tagged Msc-fusion proteins from *B. subtilis*, Wahome and Setlow (2008) found that the cellular levels of the MscL, YhdY, YfkC, YkuT proteins varies with growth phase, suggesting that some of these mechanosensitive channels might play not yet understood roles during certain periods of the growth cycle of individual cells.

The central role of mechanosensitive channels for the challenging transition from high to low osmolarity surroundings is reflected in the speed in which osmotically down-shifted *B. subtilis* cells can jettison radiolabeled glycine betaine that was pre-accumulated under high salinity (0.8 M NaCl) growth conditions. Both the osmotic properties of the *B. subtilis* cell (Whatmore and Reed 1990) and the elastic properties of the peptidoglycan sacculus (Vollmer and Seligman 2010) appear to contribute to the timing and the speed by which glycine betaine is released from the cells through the transient opening of mechanosensitive channels. Moderate osmotic downshifts (equivalent to the withdrawal of about 0.285 mM NaCl) elicit little release of glycine betaine. This finding indicates that the elastic properties of the peptidoglycan sacculus allow the *B. subtilis* cell to restrain moderate increases in turgor and thereby obviate the need for the opening of mechanosensitive channels. Osmotic downshifts that exceed this threshold value lead to a progressive increase in the amount of the released glycine betaine and the extent of the release correlates with the severity of the imposed osmotic downshift. Upon an osmotic downshift equivalent to the withdrawal of about 0.57 mM NaCl, almost the entire glycine betaine content of the cells was released. Expulsion of glycine betaine under these conditions was very rapid (in less than 30 s) and was definitely not caused by cell lysis. A wild-type *B. subtilis* culture survives such a severe osmotic down-shift without any reduction in viability, whereas the cells of a MscL-YkuT double mutant strain were almost all killed under such a regimen (Hoffmann et al. 2008).

Recently reported data for the soil bacterium *Corynebacterium glutamicum* indicate that an MscS-type mechanosensitive channel (MscCG) also contributes to the fine-tuning of the cellular solute pool and turgor of high-osmolarity-grown cells that are not subjected to rapid osmotic downshifts. The data obtained by Börngen et al. (2010) indicate that MscCG may act in the osmoadaptation process of *C. glutamicum* by fine-tuning the steady state level of the glycine betaine solute pool resulting from import of this osmoprotectant from environmental sources. Hence, *C. glutamicum* cells apparently respond to temporary imbalances in turgor through a "pump and leak" mechanism – import of the compatible solute glycine betaine through the high-affinity BetP carrier and its release via the MscCG channel – to attain a level of turgor that is "just right" for the prevailing osmotic and cellular conditions (Börngen et al. 2010). *B. subtilis* might employ similar mechanisms for the fine-tuning of its cellular compatible solute pool.

15.3 Exposure to Hyperosmolarity: Salt-In or Salt-Out

To cope physiologically with high osmolarity surroundings, prokaryotes have developed two principally different approaches. One is the so-called salt-in-cytoplasm strategy in which the internal salt concentration is actively maintained through transport processes at a higher level than that present in the environment. Primarily K^+ and Cl^- ions are pumped into the cell and cytotoxic Na^+ ions are pumped out. The salt-in cytoplasm strategy is energetically favorable (Oren 2010), but it requires far reaching adaptations of the entire intracellular enzyme machinery and cellular physiology to the permanently high ion content of the cell. The evolutionary adjustment to a high ion content of the cytoplasm has left an "acid signature" on the entire proteome of those Archaea and Bacteria that have adopted the salt-in-cytoplasm strategy in order to maintain proper protein solubility, stability and catalytic activity in a cytoplasm of high ionic strength (Coquelle et al. 2010; Rhodes et al. 2010). Although energetically favorable (Oren 2010), the salt-in cytoplasm strategy is not prevalent among the different phylogenetic and physiological groups of halophilic Archaea and Bacteria (Oren 2008; Ventosa et al. 1998). This is probably because considerable amendments in protein composition and cellular physiology had to be made by cells in the course of evolution to cope on a sustained basis with molar concentrations of KCl in their cytoplasm.

No such evolutionary adjustments in protein composition or cellular physiology are imposed on those microorganisms that use a salt-out cytoplasm strategy and that employ a selected class of organic osmolytes, the compatible solutes, to balance turgor (Kempf and Bremer 1998; Empadinhas and da Costa 2008; Bremer and Krämer 2000; Wood et al. 2001). The amassing of compatible solutes through synthesis is energetically more costly then the import of ions (Oren 2010), but it provides great flexibility to the osmotic stress response of those microorganisms that frequently encounter fluctuating osmotic conditions. It also does not tie their wellbeing and cellular integrity to permanently high salinity habitats, as the salt-in

cytoplasm strategy often does (Ventosa et al. 1998; Oren 2008). Furthermore, almost all microorganisms can take up pre-formed compatible solutes, or their biosynthetic precursors, from environmental sources (Kempf and Bremer 1998; Bremer and Krämer 2000; Wood et al. 2001; Ziegler et al. 2010). This allows the bacterial cell to take advantage of precious environmental resources (Welsh 2000). Import of compatible often partially suppresses the expression of biosynthetic genes for these types of stress protectants, indicating that the uptake of compatible solutes is physiologically (or energetically) more advantageous for osmotically stressed cells than their biosynthesis.

It should, however, be stressed that there are microorganisms that can use a combination of both the salt-in and the salt-out cytoplasm strategies to cope with high salinity habitats on a sustained basis. One example is the poly-extremophile *Natranaerobius thermophilus*. It uses both high intracellular K^+ concentrations and the accumulation of compatible solutes to balance the osmotic potential of its cytoplasm and employs Na^+/H^+ antiporters to keep the intracellular sodium concentration very low (about 8 mM) in an environment that contains 3–4 M NaCl (Mesbah et al. 2009).

15.4 Properties of Compatible Solutes

Compatible solutes are operationally defined as organic osmolytes that can be amassed by cells to exceedingly high concentrations without interfering with cellular physiology and growth. They are widely employed as osmostress protectants not only by microorganisms but also by plant, animal and even human cells, attesting to the effectiveness of this strategy for coping with high osmolarity in different environmental and cellular settings (Kempf and Bremer 1998; Yancey 2005; Burg and Ferraris 2008). Compatible solutes used by members of the *Bacteria* are usually non-charged highly water soluble compounds and comprise a rather restricted number of organic osmolytes. Important representatives are the sugar trehalose, the heteroside glycosylglycerol, the imino acid proline, the trimethylammonium compound glycine betaine and the tetrahydropyrimidines ectoine and 5-hydroxyectoine (Kempf and Bremer 1998; Bremer and Krämer 2000; Empadinhas and da Costa 2008; Hagemann 2011; Ziegler et al. 2010). *B. subtilis* synthesizes proline as its primary compatible solute (Whatmore et al. 1990), whereas many other Bacilli synthesize ectoines (Kuhlmann and Bremer 2002; Zhao et al. 2006; Bursy et al. 2007; Kuhlmann et al. 2008; Rajan et al. 2008; Saum and Müller 2008a). Bacilli can also use exogenously provided proline, glycine betaine and ectoine as osmoprotectants using various types of osmotically controlled high affinity transport systems for their retrieval (Fig. 15.1) (Bremer and Krämer 2000; Bremer 2002).

A hallmark of compatible solutes is their preferential exclusion from the immediate hydration shell of proteins that is due to unfavorable interactions between the osmolytes and the protein backbone. This uneven distribution of the compatible

solutes in the cell water generates a thermodynamic driving force that promotes the proper folding and conformation of proteins and enhances their stability (Street et al. 2006). Hence, not only do compatible solutes contribute to osmotic balance by serving as water-attracting osmolytes, they also benefit for the cell by helping maintain proper protein function (Bourot et al. 2000; Diamant et al. 2003; Fisher 2006; Ignatova and Gierasch 2006). These solutes are therefore sometimes referred to as chemical chaperones.

In addition to their osmoprotective role, compatible solutes also have notable effects on the growth of microorganisms at the very upper and lower temperature boundaries. For instance, glycine betaine acts both as a thermo-stress and cold-stress protectant for *B. subtilis*, regardless of whether it is synthesized from the precursor choline or taken up from the environment (Brigulla et al. 2003; Holtmann and Bremer 2004; Hoffman and Bremer 2011). The biochemical and biophysical mechanisms that underlie the temperature-stress protective functions of compatible solutes are far from being understood and might not necessarily be connected with their well-studied function as water-attracting osmolytes. However, the combined osmotic-stress and temperature-stress protective properties of compatible solutes can have undesired consequences for human health. It allows food-pathogens such as *Bacillus cereus* and *Bacillus weihenstephanensis* to counteract commonly used food-preservation measures such as salting and refrigeration since plant and animal materials present in foodstuff contain osmoprotectants such as choline, glycine betaine and carnitine. The same compatible solute transporters (Opu) are used under osmotic and temperature stress conditions by *B. subtilis* to acquire these compounds from environmental sources (see below).

15.5 *B. subtilis*: A Genetically Tractable Model System to Study Cellular Adjustment to High Salinity

Although *B. subtilis* is certainly not a salt-loving bacterium, it can grow, albeit slowly, in minimal and rich media containing a substantial amount of salt (e.g., 1.2 M NaCl) (Boch et al. 1994). The development of such a considerable degree of salt stress resistance probably stems from the exposure of *B. subtilis* cells to prolonged periods of desiccation in natural habitats; e.g., the well-oxygenated upper layers of the soil. *B. subtilis* does not respond to high salinity conditions by initiating the sporulation process (Ruzal et al. 1998) that under nutrient-limited growth conditions leads to the formation of highly desiccation resistant endospores. Hence, *B. subtilis* needs to engage in defense mechanisms that allow the vegetative cell to confront high salinity environments effectively.

One facet in the osmostress response system of *B. subtilis* and other Bacilli is the salt-induction of the SigB-controlled general stress regulon (Hecker et al. 2007). However, induction of the SigB-regulon of *B. subtilis* by a salt-shock is only transient and a *sigB*-mutant grows like a wild-type strain in minimal medium

with 1.2 M NaCl (Spiegelhalter and Bremer 1998). Therefore, the general stress regulon is certainly not the central osmotic stress response system for vegetative *B. subtilis* cells, although the disruption of the structural gene for *sigB* itself and of many members of the SigB regulon frequently causes a salt-sensitive phenotype (Höper et al. 2005).

Proteome and genome-wide transcriptional profiling studies of high salinity-challenged cells have highlighted the complexity and multifaceted nature of the osmotic stress response systems of *B. subtilis* (Steil et al. 2003; Höper et al. 2006; Hahne et al. 2010). However, detailed physiological and molecular studies have shown beyond doubt that the effective water-management is the cornerstone of the cell's acclimatization to either sudden or sustained rises in the environmental osmolarity and salinity (Bremer and Krämer 2000; Bremer 2002). The accumulation of compatible solutes, either via synthesis or uptake, plays a key role in this process. This is evident by the salt-sensitive growth phenotypes of *B. subtilis* mutants that cannot synthesize the compatible solute proline (Brill J. and Bremer E.; unpublished results) or by the strongly impaired ability of mutants with defects in compatible solute uptake system, to grow efficiently in high-salt minimal media (Kappes et al. 1996, 1999).

As in other bacteria, the cellular reaction of *B. subtilis* to a sudden rise in the external osmolarity is multiphasic (Kempf and Bremer 1998; Bremer and Krämer 2000; Wood et al. 2001; Booth et al. 2007). A rapid rise in the cellular K^+ content through transport processes is typically the initial cellular response that follows the loss of cell water subsequent to an osmotic up-shift (Whatmore et al. 1990). In Gram-negative bacteria, glutamate is used as the counter-ion for K^+ to maintain electro-neutrality. However, the nature of the counter-ion used by *B. subtilis* to balance the positive electric charge of the accumulated K^+ ion has not been rigorously established. The size of the glutamate-pool increases only moderately, whereas the K^+ content of osmotically challenged cells increases strongly from a basal level of about 350 mM to about 650 mM within 1 h subsequent to the osmotic up-shock (Whatmore et al. 1990). The Ktr-type potassium uptake systems KtrAB and KtrCD play a central role for K^+ uptake, both in osmotically non-stressed and in osmotically stressed *B. subtilis* cells (Holtmann et al. 2003). This type of transport system consists of a dimer of the K^+-translocating membrane-embedded subunit (KtrB), probably evolutionarily derived from a subunit of an ancestral K^+ channel, and a soluble regulatory subunit that can bind both ATP and NAD^+/NADH via its RCK/KTN domain; an octameric ring structure has been proposed for the RCK/KTN domain-containing KtrA subunit (Albright et al. 2006). Ktr-systems are Na^+-coupled K^+ uptake systems (Corratge-Faillie et al. 2010) and hence, the massive import of K^+ that follows an osmotic up-shift (Whatmore et al. 1990) will simultaneously lead to a large-scale import of cytotoxic Na^+ ions into the *B. subtilis* cell.

Concomitant with the rise in the K^+ pool in osmotically up-shocked cells, *B. subtilis* starts to synthesize the compatible solute proline on a very large scale (Whatmore et al. 1990). This leads eventually to a reduction in the K^+ pool, and hence, of the ionic strength of the cytoplasm. Such a reduction in K^+ content can also be detected when the osmotically stressed cells are allowed to take up glycine betaine

from exogenous sources (Whatmore et al. 1990). It is obvious that K^+ extrusion systems must function in the second acclimatization stage of *B. subtilis* to high-salinity surroundings, a time when primarily compatible solutes are amassed to raise the osmotic potential of the cytoplasm (Fig. 15.1). Indeed, one such K^+ export system (YhaTU) has already been identified and characterized biochemically (Fujisawa et al. 2007). The expression of the structural genes for the YhaTU system is strongly induced in response to an osmotic up-shock (Hahne et al. 2010). Hence, during the initial acclimatization reactions of *B. subtilis* to a sudden rise in the external osmolarity, fluxes of K^+ ions in and out of the cell apparently play an important role. This must be certainly also true for the Na^+ ions that enter the cell in co-transport with K^+ via the Ktr transporters (Corratge-Faillie et al. 2010) and that need to be effectively exported from the cell due to the cytotoxicity of Na^+ ions. Na^+ ions will also enter the *B. subtilis* cell as a by-product of the import of compatible solutes through secondary transport systems. For instance, the BCCT-type glycine betaine uptake system OpuD and the SSSF-type proline uptake system OpuE (see below) of *B. subtilis* are Na^+-coupled transporters (von Blohn et al. 1997; Kappes et al. 1996; Ziegler et al. 2010). Interestingly, the expression of the operon encoding the multi-subunit Mrp Na^+ extrusion system and the genes that encoding the single-component NhaK and NhaC Na^+ exporter are all up-regulated in salt-shocked *B. subtilis* cells (Hahne et al. 2010). However, *B. subtilis* cannot rely exclusively on the cycling of ions in and out of the cell to adjust to truly high salinity environments. This is deducible from the phenotype of a mutant strain (*proHJ*) that cannot synthesize the compatible solute proline. Such a strain exhibits a salt-sensitive growth defect in a minimal medium containing 1.2 M NaCl; however, this phenotype can be effectively rescued by the addition of a compatible solute (e.g., 1 mM glycine betaine) to the growth medium (J. Brill and E. Bremer; unpublished results).

15.5.1 Synthesis and Uptake of Compatible Solutes by **B. subtilis**

The above reported findings highlight the importance of compatible solute accumulation for the sustained salt-stress response of *B. subtilis*. *B. subtilis* can synthesize the compatible solute proline *de novo* from its considerable glutamate pool (Whatmore et al. 1990), and it can synthesize glycine betaine by first importing the precursor choline and then oxidizing it to glycine betaine (Boch et al. 1994, 1996). Furthermore, *B. subtilis* can take up a large number of pre-formed compatible solutes from environmental sources as effective osmo-stress protectants (Bremer 2002).

15.5.1.1 Synthesis of Proline

Since the pioneering studies of Measures in 1975, it has been known that *B. subtilis* synthesizes large quantities of the compatible solute proline when it is subjected to

high salinity environments (Measures 1975). Subsequent detailed physiological studies conducted by Reed and co-workers (Whatmore et al. 1990) revealed that the cellular proline pool build-up by de novo synthesis rises from a basal level of 16 mM to about 500–700 mM within 7 h of growth subsequent to a moderate osmotic up-shift with 0.4 M NaCl. Since *B. subtilis* can grow in a minimal medium with 1.2 M NaCl (Boch et al. 1994), one can expect that these severely osmotically stressed cells will amass proline in excess of 1 M through de novo synthesis. We have elucidated the genetic basis for the production of the vast quantities of proline produced by *B. subtilis* as an osmostress protectant and found that the anabolic proline biosynthetic route and that of the osmoadaptive route are interconnected via the γ-glutamyl phosphate reductase (ProA) (Brill J. and Bremer E.; unpublished results).

In many microorganisms, proline biosynthesis proceeds from the precursor glutamate and involves three enzyme-catalyzed steps. It begins with the ATP-dependent phosphorylation of glutamate by the γ-glutamyl kinase (ProB). The resulting γ-glutamyl phosphate is then reduced to γ-glutamic semialdehyde by the γ-glutamyl phosphate reductase (ProA). The formed γ-glutamic semialdehyde spontaneously cyclizes to Δ^1-pyrroline-5-carboxylate, which is then further reduced by the Δ^1-pyrroline-5-carboxylate reductase (ProC) to the end product proline. This pathway for the production of proline as a building block for protein biosynthesis is also present in *B. subtilis* (Belitsky et al. 2001) and comprises the ProB-ProA-ProI enzymes (several ProC-type enzymes are present; hence, the designation ProI) (Belitsky et al. 2001). The anabolic proline biosynthesis route from the precursor glutamate is frequently regulated in microorganisms though allosteric feedback inhibition of the biochemical activity of first proline-biosynthetic enzyme (ProB) by the end product proline. This is also the case in *B. subtilis* (Chen et al. 2007). Furthermore, the expression of the anabolic *proBA* and *proI* genes is genetically controlled via a proline-responsive T-box system, a regulatory device (Green et al. 2010) that allows the induction of the *proBA* and *proI* genes only when the *B. subtilis* cells starve for proline (Brill et al. 2011). Hence, the anabolic ProB-ProA-ProI proline biosynthetic route in *B. subtilis* is genetically and biochemically wired in such a fashion that a wasteful overproduction of proline is strictly prevented when it is synthesized as a building block for protein biosynthesis. The content of free proline in the cytoplasm of osmotically non-stressed *B. subtilis* cells is about 16 mM (Whatmore et al. 1990). It is immediately apparent that the above described biochemical and genetic control mechanisms make the anabolic ProB-ProA-ProI proline biosynthetic route unsuitable to provide the *B. subtilis* cell with the very large amounts (0.5–1 M) of proline it needs as an osmostress protectant (Bremer 2002).

A second proline biosynthetic route formed by the ProJ-ProA-ProH enzymes, accomplishes this task (Brill J. and Bremer E.; unpublished results). ProJ and ProH are iso-enzymes of ProB and ProI, respectively, and catalyze the first and last steps of the osmo-adaptive proline biosynthetic route. The implication of this finding is that the activity of ProJ enzyme, in contrast to that of ProB, is not (or at least not very strongly) subjected to feedback inhibition by proline, although this prediction

still needs to be confirmed biochemically. The transcription of the *proHJ* structural genes is strongly induced both subsequent to an osmotic up-shock and during continued growth of the *B. subtilis* cells in high salinity media (Steil et al. 2003; Hahne et al. 2010). As a matter of fact, the *proHJ* operon has the highest induction ratio of all of the approximately 100 high-salinity-induced genes of *B. subtilis* cells cultivated under sustained high osmolarity conditions (Steil et al. 2003). Attesting to the central role of the ProJ and ProH enzymes for the adjustment of the cell to high salinity habitats is the finding that the disruption of the *proHJ* operon causes osmotic sensitivity (Brill J. and Bremer E.; unpublished data).

Since no paralogous enzyme for the γ-glutamyl phosphate reductase (ProA) is present in *B. subtilis*, the anabolic (ProB-ProA-ProI) and the osmostress adaptive (ProJ-ProA-ProH) proline biosynthetic routes are interconnected. It is not immediately obvious why this interconnection has occurred in the course of evolution since the *B. subtilis* cell has to rely under osmotic stress conditions on the basal level of the T-box-controlled expression of the *proBA* operon for the supply of the ProA enzyme. Such an interconnection of the anabolic and osmoadaptive proline biosynthetic routes does not always occur in those Bacilli that produce proline as a compatible solute. For instance, in the industrial workhorse *Bacillus licheniformis*, two complete proline biosynthetic routes are present whose regulation is precisely tailed to two different tasks: proline production for protein biosynthesis and proline production for osmostress protection. As in *B. subtilis*, the expression of the genes (*proBA* and *proI*) for the anabolic route (ProB-ProA-ProI) is controlled via T-box systems (Bleisteiner M., Putzer H. and Bremer E.; unpublished results) and consequently these genes are only strongly induced under proline-starvation conditions. However, in contrast to *B. subtilis*, a full set of osmoadaptive proline biosynthetic enzymes (ProJ-ProAA-ProH) is present in *B. licheniformis*. The expression of the corresponding gene cluster (*proH-proJ-proAA*) is strongly induced at high salinity (M. Bleisteiner, T. Hoffmann, and E. Bremer; unpublished results). Likewise, in the proline-producing moderate halophile *Bacillus halophilus*, an osmotically inducible gene cluster (*proJ-proH-proA*) is present that encodes an entire set of proline biosynthetic enzymes (Saum and Müller 2008b). Interestingly, the solute glutamate serves as a messenger to turn on the expression of *proJ-proH-proA* gene cluster and thereby switch the osmolytes strategy of *B. halophilus* from glutamate production to proline production during the transition from moderate to high salinity environments (Saum and Müller 2008b).

15.5.1.2 Synthesis of Glycine Betaine

Microorganisms can synthesize glycine betaine by two different routes: (a) through a step-wise methylation of the amino acid glycine or (b) through the oxidation of choline using a variety of enzymes. The de novo synthesis of glycine betaine from glycine is catalyzed by several methylases with overlapping substrate specificities that use *S*-adenosylmethionine (AdoMet) as the methyl donor. This is an energetically very expensive way to produce glycine betaine since the reduction of a single

methyl group and its activation via AdoMet requires 12 ATP equivalents (Nyyssölä et al. 2000). Consequently, the de novo synthesis pathway for glycine betaine production is not prevalent in the microbial world. Instead, most microorganisms that are capable of glycine betaine synthesis produce it via oxidation of choline that is acquired through transport processes from external sources. This is also the way though which *B. subtilis* synthesizes glycine betaine as an osmostress protectant (Boch et al. 1994; Kappes et al. 1999). Choline is a component of the lipids of plant cells and it can be liberated from phosphatidylcholine by the action of lipases. Hence, the precursor for glycine betaine is introduced, albeit at low and variable concentrations, into the soil habitat of *B. subtilis*. Choline is taken up by *B. subtilis* via two osmotically inducible ABC-type importers: the OpuC and OpuB systems (Kappes et al. 1999). Both transporters exhibit high-affinity for choline with K_m values in the low μM range, but only OpuB is highly substrate specific; in contrast, the OpuC possesses a broad substrate specificity for compatible solutes (Bremer 2002). Once imported via OpuB and OpuC, choline is oxidized by the type III alcohol dehydrogenase (GbsB) to glycine betaine aldehyde, and this chemically highly reactive and toxic intermediate is then further oxidized to glycine betaine via the glycine betaine aldehyde dehydrogenase (GbsA) (Boch et al. 1996). In this way, *B. subtilis* can achieve a considerable degree of osmotic stress tolerance, but the synthesis of glycine betaine from choline is somewhat less effective in terms of stress protection than the uptake of pre-formed glycine betaine (Boch et al. 1994). It should be noted in this context, that choline has no osmoprotective properties per se in *B. subtilis*.

15.5.1.3 Uptake of Compatible Solutes

The cellular content of compatible solutes is sensitively determined by the degree of the osmotic stress perceived by the microbial cell (Bremer and Krämer 2000; Ziegler et al. 2010). These compounds can reach molar concentrations in severely stressed cells. Sources of compatible solutes in natural settings are osmotically down-shocked or decaying microbial and eukaryotic cells and excretion products of animals and plants (Welsh 2000). Since these compounds are typically found in very low concentrations (nM or μM) in the environment, microbial cells must possess effective transport systems to scavenge them (Bremer and Krämer 2000; Wood et al. 2001; Ziegler et al. 2010). *B. subtilis* possesses five osmotically inducible transport systems for compatible solutes (Bremer 2002). We have christened these transporters Opu: *o*smo*p*rotectant *u*ptake (Kempf and Bremer 1995; Kappes et al. 1996, 1999; von Blohn et al. 1997). These systems have homologues in many microbial species and our genetic, physiological and structural characterizations of these transporters have served as a blueprint for the functional annotation of compatible solute uptake systems in many microbial genome projects and in databases.

Glycine betaine is a particularly important osmostress protectant for *B. subtilis*. Not only can *B. subtilis* synthesize it from an exogenous supply of the precursor

choline (see above), but it also can take it up via three osmotically inducible transport systems: the ABC transporters OpuA and OpuC and the BCCT-type carrier OpuD (Kempf and Bremer 1995; Kappes et al. 1996, 1999). Each of these systems exhibits a high affinity for its substrate with K_m values in the low μM range, and each of them possess considerable transport capacity. Furthermore, each of these transporters is sufficient to mediate effective osmostress protection for *B. subtilis* cells in the presence of glycine betaine but the OpuA transporter is the dominant uptake system due to its high V_{max} (Kappes et al. 1996). The OpuA system also has a considerable transport activity for glycine betaine in cells that are not osmotically stressed, resulting in a substantial cellular glycine betaine pool of 130–170 mM when *B. subtilis* is grown in standard laboratory minimal media (Whatmore et al. 1990; Holtmann and Bremer 2004). Considerable glycine betaine pools are also expected to be present in *B. subtilis* cells grown in rich media containing yeast extract (e.g., LB medium), since yeast extract is a rich source for glycine betaine (Dulaney et al. 1968) and LB-medium is routinely prepared with a considerable amount of NaCl (5 g/l).

The OpuA, OpuC, and OpuD transporters from *B. subtilis* not only function in the uptake of glycine betaine but also for the transport of other compatible solutes, most of which are structurally related to glycine betaine (Bremer 2002). Some of these compatible solutes are substrates for more than one of these uptake systems, whereas others are taken up only through a single Opu transporter. Examples for this latter type of uptake profile are the osmoprotectants L-carnitine, crotonobetaine, γ-aminobutyrobetaine, choline-*O*-sulfate, and ectoine which are all exclusively acquired by the osmotically stressed *B. subtilis* cell via the OpuC ABC transporter (Jebbar et al. 1997; Kappes and Bremer 1998; Nau-Wagner et al. 1999). The OpuC system is noteworthy because it can transport with high affinity (K_m values in the low μM range) in total 11 osmoprotectants that are all structurally related to glycine betaine, and with very low-affinity ectoine (K_i of approximately 1.6 mM), a compatible solute structurally unrelated to glycine betaine (Jebbar et al. 1997).

It should be noted, that from the 12 currently known osmoprotectants acquired by *B. subtilis* through transport processes, 11 are metabolically inert (Bremer 2002). Hence, they are amassed for a sole purpose: stress protection! The only exception is the amino acid proline that can be efficiently used both as a sole carbon and nitrogen source (S. Moses and E. Bremer; unpublished results).

15.5.1.4 Structural Analysis of the Glycine Betaine-Binding Protein OpuAC

High-affinity interactions between compatible solutes and transport proteins must take place in order to achieve effective import. However, as mentioned above, the preferential exclusion from protein surfaces is a hallmark of these types of solutes (Street et al. 2006). Since transport systems for compatible solutes have been

detected in practically every microorganism studied in the context of adaptation to high osmolarity environments, a fundamental problem arises: how can a compound be bound by a protein with high affinity and specificity when this compound is typically excluded from the immediate hydration shell of the very same protein? What are the structural determinants for the selective binding of compatible solutes by components of transport systems?

The answer, at least for the sub-group of compatible solutes chemically related to glycine betaine, has recently come through crystallographic and mutational studies of soluble ligand-binding proteins from several microbial ABC transporters. One of the studied proteins is OpuAC, the ligand-binding protein of the OpuA transporter from *B. subtilis* (Kempf and Bremer 1995; Horn et al. 2006). This protein is tethered via a lipid anchor attached to an N-terminal Cys-residue on the outer surface of the cytoplasmic membrane of *B. subtilis* so that it can capture exogenously provided glycine betaine with high affinity (K_D of about 20 μM). The crystal structure of the OpuAC protein in complex with glycine betaine revealed a bilobal organization of the protein (Fig. 15.2; left panel), a topology that is typical for ligand-binding proteins of ABC transporters. The two lobes of the OpuAC protein are connected through a flexible hinge region that allows a rigid-body movement of the lobes between the open non-liganded form of the protein and a closed liganded form; the ligand shifts the equilibrium towards the substrate-loaded conformation. As a consequence of this movement, the ligand glycine betaine is captured and buried deep in a cleft formed by the two domains of the OpuAC protein (Fig. 15.2; left panel) (Horn et al. 2006). The open and closed crystal structures of the OpuAC protein from *Lactococcus lactis* have recently been

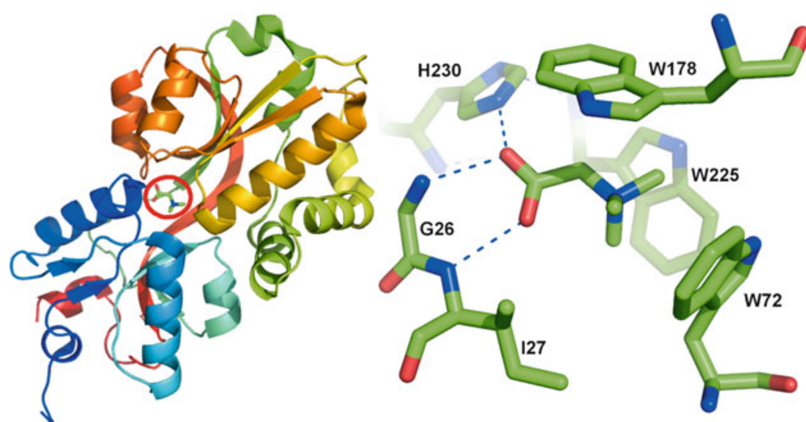

Fig. 15.2 Crystal structure of the glycine betaine binding protein OpuAC from *B. subtilis*. (*Left panel*) Overall structure of the OpuAC protein in complex with its ligand glycine betaine; the ligand is circled in *red*. (*Right panel*) Architecture of the glycine betaine-binding site present in OpuAC. The pictures were prepared with PyMol (http://www.pymol.org/) using the atomic coordinates deposited in the PDB file 2B4L (http://www.pdb.org/) (Horn et al. 2006)

reported and can thus be used to trace the movements of the two domains toward each other and the capturing of the glycine betaine ligand (Wolters et al. 2010).

The crystallographic analysis of the *B. subtilis* OpuAC protein revealed a remarkably structured ligand-binding site that consists of three Trp residues (the aromatic cage) into which the positively charged trimethylammonium headgroup of glycine betaine is wedged and is coordinated via cation–pi interactions (Fig. 15.2; right panel). The carboxylic group of the glycine betaine ligand protrudes out of the aromatic cage and forms directed hydrogen bonds with either the backbone or side chains of specific amino acid residues (Horn et al. 2006). Mutational analysis established the key contributions of cation–pi interactions for the effective binding of the glycine betaine ligand by OpuAC within the aromatic cage (Fig. 15.2; right panel) (Smits et al. 2008). The OpuAC protein from *B. subtilis* can also bind the compatibe solutes proline betaine and dimethylsulfonioacetate (DMSA), a sulfur analog of glycine betaine, with reasonable affinities (Horn et al. 2006; Smits et al. 2008).

Crystallographic studies of the glycine betaine-binding proteins from *E. coli* (ProX), the hyperthermophilic archaeon *Archaeoglobus fulgidus* (ProX), the OpuAC proteins from *L. lactis*, and the choline/acetylcholine-binding protein ChoX from the root-associated soil bacterium *Sinorhizobium meliloti* all revealed similarly structured ligand-binding sites. Although the precise architecture of the aromatic cage in the ligand-binding sites varies between these proteins, common denominators for substrate binding have emerged. In each substrate-binding protein, the bulky and positively charged head groups of glycine betaine, choline, acetylcholine, proline betaine, and DMSA are similarly accommodated within the aromatic ligand-binding site via cation–pi interactions. The tails of the various substrates protrude from the aromatic cage and are coordinated through H-bonds, salt-bridges, and water-networks by residues stemming from the two domains of the ligand-binding proteins. The ProX proteins from *E. coli* and *A. fulgidus*, the OpuAC proteins from *B. subtilis* and *L. lactis*, and the ChoX protein from *S. meliloti* have homologues in many microbial species, and in almost all of these sequence-related proteins those residues forming the aromatic cage are conserved.

The aromatic cages discussed so far are all present in soluble ligand-binding proteins of ABC transport systems. What about the ligand-binding sites in membrane-embedded carriers for compatible solutes? The crystal structure of the glycine betaine transporter BetP from *C. glutamicum*, a member of the ubiquitously found BCCT carriers (Ziegler et al. 2010), revealed a glycine betaine-binding site (Ressl et al. 2009) that is virtually super-imposable onto that present in the ProX protein from *E. coli* (Schiefner et al. 2004). Since the soluble periplasmic binding protein ProX and the integral membrane protein BetP are certainly not closely evolutionarily related, nature has apparently adopted common design principles to construct a high-affinity ligand-binding site for a solute (glycine betaine) that typically is preferentially excluded from the surface of proteins.

15.6 Osmotically Controlled Synthesis of Compatible Solutes by Bacilli Other Than *B. subtilis*

Having focused on the synthesis of proline in *B. subtilis*, we wondered whether most other Bacilli would also produce proline as their primary compatible solute. We therefore embarked on a study that evaluated the synthesis of compatible solutes of a large group of Bacilli (26 species altogether) via natural abundance ^{13}C-NMR spectroscopy (Kuhlmann and Bremer 2002; Bursy et al. 2007). Inspection of the recorded data revealed that the studied 26 Bacilli could be grouped into three main classes (a) those that used only L-glutamate as their primary compatible solute [6 species], (b) those that produced proline in response to osmotic stress [5 species], and (c) those that synthesized ectoine [15 species] when they were osmotically challenged. This later class can be further sub-grouped into microorganisms that produce ectoine alone, or in combination with hydroxyectoine, or in combination with proline. Our studies therefore revealed that the most widely synthesized compatible solute within the studied Bacilli is the tetrahydropyrimidine ectoine (Fig. 15.3). The ectoine biosynthetic gene cluster has been studied at the molecular level in several Bacilli (Kuhlmann and Bremer 2002; Zhao et al. 2006; Bursy et al. 2007; Kuhlmann et al. 2008; Rajan et al. 2008; Saum and Müller 2008a).

Although this was not systematically studied by us, those species that rely on L-glutamate production alone tend to be rather salt-sensitive (e.g., *B. cereus*), those that synthesize proline exhibit an intermediate osmotic stress resistance (e.g., *B. subtilis* and *Bacillus licheniformis*), and those species that produce ectoines (e.g., *Virgibacillus salexigens*) or a combination of ectoine and proline (e.g., *Halobacillus halophilus*) typically are rather salt tolerant. With the exception of *Paenibacillus polymyxa*, the sugar trehalose was not detected in the compatible solute pools of the studied Bacilli; furthermore, trehalose was only produced in stationary-phase cultures of *P. polymyxa*, indicating that it might be produced as a stress protectant for purposes other than osmotic balance. Hence, our findings indicate that Bacilli do not usually produce trehalose as an osmostress protectant, whereas this non-reducing sugar is the dominant compatible solute synthesized by *E. coli* (Wood et al. 2001).

Inspection of the spectrum of compatible solutes synthesized by the Bacilli studied by us experimentally and of additional data compiled from the literature or derived from genome sequencing projects revealed that the type of compatible solute(s) produced does not always closely follows phylogenetic constraints (Fig. 15.3). For instance, *B. licheniformis*, *B. subtilis*, *Bacillus amyloliquefaciens*, *Bacillus vallismortis* and *Bacillus mojavensis* are phylogenetically closely related and all synthesize proline as a compatible solute. However, *B. mojavensis* is capable to synthesize ectoine as well (Fig. 15.3). The type(s) of compatible solute produced is therefore not a very informative taxonomic marker and the inspection of the data compiled by us in Fig. 15.3 suggest that lateral gene transfer events might have significantly shaped the compatible solute profile of Bacilli. This seems

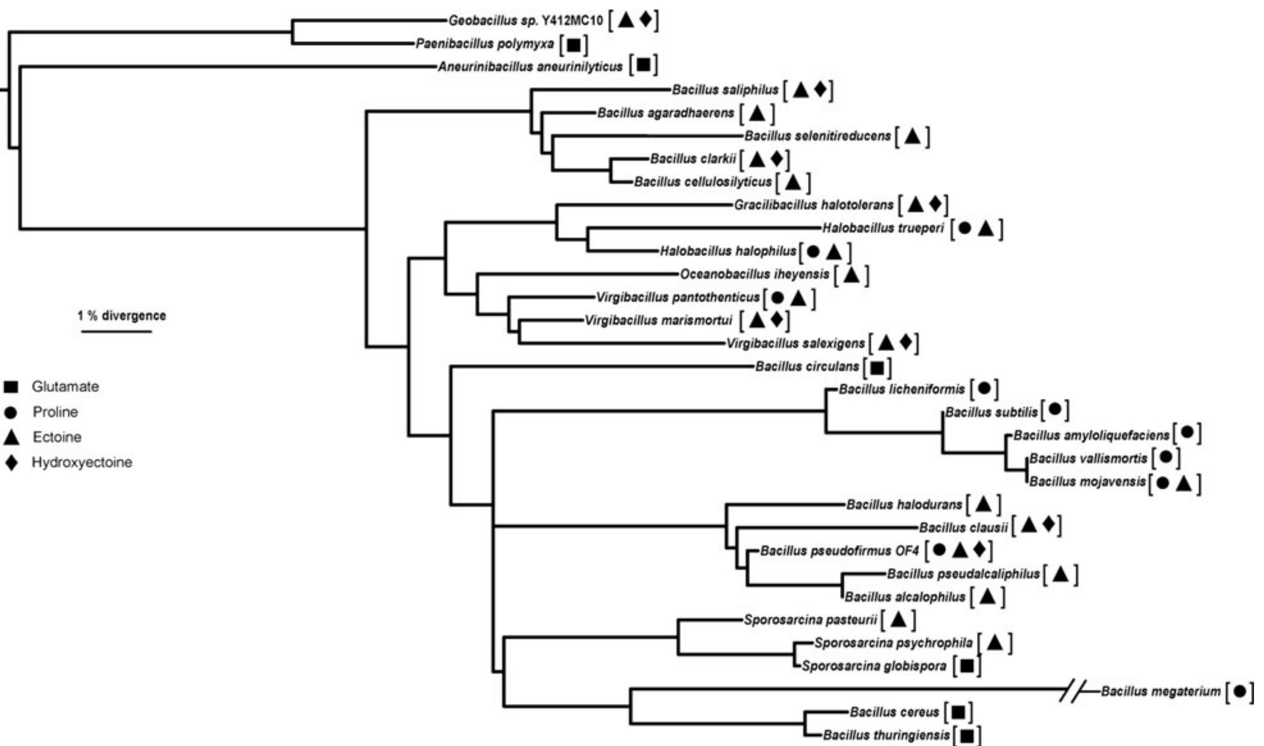

Fig. 15.3 Pattern of the synthesis of the compatible solutes glutamate, proline, ectoine, and hydroxyectoine in various Bacilli. The data on the synthesis of compatible solutes of the 32 listed Bacilli were compiled either from reports in the literature (Kuhlmann and Bremer 2002; Kuhlmann et al. 2008; Bursy et al. 2007; Saum and Müller 2008a; Romano et al. 2005; Rajan et al. 2008) or gleaned from the genome sequences of *Geobacillus* sp. Y412MC10, *Bacillus clausii* KSM K16, *Oceanobacillus iheyensis*, *Bacillus cellulosilyticus* and *Bacillus selenitireducens*. The phylogenetic tree was constructed using the ARB software package and is derived from a distance matrix of 16S rRNA sequences using the neighbor-joining method

in particular to be the case when the pattern of ectoine and hydroxyectoine production is considered (Fig. 15.3). One should take note that none of the 26 Bacilli studied by us for the production of compatible solutes with ^{13}C-NMR-spectroscopy actually synthesized glycine betaine de novo (Kuhlmann and Bremer 2002; Bursy et al. 2007). Romano et al. (2005) evaluated the compatible solute pool of the highly salt tolerant haloalkaliphic *Bacillus saliphilus* and reported that this *Bacillus* species accumulates glycine betaine as its dominant osmoprotectant (81% of the compatible solute pool) and only minor amounts of ectoine (12%) and hydroxyectoine (3%) (Romano et al. 2005). This finding can in all likelihood be explained by the fact that the cells were grown in a minimal medium that contained yeast extract, a well-known rich source of glycine betaine (Dulaney et al. 1968). We therefore stress the need to use truly chemically defined media when the spectrum of compatible solutes produced by a given microorganism is evaluated to clearly distinguish de novo synthesized compatible solutes from those that are imported. In this way, one also can avoid the pitfall that exogenously provided compatible solutes suppress the expression of compatible solute biosynthetic genes and thereby skew the profile of the composition of the cellular compatible solute pool.

15.6.1 Synthesis of Ectoine and Hydroxyectoine

Although our evaluation of the synthesis of compatible solutes by Bacilli (Kuhlmann and Bremer 2002; Bursy et al. 2007) is certainly not representative for all members of this very large order, it is apparent from our study of 26 type strains that ectoine and its derivative 5-hydroxyectoine are widely synthesized by Bacilli as osmostress protectants. Ectoine and 5-hydroxyectoine were initially regarded as rather uncommon compatibe solutes when they first were discovered in *Ectothiorhodospira halochloris* and *Streptomyces parvulus*, respectively. However, improved ^{13}C-NMR- and HPLC-based screening procedures and finally the discovery of the ectoine/hydroxyectoine biosynthetic genes (Louis and Galinski 1997) revealed their widespread occurrence in the microbial world. Our recent database searches of finished genome sequences of *Bacteria* (1,391 genome sequences) and Archaea (80 genome sequences) identified 241 putative ectoine producers (based on searches with the amino acid sequence of the ectoine synthase EctC, a search criterion that might slightly overestimate the number of actual ectoine producers). With the single exception of the archaeon *Nitrosopumilus maritimus* SCM1, all these putative ectoine producers are members of the *Bacteria*. There are currently no *Eukarya* that are known to synthesize ectoines naturally.

Three enzymes mediate ectoine biosynthesis from L-aspartate-β-semialdehyde, a central intermediate in amino acid metabolism, through the sequential enzymatic reactions of the L-2,4-diaminobutyrate transaminase (EctB), the L-2,4, diaminobutyrate acetyltransferase (EctA), and the ectoine synthase (EctC), an enzyme of the cupin protein family that mediates the ring-closure of the ectoine biosynthetic intermediate N-acetyl-L-2,4-diaminobutyrate. 5-hydroxyectoine is formed from

ectoine in a subset of the ectoine producers though the catalytic activity of the ectoine hydroxylase (EctD). In all ectoine- and hydroxyectoine-producing bacteria analyzed so far, biosynthesis of these compatible solutes is strongly enhanced under high osmolarity growth conditions. This is largely due to the osmotic induction of the expression of the ectoine biosynthetic genes, although stimulation of the activity of the ectoine biosynthetic enzymes by high salinity or high ionic strength might also contribute to ectoine production in osmotically stressed cells. The genetic disruption of the ectoine biosynthetic gene cluster typically causes an osmosensitive growth phenotype.

The genes (*ectABC*) for the ectoine biosynthetic enzymes are typically organized as an operon (Louis and Galinski 1997; Kuhlmann and Bremer 2002; Bursy et al. 2007; Kuhlmann et al. 2008; Schwibbert et al. 2010) that also can contain in the hydroxyectoine producers the gene (*ectD*) for the ectoine hydroxylase (Prabhu et al. 2004). However, the *ectD* gene is not always part of the *ectABC* gene cluster and can be frequently found at a separate position on the genome (Garcia-Estepa et al. 2006; Bursy et al. 2007). Some of the *ectABC* or *ectABCD* gene clusters also contain a gene for an aspartokinase (*ask_ect*) (Reshetnikov et al. 2006), a genetic configuration that might serve to boost the production of the ectoine precursor L-aspartate-β-semialdehyde under osmotic stress conditions. The aspartokinase (Ask) enzyme is often subjected to complex feed-back regulatory mechanisms which might generate a bottle-neck for an adequate supply of L-aspartate-β-semialdehyde to meet the considerable demands of this precursor for ectoine biosynthesis (Bestvater et al. 2008). Hence, the Ask encoded by the *ask_ect* gene might encode an enzyme with special biochemical properties to aid ectoine production under osmotic stress conditions.

Transcription of the *ect* gene cluster is typically strongly up-regulated in cells challenged by high salinity. Osmotically controlled promoters for *ect* gene clusters have been identified in several microorganisms (Kuhlmann and Bremer 2002; Calderon et al. 2004; Bursy et al. 2007; Kuhlmann et al. 2008; Schwibbert et al. 2010). It is worth noting that a subgroup of ectoine producers possesses a regulatory protein, termed EctR, which acts as a repressor of the ectoine biosynthetic genes. This regulatory protein was first discovered in the halotolerant methanotroph *Methylomicrobium acidiphilum* 20Z and is a member of the MarR family of transcriptional regulators (Mustakhimov et al. 2010). EctR contains a predicted winged helix-turn-helix motive for DNA binding, and DNA footprinting analysis has shown that EctR binds to a region overlapping the promoter for the *ect* gene cluster. Disruption of the *ectR* gene in *M. acidiphilum* 20Z substantially de-represses the transcriptional activity of the promoter for the ectoine biosynthetic genes, but this promoter remained osmotically controlled in the *ectR* mutant (Mustakhimov et al. 2010). Hence, the EctR regulator functions as a repressor, but it is apparently not a sensor for high salinity or high ionic strength of the cytoplasm. Its potential effector ligand (or covalent modification) that would trigger the displacement of EctR from its operator sequence at the *ect* promoter remains to be discovered.

There are many speculations in the literature on the genetic regulation of the ectoine biosynthetic genes in response to osmotic stress. However, in no case has the signal transduction cascade been worked out experimentally at a level that would give a firm understanding of those molecular mechanisms that enable the cell to detect increases in the environmental osmolarity. Furthermore, it is unclear how such an osmotic stimulus might be processed and how this physical signal might be converted into a genetic signal that would allow the cell to set the activity of the promoter(s) for ectoine biosynthetic gene clusters. It is worth noting that signals other than increases in the environmental osmolarity can contribute to the level of *ect* expression (Calderon et al. 2004). For instance, in *V. pantothenticus* expression of the *ectABC* genes can be triggered not only by high salinity but also by decreasing growth temperature, suggesting that the observed ectoine production in the cold-stressed cells serves protective function against chill-stress (Kuhlmann et al. 2008).

15.6.2 The Ectoine Hydroxylase EctD: Biochemistry and Structural Analysis

Despite the fact that ectoine and hydroxyectoine are chemically very closely related, the properties and physiological functions of these solutes differ. This is evident from differences in vitro assays addressing the protein stabilization function of both ectoines and from physiological studies with *Chromohalobacter salexigens*, where the EctD-mediated formation of hydroxyectoine was found to be critical for the development of full heat stress resistance (Garcia-Estepa et al. 2006). Likewise, exogenously provided ectoine and hydroxyectoine confer heat stress protection to *Streptomyces coelicolor*, with a mixture of ectoine and hydroxyectoine being the superior thermoprotectant (Bursy et al. 2008). One therefore wonders why not all ectoine-producing bacteria also synthesize hydroxyectoine. But this is clearly not the case: our genome-based database searches indicate that from the 241 potential ectoine producers, only 93 species are also potential hydroxyectoine producers as judged by the presence of the structural gene for the ectoine hydroxylase (EctD). Again, *N. maritimus* SCM1 is the only archaeon that is a potential hydroxyectoine producer, indicating that this marine microorganism has acquired the *ectABCD* gene cluster via a lateral gene transfer event from a member of the *Bacteria* that shares its aquatic habitat.

The structural gene (*ectD*) for the ectoine hydroxylase was discovered through genetic and physiological approaches in *Streptomyces chrysomallus*, *Chromohalobacter salexigens* and *Virgibacillus* (*Salibacillus*) *salexigens* (Prabhu et al. 2004; Garcia-Estepa et al. 2006; Bursy et al. 2007). Biochemical studies with the purified EctD protein from *V. salexigens* and *S. coelicolor* revealed that the ectoine hydroxylase is a member of the non-heme iron(II)-containing and 2-oxoglutarate-dependent dioxygenases (Bursy et al. 2007, 2008).

Recently, the high-resolution crystal structure of the EctD protein from *V. salexigens* in complex with a Fe^{3+} ligand was solved (Reuter et al. 2010). It has not yet been possible to obtain an EctD crystal structure containing the substrate ectoine, or the co-substrate 2-oxoglutarate or the reaction product, 5-hydroxyectoine. The EctD protein has a β-barrel fold commonly found in cupin-type proteins ("*cupa*" is the Latin form for small barrel). As typically observed for members of the non-heme iron(II)-containing and 2-oxoglutarate-dependent dioxygenase super-family, EctD consists of a double-stranded β-helix core decorated with and stabilized by a number of α-helices (Fig. 15.4; left panel). The enzymatic function of the dioxygenases depends on a highly reactive iron species. The iron ligand (Fe^{2+}) is most-often coordinated by side chains of three amino acid residues, the so-called 2-His-1-carboxylate facial triad. This iron-ligand-binding motive is also present in the ectoine hydroxylase and is formed in the *V. salexigens* EctD enzyme by His-146, Asp-148, and His-248 (Fig. 15.4; left panel) and the iron-ligand is well resolved in the high-resolution (1.85 Å) EctD crystal structure (Reuter et al. 2010). As mentioned above, the EctD crystal structure does not contain 2-oxoglutarate but educated guesses for the binding of this co-factor can be made by inspecting crystal structures of other members of dioxygenase superfamily that are structurally closely related to EctD, in particular the human phytanoyl-CoA 2-hydroxylase PhyH and the halogenase SyrB2 from *Pseudomonas syringae*. In this way, Phe-143, Ser-250, and Arg-259 were implicated in 2-oxoglutarate binding (Reuter et al.

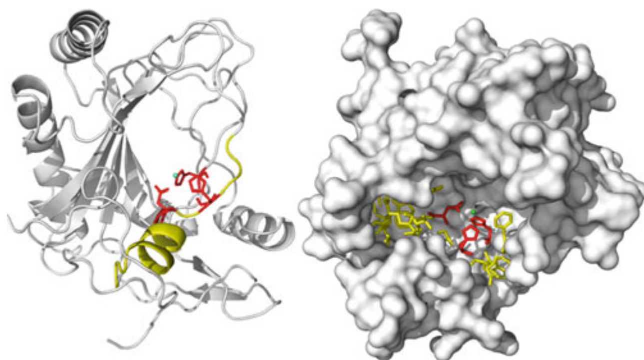

Fig. 15.4 Crystal structure of the ectoine hydroxylase EctD from the moderate halophile *Virgibacillus salexigens*. The EctD protein is a member of the super-family of the non-heme-containing ferrous iron and 2-oxoglutarate dependent oxygenases that typically contain a double-stranded β-helix (DSBH) fold at its core. (*Left panel*) Ribbon diagram of the EctD protein highlighting the side-chains of those three residues (in *red*) that coordinate the iron co-factor (in *green*). The position of the 17 amino acid long segment that serves as the signature-sequence motive for EctD-type ectoine hydroxylases is highlighted in *yellow*. (*Right panel*) Surface representation of the EctD protein. The position of the iron-binding residues, the coordinated iron ligand and the spacial orientation of the ectoine hydroxylase signature-sequence motive are highlighted. The pictures of the EctD crystal structure were prepared with PyMol (http://www.pymol.org/) using the atomic coordinates deposited in the PDB file 3EMR (http://www.pdb.org/) (Reuter et al. 2010)

2010). The crystal structure of EctD does not immediately reveal firm clues for the identity of those residues that position the substrate ectoine within the ligand-binding cavity.

Using the *V. salexigens* EctD enzyme as a search template, more than 90 EctD-type proteins can be currently identified in the databases of finished and unfinished microbial genome sequences. An alignment of the amino acid sequences of these proteins revealed a closely related protein sub-family with amino acid sequence identities that range from 72% for the EctD protein from *B. pseudofirmus* to 44% for the EctD protein from *Kytococcus sedentarius*. The most conserved region of the compiled and aligned 93 EctD-type proteins is a 17-amino-acid segment (F^{143}-X-W-H-S-D-F-E-T-W-H-X-E-D-G-M/L-P^{159}) that we suggested as a signature sequence for bona fide ectoine hydroxylases (Reuter et al. 2010). In the meantime, we have purified and biochemically characterized additional representatives of EctD-type proteins, and each of these enzymes exhibited ectoine hydroxylase activity (our unpublished data) with kinetic parameters resembling those of the *V. salexigens* and *S. coelicolor* enzymes (Bursy et al. 2007, 2008). The EctD signature sequence motive contains two (His-146 and Asp-148) of the three residues involved in iron binding (Fig. 15.4) and one of the residues (Phe-143) implicated in the binding of the 2-oxoglutarate co-factor. The two other residues (Ser-250 and Arg-259) considered in the binding of 2-oxoglutarate as well, are completely conserved in our compilation of 93 EctD-type proteins, lending credence to our suggestion for a functional role of these residues in the functioning of the EctD enzyme (Reuter et al. 2010). When one considers the topological organization of those 17 residues forming the ectoine hydroxylase signature sequence motive within the framework of the available EctD crystal structure from *V. salexigens* (Fig. 15.4; left panel), one finds that this segments lines one side of the ligand-binding cavity of the EctD enzyme (Fig. 15.4; right panel). It thus appears that this segment of the EctD protein is of both structural and functional relevance because it not only contributes to the formation of the overall architecture of the ligand-binding cavity but it also positions the side chains of functionally important residues within the active site of the enzyme (Fig. 15.4; right panel). The strict conservation of the 17-residue signature sequence can thus be rationally understood within the framework of the overall fold of the EctD protein and the architecture of its catalytic core.

15.7 Conclusions, Perspectives and Challenges

The complexity and multifaceted nature of the osmostress response systems of *B. subtilis* have been highlighted by proteome and genome-wide transcriptional profiling studies (Steil et al. 2003; Höper et al. 2006; Hahne et al. 2010). As exemplified by a comprehensive gene disruption study of members of the

salt-inducible SigB general stress regulon of *B. subtilis*, physiological function can often not be gleaned with certainty from bioinformatic approaches and data base searches, despite the fact that many of the disrupted SigB-controlled genes exhibited a salt-sensitive phenotype (Höper et al. 2005). Further complications arise when one considers that osmotic stress caused by salt shock or continued cultivation of cells in high-salinity media apparently requires different, but partially overlapping, cellular stress responses (Steil et al. 2003; Höper et al. 2006; Hahne et al. 2010). Hence, detailed physiological studies are required to uncover the function of individual osmotic inducible genes within the wider osmostress response network of the *B. subtilis* cell. Genome-wide assessments of the transcriptional and protein-biosynthetic profile of osmotically stressed cells continue to provide clues and inspiration for such gene-by-gene functional studies.

As outlined in this chapter with a focus on *Bacillus subtilis* and other Bacilli, effective water management by the microbial cell is without doubt the cornerstone of its acclimatization to either sudden or sustained rises in the environmental osmolarity and the osmotic downshift that inevitably will follow hyperosmotic growth conditions. Although much has already been learned about the genetics, biochemistry, and physiology of salt-stressed *B. subtilis* cells (Bremer and Krämer 2000; Bremer 2002), one issue has been a very hard nut to crack: how do microorganisms sense fluctuations in the external salinity and osmolarity? Do microorganisms actually sense differences in the external osmolarity or do they deduce changes in the environmental conditions by monitoring changes in intracellular parameters such ionic strength or the size and composition of their solute pool? How do bacteria transform the information gleaned about osmotic changes in their environment into a genetic signal that allows the cell to sensitively set gene expression of osmoadaptive systems? Molecular studies of the regulation of the osmotic control of the activity of transport systems for osmoprotectants (Mahmood et al. 2009; Keates et al. 2010; Ziegler et al. 2010) and studies focusing on the osmotic control of the transcription of genes encoding synthesis and uptake systems for compatible solutes (Krämer 2010; Hagemann 2011) will certainly continue to enlighten and inform us about the ins-and outs of microbial salt-stress responses in the coming years.

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