CHAPTER 11.2

Management of osmotic stress by *Bacillus subtilis*: genetics and physiology

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11.2.1 Counteracting environmental osmotic changes is key for cell integrity and growth

Water is the foundation of life (Stevenson *et al.*, 2015). The development of a semipermeable membrane through which water can pass freely, but ions, nutrients, and metabolites cannot, was a key event in the evolution of microbial proto-cells (Booth *et al.*, 2015). Due to the considerable osmotic potential of the cytoplasm caused by nucleic acids, proteins, and metabolites, water enters the cell and creates an outward-directed hydrostatic pressure, the turgor (Bremer and Krämer, 2000; Wood, 2011). Turgor is rather difficult to measure experimentally, and values between 3 and 5 atm have been reported for Gram-negative bacteria such as *Escherichia coli* and between 20 and 30 atm for Gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus*. However, recently published data suggest that the magnitude of turgor, at least for *E. coli*, might have been substantially overestimated (Deng *et al.*, 2011).

Essentially, all free-living bacteria have to cope in their natural habitats with fluctuations in the osmolarity of their surroundings. Caused by the biophysical properties of the cytoplasmic membrane, fluctuations in the environmental osmolarity will inevitably trigger water fluxes along the osmotic gradient into or out of the cell; hence, the magnitude of turgor will be affected (Booth, 2014; Booth *et al.*, 2015). Turgor is considered to be critical for the expansion of microbial cells during growth and for their viability. Consequently, cellular adjustment processes that aim to maintain turgor and the hydration of the cytoplasm within physiological acceptable boundaries are cornerstones of the stress response to osmotic changes (Bremer and Krämer, 2000; Wood, 2011). This is true for members of the Bacteria and Archaea alike (Csonka, 1989; Kempf and Bremer, 1998; Roesser and Müller, 2001; Wood *et al.*, 2001).

Water influx at low external osmolarity can potentially drive up turgor in milliseconds to such an extent that the stability of the stress-bearing peptidoglycan sacculus is no longer sufficient to resist the internal hydrostatic pressure; hence, the integrity of the cell is threatened (Booth, 2014). Conversely, water efflux in high-osmolarity habitats causes dehydration of the cytoplasm, and an ensuing drop in turgor, and the cell will experience plasmolysis; hence, growth is restricted or even prevented (Bremer and Krämer, 2000; Wood, 2011). Although a considerable number of microorganisms possess AqpZ-type aquaporins that mediate accelerated water fluxes across the cytoplasmic membrane (Calamita, 2000; Delamarche *et al.*, 1999), it is important to recall that no bacterium can actively and vectorially transport water. Accordingly, microorganisms can only achieve control of water fluxes across their cytoplasmic membrane indirectly. They do so by actively modulating the intracellular concentration of osmotically active solutes (Bremer and Krämer, 2000; Csonka, 1989; Wood, 2011), and corresponding osmotically driven water fluxes will then ensue (Booth, 2014). Under hypotonic conditions, the cell rapidly jettisons ions and organic compounds through the transient opening of mechanosensitive channels (Naismith and Booth, 2012) and thereby reduces the osmotic potential of the cytoplasm; as a consequence, water influx and the concomitant raise in turgor is curbed. Conversely, it actively accumulates ions or organic solutes to increase the osmotic potential of the cytoplasm and thereby creates a driving force for water influx to stabilize turgor (Csonka, 1989; Galinski and Trüper, 1994; Kempf and Bremer, 1998).

Here, we present an overview on the genetic and cellular adaptation mechanisms of *B. subtilis*, the model organism for Gram-positive bacteria, to fluctuating osmolarities (Figure 11.2.1). *B. subtilis* can be found widely in nature and in many different habitats (Logan and De Vos, 2009), but the upper layers of the soil comprise one of the prime ecological niches in which

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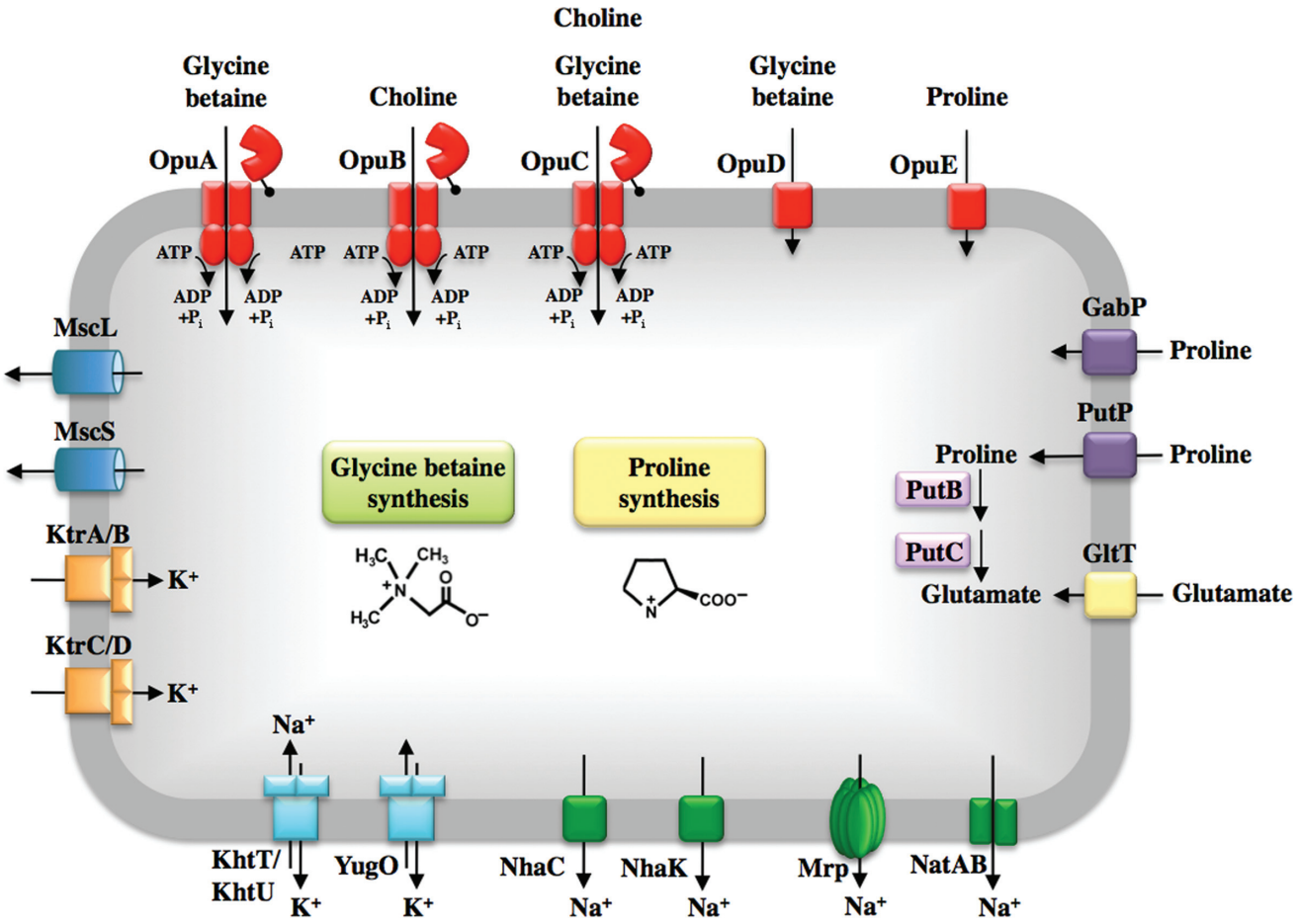

Figure 11.2.1 Overview of the cellular components involved in osmoadaptation in *B. subtilis*.

B. subtilis cells prevail (Belda *et al.*, 2013; Earl *et al.*, 2008). The soil is a very challenging ecosystem with respect to both the variability in the supply of nutrients and the fluctuations in various abiotic parameters. Changes in the osmotic conditions occur frequently through flooding and desiccation, and one can therefore readily envision that adjustments to these changes are critical for growth and survival (Bremer, 2002).

11.2.2 Sporulation is not an escape route from challenges imposed by high osmolarity

B. subtilis is well-known for its ability to form a highly desiccation-resistant endospore (Higgins and Dworkin, 2012; Setlow, 2014) that can last for extended time periods in a dormant state before it can be awakened again to a vegetative cell (Setlow, 2014; Sinai *et al.*, 2015). Sporulation is typically triggered by nutrient depletion and involves a complex developmental process involving several hundred genes (Higgins and Dworkin, 2012; Nicolas *et al.*, 2012). It is regulated through phosphorylation and de-phosphorylation events of components of a signal perception and transduction (see Section 3) cascade that ultimately lead to the activation of the central transcriptional regulator of sporulation, Spo0A, and an increase in its cellular level (Higgins and Dworkin, 2012). The phosphorylated form of Spo0A in turn activates, through a genetically finely tuned system of checks and balances, an intricate network of alternative sigma factors that direct stage-specific gene expression when the sporulation process proceeds (Higgins and Dworkin, 2012). In this process, the mother cell dies, but the survival of the species within a given habitat (e.g., the soil) is ensured through the spore and the subsequent resurrection of a vegetative *B. subtilis* cell (Setlow, 2014).

By thinking about sporulation and the pronounced desiccation-resistant properties of the endospores, one would intuitively assume that spore formation would be an effective route for the *B. subtilis* cell to escape from the unfavorable high-osmolarity conditions caused by the drying of the soil. Contrary to expectations, high salinity is actually an inhibitor of spore formation by *B. subtilis* (Kunst and Rapoport, 1995; Ruzal and Sanchez-Rivas, 1998). How can this observation be interpreted within the physiological context of salt-stressed

cells? The commitment of the cell to sporulate is a dicey decision since sporulation is an energy-demanding and complex developmental process that requires, even under favorable laboratory conditions, several hours for its completion (Higgins and Dworkin, 2012). Increases in the external salinity negatively impinge on many cellular processes and slow the growth of *B. subtilis* considerably (Boch *et al.*, 1994). One is thus tempted to speculate that the stressed *B. subtilis* cell might not possess the energetic and biosynthetic resources required to complete spore formation under high-osmolarity conditions. Since progression of the sporulation process is irreversible after a certain time point (Higgins and Dworkin, 2012), committing the *B. subtilis* cell to this developmental pathway under high-salinity conditions bears the risk of the death of the mother cell without having produced a fully stress-resistant spore. It would therefore make sense physiologically to block the sporulation process at an early stage, and this is actually what has been observed under high-salinity conditions (Ruzal and Sanchez-Rivas, 1998; Widderich *et al.*, 2016).

The activity of the master regulator of sporulation in *B. subtilis*, the phosphorylated form of Spo0A, is regulated by a sophisticated phosphorelay integrating multiple positively and negatively acting signals through the activities of kinases and phosphatases. One of these phosphatases (Spo0E) specifically targets Spo0A–phosphate and thereby abrogates the sporulation process (Higgins and Dworkin, 2012). Recently, an interesting connection between the genetics of the sporulation network and the operation of the SigB-controlled general stress response system of *B. subtilis* (Hecker *et al.*, 2007; Price, 2011) has been uncovered by the finding that a SigB-dependent promoter contributes to *spo0E* expression (Reder *et al.*, 2012). Suddenly imposed severe salt stress is a strong inducer of the SigB regulon (Nannapaneni *et al.*, 2012; Young *et al.*, 2013), and SigB activity is therefore integrated via Spo0E into the finely tuned decision-making process during the onset and progression of sporulation (Reder *et al.*, 2012). Since SigB activity increases only transiently after a severe salt shock (Young *et al.*, 2013) and is not triggered by sustained high salinity (Spiegelhalter and Bremer, 1998), the observations made by Reder *et al.* (2012) address a phenomenon and regulatory circuit that must be different from the strong decrease in sporulation frequency that has been observed in *B. subtilis* cells exposed to sustained high salinity (Kunst and Rapoport, 1995; Lopez *et al.*, 1998).

In addition to the repressing influence on sporulation, various effects of high salinity on the germination of spores have also been reported (Nagler *et al.*, 2014). Increases in salinity cause progressive, albeit reversible, inhibition of germination efficiency and heterogeneity of germination initiation in a given spore population, and it slows germination kinetics of individual spores. Interestingly, part of the spore population can be triggered to initiate spore germination even in solutions containing near-saturated NaCl concentrations (about 5.4 M). This observation by Nagler *et al.* suggests that spores lack a sensory system that would prevent them from germinating under conditions where the awakened vegetative cells cannot grow at all (Boch *et al.*, 1994; Nagler *et al.*, 2014).

11.2.3 Contributions of the SigB-controlled general stress regulon to cellular adjustment to acute osmotic stress

High-salinity shocks are one of the most effective triggers to onset the general stress response of *B. subtilis* (Nannapaneni *et al.*, 2012; Young *et al.*, 2013). This emergency response system comprises several hundred genes (Nannapaneni *et al.*, 2012; Nicolas *et al.*, 2012) and provides the cell with a preemptive resistance against a multitude of environmental insults and against energy stress. It is controlled through the biochemical activation of the alternative transcription factor SigB (Hecker *et al.*, 2007; Price, 2011). Disruption of *sigB* causes sensitivity against severe salt shocks. Indeed, a systematic inactivation of a large subset of SigB-controlled genes of *B. subtilis* revealed a salt-sensitive phenotype for many of them under conditions where the imposed osmotic stress (with 1.5 M NaCl) was severe and acute (Höper *et al.*, 2005, 2006). Unfortunately, thus far a bioinformatics analysis of the predicted functions of the genes with a salt-sensitive phenotype has not provided truly informative clues about how the encoded proteins might participate in the development of salt-shock resistance. While SigB is critical for the ability of the *B. subtilis* cell to withstand growth-restricting salt shocks (Höper *et al.*, 2005), it is dispensable for cells that are growing under sustained high-salinity conditions (e.g., in a chemically defined medium with 1.2 M NaCl).

In nonstressed cells, SigB is held in an inactive form through interactions with the antisigma factor RsbW. SigB activity is regulated by a complex signaling network that mediates the release of SigB from the activity-inhibiting RsbW–SigB complex through a series of partner-switching modules that trap RsbW in a complex with the anti-antisigma factor RsbV. These protein–protein interactions between alternative binding partners of RsbW are governed by phosphorylation and dephosphorylation events (Hecker *et al.*, 2007; Price, 2011). Perception of environmental stress and the transduction of this information into the partner-switching modules are mediated by a set of regulatory proteins that are organized in a 1.8 MDa macromolecular complex, the stressosome (Figure 11.2.2a) (Marles-Wright *et al.*, 2008). With respect to the induction of the SigB regulon by salt shocks, the phosphatase activator protein RbsT is released from the stressosome and interacts with the environmental phosphatase RsbU that in turn then de-phosphorylates the anti-antisigma factor RsbV, leading to the entrapment of RsbW in a RsbV–RsbW protein complex. SigB is thus free to interact with core RNA polymerase to direct transcription on a genome-wide scale of those genes that possess promoters recognized specifically by this alternative transcription factor (Hecker *et al.*, 2007; Marles-Wright *et al.*, 2008; Nannapaneni *et al.*, 2012; Price, 2011).

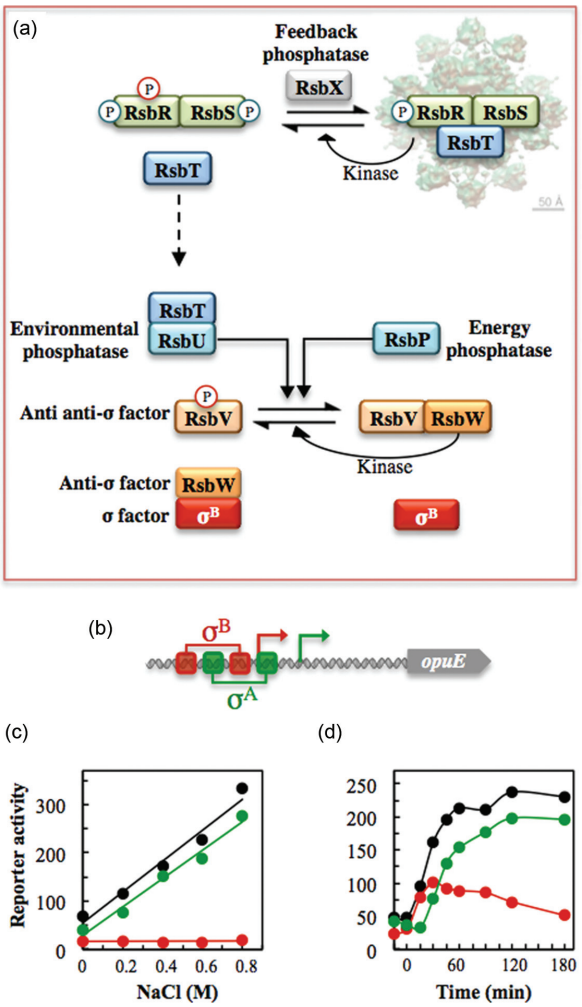

Figure 11.2.2 The SigB-controlled general stress response system of *B. subtilis* and its influence on the osmotic control of the *opuE* proline uptake gene. (a) The stressosome, composed of the RsbR–RsbS–RsbT proteins (Marles-Wright *et al.*, 2008), acts as a sensor for environmental stress (e.g., high salinity). The perceived signal is transduced via the phosphatase activator protein RsbT and the environmental phosphatase RsbU into a partner-switching module of antisigma factors that eventually free the transcription factor SigB and thereby allow its association with core RNA polymerase (Hecker *et al.*, 2007; Price, 2011) to direct gene expression of the entire SigB regulon (Nannapaneni *et al.*, 2012). (b) Architecture of the SigA- and SigB-controlled regulatory region of the *opuE* gene (Spiegelhalter and Bremer, 1998). (c) A 390 bp *opuE* promoter fragment was fused to a promoter-less reporter gene (*treA*; encoding a salt-tolerant phosphotrehalase), and this DNA construct was inserted into the *B. subtilis* chromosome as single copy into the non-essential *amyE* gene. TreA reporter enzyme activity (given in units [mg protein^{-1}]) was measured as a readout of *opuE* promoter activity. *B. subtilis* strains harboring the *opuE*–wild-type promoter fusion (black circles), a promoter fusion fragment where only the SigA-type promoter was active (green circles), or a promoter fusion fragment where only the SigB-type promoter was active (red circles) were grown to mid-exponential growth phase in minimal medium (SMM) supplemented with the indicated NaCl concentrations. (d) The same reporter fusion strains were grown in minimal medium and were subjected to a NaCl up-shock (with 0.4 M NaCl) at time point zero. The data shown in (c) and (d) were replotted from previously published data (Spiegelhalter and Bremer, 1998).

The signal output of the stressosome leads to a single pulse of SigB activation, whose amplitude is determined by the rate at which the stress increases over time (Young *et al.*, 2013). As a consequence, the general stress system responds only to rapidly increasing stresses (e.g., a salt shock) and typically not to sustained stresses, with the notable exception of temperature stress in actively growing cells (Brigulla *et al.*, 2003; Holtmann *et al.*, 2004). Hence, the SigB system generally permits only a temporary relief from stress (Hecker *et al.*, 2007; Price, 2011). An interesting question in this context is how long the newly synthesized proteins of the SigB regulon might persist, despite the rather short-lived pulse in gene expression (Young *et al.*, 2013), and thereby might provide prolonged protection to stressed cells, a question that could be addressed by time-resolved proteomics.

The stressosome is a cytoplasmic macromolecular protein complex (Marles-Wright *et al.*, 2008), and no membrane-associated or membrane-embedded regulatory components of the systems controlling the activity of SigB in *B. subtilis* are known (Hecker *et al.*, 2007; Price, 2011). Hence, changes in the environment must be perceived by the stressosome in an indirect fashion as a consequence of changes that occur in the cytoplasm. However, it is unclear how the various types of stresses that are triggered by diverse environmental cues are actually sensed by the stressosome. With respect to the perception of salt shocks, two possible signal input routes into the stressosome come readily to mind: (i) Salt shocks cause a rapid, but only transient, increase in the potassium concentration of the *B. subtilis* cell (Whatmore *et al.*, 1990). Hence, an altered ionic composition of the cytoplasm could potentially be detected by the stressosome. Indeed, ionic strength has been suggested as the cellular cue that determines the DNA-binding activity of BusR, a repressor controlling the expression of the *opuA* operon encoding a glycine betaine import system (OpuA) of *Lactococcus lactis* and of CosR, a repressor controlling synthesis of the compatible solute ectoine and systems for osmoprotectants uptake in the pathogen *Vibrio cholerae* (Romeo *et al.*, 2007; Shikuma *et al.*, 2013). (ii) The rapid outflow of water subsequent to a salt shock will lead to an increase in the concentration of cytoplasmic solutes and hence to an increase in molecular crowding (Boersma *et al.*, 2015; Gnutt *et al.*, 2015). Crowding effects on protein conformation are well known and could potentially be perceived by the stressosome.

11.2.4 Mechanosensitive channels: microbial safety valves

Influx of water under hypotonic conditions will increase turgor. The elastic murein sacculus can withstand increased hydrostatic pressure to a certain degree, but beyond a certain threshold value it will rupture (Reuter *et al.*, 2014). As a consequence, the cell will lyse since the cytoplasmic membrane is not a very stable structure and is unable to restrain turgor. To avoid

such a catastrophic event, most microorganisms have developed safety valves whose gating is triggered by an upshot in turgor. Mechanosensitive channels embedded in the cytoplasmic membrane are the molecular basis of these safety valves, and their transient opening allows the cell to rapidly jettison water-attracting ions and organic compounds to reduce the driving force for the osmotically instigated rapid water influx that follows an osmotic downshift. The ability of different types of these channels to gate at different pressure thresholds allows a graded response of the cell coupled to the severity of the osmotic downshift (Booth, 2014; Naismith and Booth, 2012). Electrophysiological studies with giant spheroplasts derived from bacterial cells and reconstituted *in vitro* systems set up for patch-clamp analysis allowed the classification of mechanosensitive channels into three classes: MscM (*mini*), MscS (*small*), and MscL (*large*). The MscS and MscL channels are best understood with respect to their gating behavior, biochemistry, and crystal structure (Booth, 2014; Naismith and Booth, 2012). MscL is the measure of last resort; it gates just shortly before the osmotically downshifted cell would rupture and thereby transiently opens a channel in the cytoplasmic membrane with a diameter of about 30 Å (Booth, 2014; Naismith and Booth, 2012), substantially larger than the permanently open pores (porins) in the outer membrane of Gram-negative bacteria. Fully opened MscS-type channels possess a diameter of about 6 Å (Booth, 2014; Naismith and Booth, 2012). Mechanosensitive channels typically do not possess substrate specificity, and hence both ions and organic compounds can pass freely. It is obvious that the cell must very carefully control their numbers and gating behavior.

For a soil bacterium such as *B. subtilis*, a rapid osmotic downshift can simply be caused through the wetting of the dried-out upper layers of soil by rainfall. Electrophysiological studies revealed that different types of mechanosensitive channels must operate in *B. subtilis,* and the inspection of its genome sequence suggested the presence of one MscL- and three MscS-type channels (Figure 11.2.1) (Hoffmann *et al.*, 2008; Wahome *et al.*, 2009). Nothing is known about the potential operation of MscM-type channels in *B. subtilis;* the molecular basis of these channels has only recently been discovered in *E. coli* (Booth, 2014; Booth *et al.*, 2015). The systematic inactivation of the *mscL* and the three *mscS* channel genes (*ykuT*, *yfkC*, and *yhdY*) reveals that neither the single mutants nor the quadruple mutant strain (*mscL*, *ykuT*, *yfkC*, and *yhdY*) have a growth disadvantage in either low-osmolarity or high-osmolarity media in comparison to the wild-type strain. However, osmotic downshock experiments demonstrated that the MscL channel is the principle solute release system of *B. subtilis*. The activity of the YkuT MscS-type channel aids MscL in protecting the cells from the detrimental effects of a severe osmotic downshift. Indeed, cells of an *mscL ykuT* double mutant are almost all killed when cells grown at high osmolarity are rapidly shifted to low-osmolarity conditions (Hoffmann *et al.*, 2008). No evidence for mechanosensitive channel activity has been found with this assay for the MscS-type proteins YfkC and YhdY.

Interestingly, in a genome-wide transcriptional profiling study of *B. subtilis*, cells subjected to a severe osmotic upshift express the *mscL* and *ykuT* genes at a significantly higher level than in their non-osmotically stressed counterparts; in contrast, expression of the *mscS*-type genes *yfkC* and *yhdY* is not affected (Hahne *et al.*, 2010). The observation of the transcriptional induction of the *mscL* and *ykuT* genes by high salinity could potentially be rationalized by invoking the idea that cells subjected to high-osmolarity stress already prepare themselves for an osmotic downshock that inevitably will follow at one point in time. One can thus summarize that tension-activated mechanosensitive channels of the MscS and MscL types (Booth, 2014; Naismith and Booth, 2012) are key for managing the transition of *B. subtilis* cells from high- to low-osmolarity environments (Hoffmann *et al.*, 2008; Wahome *et al.*, 2009).

11.2.5 *Salt-in* and *salt out*: strategies to cope with high osmolarity

Selected groups of Archaea (representatives of Halobacteria, halophilic methanogenic Archaea, and Nanohaloarchaea) and a few Bacteria (representatives of Bacterioidetes and Haloanaerobiales) that live permanently in high-salinity environments preferentially accumulate molar concentrations of potassium and chloride, to balance the osmotic gradient between the cell's interior and that of the surroundings (Oren, 2011). The lasting accumulation of ions is an energetically favorable cellular adjustment to cope with the challenges of high osmolarity (Oren, 2011). However, it comes at an evolutionary price since the high ionic strength of the cytoplasm requires the adaptation of macromolecular structures and biochemical processes on a genome-wide scale to maintain the solubility and structural integrity of both cell surface exposed and cytoplasmic proteins. The solute-exposed surfaces of proteins become more acidic, and at the same time their hydrophobicity is reduced (Coquelle *et al.*, 2010; Talon *et al.*, 2014).

Microorganisms that use this *salt-in* strategy (Galinski and Trüper, 1994; Oren, 2011) typically can only tolerate moderate fluctuations in the osmolarity of their environment. Thus, a more flexible osmostress response is needed for bacteria that live in environments exhibiting either more frequent or more extreme changes in the external osmolarity. These microorganisms pursue an osmostress adaptation strategy that aims at keeping the permanent intracellular ion concentrations low (*salt-out*) (Galinski and Trüper, 1994). To accomplish this, a selected class of organic osmolytes, the compatible solutes, are amassed, and their intracellular concentration is set by the osmolarity prevalent at a given time in the environment of the microbial cells (Csonka, 1989; Kempf and Bremer, 1998; Roesser and Müller, 2001; Wood, 2011; Wood *et al.*, 2001). Since compatible solutes are highly compliant with cellular functions (Ignatova and Gierasch, 2006; Street *et al.*, 2006), this obviated, on an evolutionary timescale, the need to adapt cellular components to a

high-ionic-strength cytoplasm in the way that was dictated by the *salt-in* strategy (Oren, 2013; Talon *et al.*, 2014). It suffices to state here that *B. subtilis* uses the *salt-out* strategy to physiologically cope with prolonged high-osmolarity conditions.

11.2.6 Uptake of potassium and extrusion of sodium: the first line of defense

As in many other bacteria (Csonka, 1989; Kempf and Bremer, 1998; Wood *et al.*, 2001), in *B. subtilis*, osmotic upshifts trigger a rapid import of potassium ions (Whatmore *et al.*, 1990). The buildup of this elevated potassium pool serves as the first line of cellular defense against loss of water and a reduction in turgor (Holtmann *et al.*, 2003). Studies conducted by Whatmore *et al.* (1990) have revealed a potassium pool of about 350 mM in cells grown in a minimal medium, which increases about twofold (up to about 720 mM) over 3 h subsequent to a moderate salt shock with 0.4 M NaCl. The slowness of this increase is probably due to the use of cells for these measurements that had been grown at a suboptimal temperature (25 °C) for *B. subtilis* (Whatmore *et al.*, 1990) and the fact that the expression of the genes encoding its major potassium uptake system (KtrAB) (Holtmann *et al.*, 2003) (discussed further in this chapter) is downregulated at low-growth temperatures (Nicolas *et al.*, 2012).

B. subtilis lacks a true high-affinity potassium transport system and instead possesses two representatives of the Ktr potassium uptake family (Figure 11.2.1) (Hänelt *et al.*, 2011). The KtrAB and KtrCD systems of *B. subtilis* possess only moderate affinities for the potassium ion and exhibit K_m values of approximately 1 mM and 10 mM, respectively (Holtmann *et al.*, 2003). Loss of the KtrAB system causes a severe salt sensitivity in cells continuously challenged by high osmolarity, a phenotype that is augmented by the simultaneous inactivation of the KtrCD transporter. In a salt shock experiment, cells lacking KtrAB cannot recover from an increase in salinity (with 0.6 M NaCl) in a growth medium containing 2 mM potassium. Higher concentrations of potassium (50 mM), however, allow recovery from the suddenly imposed salt stress, as the KtrCD transporter will permit potassium uptake under these conditions (Holtmann *et al.*, 2003). Collectively, these experiments illustrate how critical potassium uptake is for cells that are either suddenly exposed to high salinity or continuously challenged by it.

Ktr-type potassium transporters consist of a membrane-embedded protein (e.g., KtrB and KtrD) and a regulatory component (e.g., KtrA and KtrC) (Figure 11.2.1) that is peripherally associated with the membrane and interacts with the membrane-embedded potassium translocation subunit (Hänelt *et al.*, 2011). The KtrA and KtrC proteins serve to control the activity of the potassium translocating subunit and possess each two RCK domains (***r***egulator of ***c***onductance of $\boldsymbol{K^+}$), modules that bind nucleotides and thereby affect potassium import (Corrigan *et al.*, 2013). The crystal structure of the *B. subtilis* KtrAB system has recently been solved and reveals a striking arrangement of a dimer of the membrane-embedded KtrB subunit that is decorated on the cytoplasmic side by an octameric ring formed by KtrA (Vieira-Pires *et al.*, 2013).

In contrast to the structural genes for the high-affinity potassium uptake Kdp system of *E. coli* (Laermann *et al.*, 2013), transcription of the *ktrAB*, *ktrC*, and *ktrD* genes from *B. subtilis* is not induced by potassium limitation or high osmolarity (Holtmann *et al.*, 2003). However, the *ktrAB* operon is regulated in a highly interesting fashion since its transcription is controlled by an *ydaO*-type riboswitch that recognizes as its effector molecule the recently discovered second messenger, cyclic diadenylate monophosphate (c-di-AMP) (Nelson *et al.*, 2013). Strikingly, c-di-AMP is also an effector for the RCK-containing KtrA subunit of *Staphylococcus aureus*, and its binding serves to downregulate KtrAB potassium transporter activity (Corrigan *et al.*, 2013). It will therefore be highly interesting in future studies to probe how the control of potassium homeostasis in *B. subtilis* is affected by the genetic and biochemically control mechanisms setting the c-di-AMP pool (Mehne *et al.*, 2013) and how this might affect the physiology of the overall osmostress adaptation process of the cell.

A mutant strain lacking both the KtrAB and KtrCD systems has a residual potassium transport activity that exhibits a K_m value of around 110 mM, suggesting the operation of other potassium uptake systems in *B. subtilis*. Indeed, the inspection of the *B. subtilis* genome sequence revealed the presence of another transporter, YugO (Figure 11.2.1), which is related to the MthK channel from *Methanobacterium thermoautotrophicum* (Ye *et al.*, 2010). The arrangements of the membrane-spanning segments in YugO are different from the potassium translocating subunit (KtrB) of the Ktr system, and it carries at its C-terminus an RCK domain. Interestingly, disruption of the YugO channel abolishes biofilm formation of *B. subtilis* in a regulatory loop that involves KinC, a histidine kinase that seems to be activated by potassium leakage (Lopez *et al.*, 2009a). Consistent with the role of YugO in biofilm formation (Lundberg *et al.*, 2013), expression of the *mstX–yugO* operon is negatively controlled by SinR, the central regulator of matrix and expolysaccharide synthesis during biofilm formation by *B. subtilis* (Lopez *et al.*, 2009b; Vlamakis *et al.*, 2013).

For microorganisms that use the *salt-out* strategy, prolonged high levels of potassium ions are detrimental to cellular physiology. Hence, the osmotically stressed cells replace part of the initially accumulated potassium with organic osmolytes (e.g., proline and glycine betaine) that are highly compliant with its biochemistry and physiology (Ignatova and Gierasch, 2006; Street *et al.*, 2006; Whatmore *et al.*, 1990). In this way, the ionic strength of the cytoplasm is reduced without compromising turgor. Potassium extrusion in *B. subtilis* is only incompletely understood, but one of the systems that mediate potassium export has been identified. It is the cation–proton antiporter KhtTU (also known as YhaTU) (Figure 11.2.1) (Fujisawa *et al.*, 2007). The involvement of this potassium extrusion system in the cell's overall osmotic adjustment process is evident from the

upregulation of the *khtSTU* (*yhaSTU*) gene cluster in response to a salt shock (Fujisawa *et al.*, 2004; Hahne *et al.*, 2010). It is highly likely that other potassium exporters exist in *B. subtilis* since a mutant carrying a deletion of the *khtSTU* (*yhaSTU*) operon is viable (our unpublished data).

Sodium ions are highly cytotoxic, and *B. subtilis* keeps its cytoplasmic concentration very low (Gorecki *et al.*, 2014). During salt challenges, Na^+ might enter the cell in various ways, one of which occurs during the import of osmostress protectants such as glycine betaine and proline via the OpuD and OpuE transporters, respectively. The proline transporter OpuE (von Blohn *et al.*, 1997) and the glycine betaine transporter OpuD (Figure 11.2.1) (Kappes *et al.*, 1996) belong to different types of transporter superfamilies, but both of them import their substrates in concert with Na^+ ions. The imported sodium must be speedily exported, and *B. subtilis* possesses four Na^+ extrusion systems that serve to keep intracellular Na^+ levels low: the multicomponent Mrp transporter, the single-component NhaC and NhaK systems, and the adenosine triphosphate (ATP)-dependent NatAB system (Figure 11.2.1). A severe salt shock triggers the expression of the *mrp* operon, of *nhaK*, and, to a lesser extent, of *nhaC* (Hahne *et al.*, 2010). The ATP-binding cassette (ABC)-type transporter NatAB is primarily involved in Na^+ extrusion at alkaline pH, and the expression of its structural genes is controlled by the two-component NatK–NatR regulatory system (Ogura *et al.*, 2007). The transcriptional induction of the Mrp, NhaK, and NhaC systems subsequent to a salt shock in cells grown at neutral pH highlights the importance of sodium homeostasis in salt-stressed cells and buttresses the physiological role of Na^+ extrusion in the cellular response of *B. subtilis* to osmotic stress. Indeed, the genetic disruption of the Mrp system causes a strong salt-sensitive growth phenotype and drives up the Na^+ content of the cells from a practically nonmeasurable level to 11 mM (Gorecki *et al.*, 2014).

11.2.7 Uptake of compatible solutes

As outlined in this chapter, the accumulation of compatible solutes is a key event in the well-orchestrated cellular responses of many Bacteria and Archaea to osmotic stress (Csonka, 1989; Kempf and Bremer, 1998; Roesser and Müller, 2001; Wood *et al.*, 2001). This is true for *B. subtilis* as well (Bremer, 2002; Bremer and Krämer, 2000). The amassing of compatible solutes by osmotically stressed cells can occur either via synthesis or by uptake (Kempf and Bremer, 1998), and both processes are used by *B. subtilis* to achieve osmostress resistance (Bremer, 2002; Bremer and Krämer, 2000). Extensive physiological studies have revealed that *B. subtilis* uses primarily compatible solutes that are chemically related to either proline or glycine betaine. So far, 14 naturally occurring compatible solutes have been identified to serve as osmostress protectants for *B. subtilis*, all of which can be found in habitats populated by this bacterium (e.g., the soil or marine sediments) (Bashir *et al.*, 2014a; Broy *et al.*, 2015; Hoffmann and Bremer, 2011; Nau-Wagner *et al.*, 1999). With the notable exception of proline (Moses *et al.*, 2012), none of these compounds can be metabolized by *B. subtilis* and are thus amassed exclusively as stress protectants, to fend off either the detrimental effects of high osmolarity on cellular physiology (Bremer, 2002), or extremes in high- and low-growth temperatures (Hoffmann and Bremer, 2011; Holtmann and Bremer, 2004).

The addition of low concentrations of compatible solutes to high-salinity minimal growth media (SMM) exerts a strong osmoprotective effect on cell growth (Boch *et al.*, 1994). This is exemplarily shown for glycine betaine in Figure 11.2.3a, probably the most widely found compatible solute in nature (Yancey, 2005). Measurements of glycine betaine pools in *B. subtilis*, built up through transport processes, revealed a linear relationship

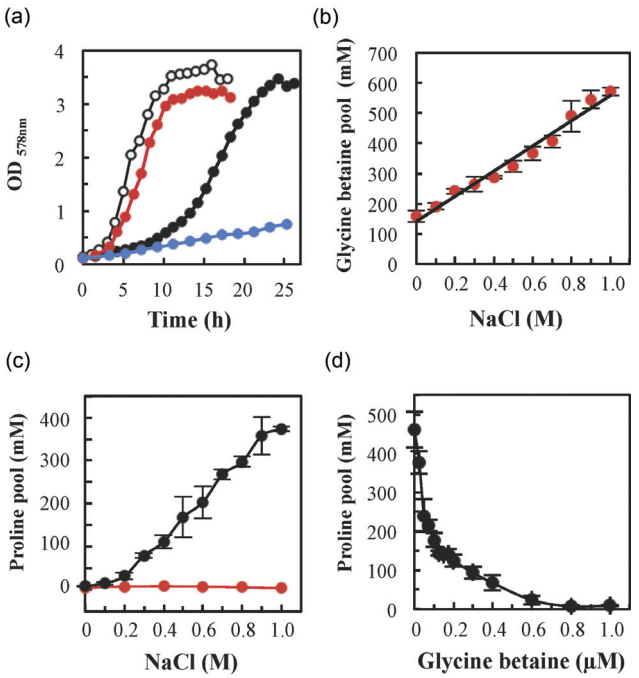

Figure 11.2.3 Growth of *B. subtilis* at high salinity in the absence or presence of the osmostress protectant glycine betaine, and measurements of the ensuing cellular proline and glycine betaine pools. (a) Growth curves of *B. subtilis* wild-type strain JH642 grown in SMM (open circles), in SMM supplemented with 1.2 M NaCl (black circles), or in SMM containing 1.2 M NaCl and 1 mM glycine betaine (red circles). Growth of a JH642-derived *proHJ* mutant strain (blue circles) that is defective in the osmoadaptive proline biosynthesis was cultivated in SMM with 1.2 M NaCl. (b) The intracellular glycine betaine pool of *B. subtilis* wild-type cells was determined by cultivating the cells in SMM with varying salinities in the presence of 1 mM glycine betaine spiked with 0.64 µM radiolabeled [1-^{14}C] glycine betaine. (c) Intracellular pools of the compatible solute proline were measured in response to different external salinities. *B. subtilis* wild-type cells were grown either in the absence (black circles) or in the presence (red circles) of 1 mM glycine betaine. (d) Intracellular proline pools were measured in *B. subtilis* wild-type cells grown at high salinity (SMM containing 1.2 M NaCl) in the presence of increasing glycine betaine concentrations. The data shown in this figure were replotted from previously published data (Hoffmann *et al.*, 2013).

between the degree of the imposed osmotic stress and the cellular levels of this solute (Figure 11.2.3b). This is quite an amazing relationship because it shows that the *B. subtilis* cell can detect rather small increases in the external salinity and then set its osmostress-protective glycine betaine pool very precisely to maintain a physiologically perfect balance between the intracellular and environmental osmolarity. In severely osmotically stressed cells (e.g., with 1 M NaCl) (2200 mOsmol kg^{-1}), the glycine betaine pool increases from a basal level of 150 mM in cells cultivated in SMM (350 mOsmol kg^{-1}) about 3.8-fold to a cellular content of about 570 mM (Figure 11.2.3b) (Hoffmann *et al.*, 2013).

The glycine betaine content of the cells is already remarkably high (about 150 mM) in cells grown in SMM, a minimal medium that is widely used in physiological studies with *B. subtilis*; its osmolarity (350 mOsmol kg^{-1}) is considered not to confer any osmotic stress onto the cells.

The uptake of glycine betaine by *B. subtilis* is mediated by three distinct transport systems that belong to the Opu (*o*smo*p*rotectants *u*ptake) family of transporters (Bremer, 2002) and that comprise the ABC-type uptake systems OpuA and OpuC and the single-component BCCT-type transporter OpuD (Figure 11.2.1) (Kappes *et al.*, 1996, 1999; Kempf and Bremer, 1995). Each of these transporters imports glycine betaine with high affinity (K_m values in the low μM range), but their transport capacity (V_{max}) varies. OpuA is the dominant glycine betaine transporter operating in *B. subtilis* due to its substantial uptake velocity (Kappes *et al.*, 1996; Kempf and Bremer, 1995), but its substrate spectrum is rather restricted (Bashir *et al.*, 2014a,b; Broy *et al.*, 2015; Hoffmann and Bremer, 2011). In contrast, the OpuC system has a very broad substrate profile and, with the exception of proline, can mediate the import of all osmostress protectants known so far for *B. subtilis*. Remarkably, it can also catalyze the import of several synthetic compatible solutes derived from the ecologically abundant marine osmolyte dimethlylsulfoniopropionate (DMSP) (Broy *et al.*, 2015) and a toxic derivative of glycine betaine (Cosquer *et al.*, 2004).

An ABC transporter (OpuB) very closely related to the OpuC system exists in *B. subtilis* (Figure 11.2.1); the genes encoding these two transporters have in all likelihood arisen through a gene duplication event (Kappes *et al.*, 1999). However, the substrate specificity of OpuB and OpuC is strikingly different. OpuB mediates only the import of choline and glycine betaine aldehyde, the precursor and intermediate, respectively, for the synthesis of glycine betaine (Boch *et al.*, 1996; Kappes *et al.*, 1999), whereas OpuC mediates the import of essentially all osmoprotectants (with the exception of proline) known to date for *B. subtilis* (Bashir *et al.*, 2014a; Broy *et al.*, 2015; Hoffmann and Bremer, 2011).

The OpuA, OpuB, and OpuC systems are all members of the ABC superfamily and possess extracellular substrate binding proteins (OpuAC, OpuBC, and OpuCC) that are tethered with a lipid anchor to the outer surface of the cytoplasmic membrane (Figure 11.2.1) (Kappes *et al.*, 1999; Kempf and Bremer, 1995). These lipoproteins serve as the primary ligand recognition component of the OpuA, OpuB, and OpuC transporters and determine their overall affinity and substrate specificity (Berntsson *et al.*, 2010). Hence, the question arose how the OpuAC, OpuBC, and OpuCC solute receptor proteins can recognize ligands with high affinity (Horn *et al.*, 2006; Pittelkow *et al.*, 2011) that are otherwise effectively excluded from protein surfaces (Street *et al.*, 2006). Crystallographic analysis has provided the answer and showed that each of them possessed a ligand-binding site that accommodates the positively charged trimethlyammonium head group of the various ligands (e.g., glycine betaine, choline, and carnitine) in a cage formed by aromatic side chains and stabilizes them via cation–π interactions. The "tails" of the various ligands protrude out of these aromatic cages and are further stabilized within the binding site via contacts with specific side chains, with the protein backbone, or through intricate water networks (Du *et al.*, 2011; Horn *et al.*, 2006; Pittelkow *et al.*, 2011). The ligand-binding site present in the OpuCC ligand-binding protein has proven to be particularly structurally flexible (Du *et al.*, 2011) and thereby provides the molecular underpinnings for the remarkably broad substrate specificity of the OpuC transport system (Bashir *et al.*, 2014a,b; Broy *et al.*, 2015; Hoffmann and Bremer, 2011).

Intensive studies with the OpuA system from *Lactococcus lactis* have shown that this glycine betaine import system (also sometimes referred to as BusA) is osmotically regulated not only at the transcriptional level through the ionic strength–dependent BusR repressor protein (Romeo *et al.*, 2007) but also at the level of its transport activity (Poolman *et al.*, 2004). Detailed studies by Poolman and coworkers have revealed that the osmotic activity control of the *L. lactis* OpuA transporter is dependent on two cystathionine β-synthase (CBS) domains present within the extended C-terminal domain of the OpuAA ATPase. These serve as sensors of the ionic strength of the cytoplasm and thereby couple information on the properties of the cytoplasm (changes in ionic strength and molecular crowding subsequent to an osmotic upshift) to conformational changes in the nucleotide-binding domain (OpuAA) of the ABC transporter, and thereby affect the overall transport activity of the OpuA system (Karasawa *et al.*, 2011). Data derived from *in silico* assessments indicate that such CBS domains are also present in the ATPases (OpuAA, OpuBA, and OpuCA) of the OpuA, OpuB, and OpuC systems of *B. subtilis* (Chen and Beattie, 2007). This finding suggests that these transporters are also subjected to activity control of their transport capacity in response to increase in the external osmolarity. Such an enhancement of transport activity would allow the cell to react instantaneously with increased uptake of compatible solutes to relieve osmotic stress (Poolman *et al.*, 2004). However, a possible activity control of the *B. subtilis* OpuA, OpuB, and OpuC systems has not yet been studied experimentally.

The third glycine betaine transporter operating in *B. subtilis* is OpuD (Figure 11.2.1), a single-component system (Figure 11.2.1) and a member of the BCCT family of permeases

(Ziegler *et al.*, 2010). These widely found types of transporters are involved in the uptake of various kinds of compatible solutes in many microbial species. OpuD is closely related to the glycine betaine transporter BetP from *Corynebacterium glutamicum*, the biochemically, mechanistically, and structurally best studied member of the BCCT family (Maximov *et al.*, 2014; Perez *et al.*, 2012; Ziegler *et al.*, 2010). BetP imports glycine betaine in symport with two Na^+ ions (Perez *et al.*, 2014) and has been crystallized in the presence and absence of its ligand. Not only is the expression of the *betP* gene upregulated in response to high osmolarity, a process that involves the osmostress-sensing two-component MtrAB regulatory system (Möker *et al.*, 2004, 2007), but also the activity of the BetP transporter itself is modulated both by a trans-membrane osmotic gradient and by the cytoplasmic potassium concentration (Maximov *et al.*, 2014). Given the close amino acid sequence identity of BetP and OpuD and the conservation of ligand- and sodium-contacting amino acid residues, it is highly likely that the two proteins function mechanistically in the same fashion with respect to the overall transport process. Strikingly, the architecture of the glycine betaine–binding site present in the membrane-embedded BetP protein (Perez *et al.*, 2012, 2014), and hence in all likelihood also in OpuD, resembles that of the soluble glycine betaine–binding protein OpuAC (Horn *et al.*, 2006). Hence, nature has found a common solution for providing a high-affinity binding site in transport proteins to a solute that is otherwise preferentially excluded from protein surfaces (Street *et al.*, 2006).

The fifth osmostress protectant uptake system found in *B. subtilis* is the proline transporter OpuE (Figure 11.2.1) (von Blohn *et al.*, 1997). It is a member of the solute sodium symporter (SSS) family, and is sequence related to the PutP transporter that is used by *E. coli* and *B. subtilis* for the acquisition of proline as a nutrient (Moses *et al.*, 2012; Olkhova *et al.*, 2011). In contrast to the osmotic transcriptional control of *opuE* (Spiegelhalter and Bremer, 1998), *putP* expression in *B. subtilis* is upregulated in response to the availability of proline in the growth medium, a genetic control mechanism that is dependent on the proline-responsive activator protein PutR (Belitsky, 2011; Moses *et al.*, 2012). Proline is the only compatible solute used by *B. subtilis* for osmostress protective purposes that can also be exploited as a nutrient through its PutBC-mediated catabolism to glutamate (Figure 11.2.1). This limits the effectiveness of exogenously provided proline as an osmostress protectant (Zaprasis *et al.*, 2013a) and requires genetic precautions to prevent the degradation of newly synthesized proline by high-osmolarity-challenged cells (Moses *et al.*, 2012).

Osmostress protective levels of proline can also be achieved by *B. subtilis* through the import of proline-containing di- and oligo-peptides via the Opp, Dpp, App, and DtpT transporters and their subsequent hydrolysis to release the compatible solute proline (Zaprasis *et al.*, 2013a). Furthermore, *B. subtilis* can replenish its osmostress-protective proline pool to derive osmostress protection by importing proteogenic (Glu, Gln, Asp, Asn, and Arg) and non-proteogenic (Orn, Cit) amino acids that can be metabolically converted into proline. One example is the import of glutamate (Glu) via the GltT transporter (Figure 11.2.1) (Zaprasis *et al.*, 2015); Glu serves as the direct precursor for proline biosynthesis in *B. subtilis* (Brill *et al.*, 2011a,b).

If one views the considerable number of osmostress adaptive transporters that are present in *B. subtilis*, the breadth of their substrate profile for compatible solutes, and the varied ways in which this bacterium can acquire osmoprotective levels of proline (Figure 11.2.1), one can readily see that *B. subtilis* is well prepared to exploit many osmoprotectants present in its varied habitats. Furthermore, the use of importers that either are energetically coupled to ATP hydrolysis (OpuA, OpuB, and OpuC) or are dependent for their functioning on ion gradient (OpuD and OpuE) provides additional flexibility to osmotically stressed *B. subtilis* cells.

With respect to the osmotically regulated OpuE proline transporter (von Blohn *et al.*, 1997), an interesting additional physiological function was discovered when its structural gene (*opuE*) was disrupted. In such a mutant strain, part of the newly synthesized proline produced as an osmostress protectant (discussed further in this chapter) is found in the supernatant of the cells grown in high-salinity medium (Hoffmann *et al.*, 2012). Because the osmostress adaptive proline pool is reduced in an *opuE* mutant, such a strain is at a significant growth disadvantage. Hence, under high-osmolarity growth condition, *B. subtilis* engages in a cycle of synthesis–release–recapture of proline. Although this cycle appears energetically wasteful at first sight, it might actually provide a useful physiological function since it could potentially aid the osmotically stressed cell to fine-tune turgor when it elongates and eventually divides (Hoffmann *et al.*, 2012). It is unclear how proline is released from the *B. subtilis* cells under steady-state high-salinity growth conditions. In *C. glutamicum*, a mechanosensitive channel of the MscS type has been implicated in the release of glycine betaine that had been imported from the environment (Börngen *et al.*, 2010). In contrast, in *B. subtilis* the involvement of MscL- and MscS-type channels (Figure 11.2.1) in the release of proline was firmly ruled out (Hoffmann *et al.*, 2012). Therefore, the question arises whether dedicated export systems for compatible solutes exist in microorganisms, similar to those mediating the efflux of amino acids (Eggeling and Sahm, 2003).

11.2.8 Synthesis of compatible solutes

Natural-abundance ^{13}C-NMR (carbon-13 nuclear magnetic resonance) spectroscopy has been used to assess the types of compatible solutes synthesized *de novo* by members of the Bacilli in response to high salinity. Three large groups were detected: (i) those that synthesized only glutamate, (ii) those that synthesized proline, and (iii) those that synthesized ectoine. In this latter group, ectoine production could be combined with the synthesis of the ectoine derivative 5-hydroxyectoine, or with the synthesis of proline (Bursy *et al.*, 2007; Kuhlmann and Bremer, 2002).

Although not studied in detail, these three groups differed in their ability to withstand salt stress: The glutamate producers were the most salt-sensitive *Bacillus* species, proline production conferred an intermediate degree of salt stress resistance, whereas those Bacilli that synthesized ectoines could withstand the highest levels of salt stress. None of the studied *Bacilli* synthesized glycine betaine *de novo,* and none produced the compatible solute trehalose (Bursy *et al.*, 2007), an osmostress protectant that is otherwise synthesized by many bacterial species in response to high salinity (Bremer and Krämer, 2000; Csonka, 1989; Kempf and Bremer, 1998; Wood *et al.*, 2001). We will not cover here the osmotically controlled synthesis of ectoine and 5-hydroxyectoine since *B. subtilis* does not produce these compatible solutes (Bursy *et al.*, 2007; Kuhlmann and Bremer, 2002), but we refer the reader to recent overviews that trace the synthesis of ectoines on genome-wide scales in members of the Bacteria and a few selected Archaea and that summarize the genetics, biochemistry, and biotechnological applications of these versatile stress protectants (Höppner *et al.*, 2014; Widderich *et al.*, 2014). Here, we will focus on the only compatible solute that *B. subtilis* can produce *de novo*, the amino acid proline, and the synthesis of glycine betaine from the precursor choline. All other compatible solutes that *B. subtilis* can acquire via transport cannot be synthesized by this bacterium (Bashir *et al.*, 2014a; Broy *et al.*, 2015; Hoffmann and Bremer, 2011).

11.2.8.1 *De novo* synthesis of proline

As in many other bacteria (Fichman *et al.*, 2015), in *B. subtilis*, proline biosynthesis proceeds from the precursor glutamate and involves three enzyme-catalyzed steps carried out by the γ-glutamyl kinase (ProB and ProJ), the γ-glutamyl-phosphate reductase (ProA), and the Δ^1-pyrroline-5-carboxylase reductase (ProI, ProH, and ProG) (Figure 11.2.4). *B. subtilis* possesses two proline biosynthetic routes that serve for the production of proline either as a building block for protein synthesis (ProB–ProA–ProI) or as an osmostress protectant (ProJ–ProA–ProH). These two pathways are needed since the cellular pools of proline required to fulfill the indicated physiological functions are strikingly different. Proline levels between 10 and 20 mM are found in osmotically nonstressed cells, whereas cells that are severely stressed by high salinity (e.g., 1.2 M NaCl) possess proline pools of about 500 mM (Hoffmann *et al.*, 2013; Whatmore *et al.*, 1990; Zaprasis *et al.*, 2013a).

The first and last steps of the anabolic and osmostress-adaptive proline biosynthetic routes in *B. subtilis* are catalyzed by iso-enzymes, but it is not obvious why these pathways are interconnected through ProA (Figure 11.2.4b). In other Bacilli (e.g., *Bacillus licheniformis*), two complete sets of proline biosynthetic enzymes are present to fulfill the cells' anabolic and osmostress adaptive needs for proline production (Schroeter *et al.*, 2013).

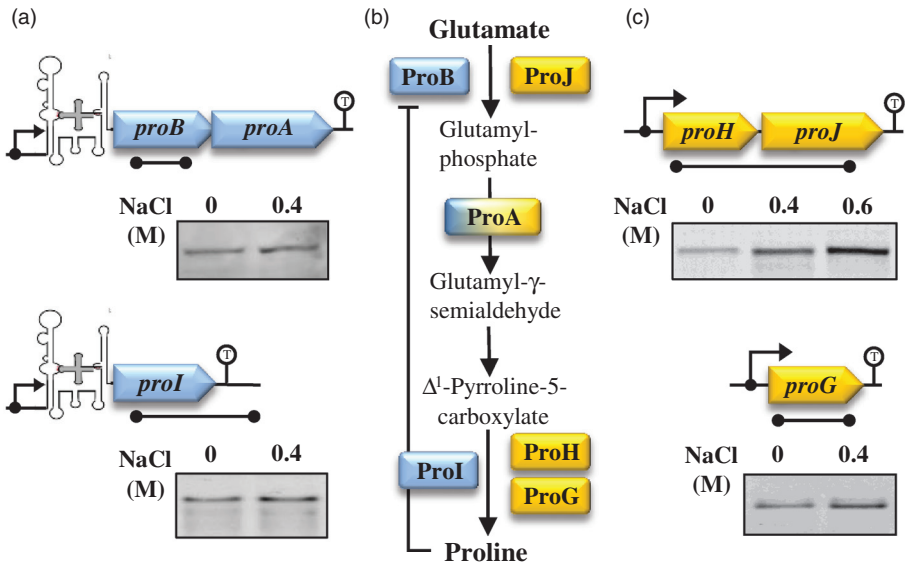

Figure 11.2.4 Interlinked anabolic and osmostress-adaptive proline biosynthetic pathways in *B. subtilis*. The ProB–ProA–ProI and ProJ–ProA–ProH enzymes mediate the anabolic and osmostress-adaptive proline biosynthetic routes, respectively; they are interlinked via the *proA*-encoded γ-glutamyl-phosphate-reductase (ProA) (Brill *et al.*, 2011a, 2011b). Like *proI* and *proH*, the *proG* gene encodes a Δ^1-pyrroline-5-carboxylate reductase, but the true physiological role of the ProG enzyme remains unclear. (a) The anabolic proline biosynthetic enzymes are encoded by the *proBA* operon and by the *proI* gene. These genes are under the regulatory control of an mRNA-based T-box control mechanism that inhibits transcription when the intracellular proline concentration has a level sufficient for anabolic purposes. Northern blot analyses of the *proBA* and *proI* transcripts in cells grown in SMM in the absence or the presence of NaCl were carried out. (b) Synthesis routes of proline under osmotic stress conditions (yellow boxes) and for anabolic purposes (blue boxes) from the biosynthetic precursor proline. Proline inhibits the anabolic glutamate-kinase (ProB) at the level of enzyme activity via a negative feedback loop. (c) Genetic organization of the osmotically controlled *proHJ* operon and of the *proG* gene, and levels of their mRNA in cells grown in the presence of various salinities (assessed by Northern blot analysis). This figure was crafted based on previously published data (Brill *et al.*, 2011a, 2011b)

The biochemistry and genetics of the anabolic ProB–ProA–ProI route in *B. subtilis* are geared to prevent a wasteful overproduction of proline and to adjust the proline pool with the ongoing protein biosynthetic activities of the cell. This is achieved (i) through a sensitive feedback regulation of the enzyme activity of the γ-glutamyl kinase (ProB) by proline (Figure 11.2.4b) (Chen *et al.*, 2007), and (ii) through transcriptional control of the *proBA* and *proI* genes through a T-box regulatory mechanism (Figure 11.2.4a) (Brill *et al.*, 2011b). T-box-controlled genes possess long nontranslated 5′ messenger RNA (mRNA) sequences that can fold in two mutually exclusive secondary structures and thereby affect the transcription of the full-length coding sequence (Gutierrez-Preciado *et al.*, 2009). When the proline pool is sufficient to fuel protein biosynthesis, *proBA* and *proI* expression is limited through the folding of the 5′UTR-mRNA region into a terminator structure that thereby prevents the transcription of the coding regions. When the cells starve for proline, the 5′UTR-mRNA region assumes an antiterminator structure that allows the transcription of the full-length *proBA* and *proI* genes. This antiterminator structure is stabilized by the specific binding of the uncharged prolyl-tRNA via a proline-specific specifier codon; hence, the loading status of a $tRNA^{Pro}$ is used by the cell as readout of an insufficient proline pool (Gutierrez-Preciado *et al.*, 2009). In contrast, the prolyl-tRNA charged with proline cannot make such interactions with the T-box device and thus promote premature transcription termination of the *proBA* and *proI* mRNAs (Brill *et al.*, 2011b).

It is obvious that the biochemistry and genetics of the ProB–ProA–ProI route are unable to provide the very large pools of proline (up to 0.5 M) needed for osmostress protection (Brill *et al.*, 2011a; Whatmore *et al.*, 1990; Zaprasis *et al.*, 2013b). Hence, *B. subtilis* has developed a second route (ProJ–ProA–ProH) (Figure 11.2.4b and 11.2.4c) that produces proline under osmotic stress conditions (Brill *et al.*, 2011a). Although not proven experimentally, the enzyme activity of the ProJ γ-glutamyl kinase, unlike that of ProB, cannot be strongly influenced by proline-mediated feedback control since this type of posttranslational control sets in already at μM concentrations of the effector molecule (Chen *et al.*, 2007). The major osmotic control of the ProJ–ProA–ProH biosynthetic route occurs at the level of transcription of the *proHJ* operon (Figure 11.2.4c) (Brill *et al.*, 2011a). It is expressed from a SigA-responsive and osmotically controlled promoter. Detailed reporter gene fusion analysis of *proH–treA* constructs has shown that *proHJ* expression is strongly increased both subsequent to sudden osmotic upshifts and during sustained increases in the external osmolarity. Once the external osmolarity has exceeded a certain threshold value, *proHJ* expression increases linearly in response to stepwise increases in the osmolarity (Brill *et al.*, 2011a) and thereby satisfies the need of the *B. subtilis* cell for an increased proline biosynthetic capacity as a defense against the detrimental effects of high osmolarity on cellular hydration and turgor (Figure 11.2.3c).

11.2.8.2 Synthesis of glycine betaine from the precursor choline

The trimethylammonium compound glycine betaine is, without any doubt, the most widely employed compatible solute in nature since members of each domain of life use it as an osmostress protectant (Yancey, 2005). In bacteria, glycine betaine can be synthesized either through the stepwise methylation of glycine or through the oxidation of the precursor molecule choline. The *de novo* synthesis of glycine betaine through the methylation route requires regeneration of the cofactor of the biosynthetic enzymes, *S*-adenosyl-methionine, a process that demands high energy. The methylation route is therefore infrequently used by microorganisms to produce glycine betaine (Nyyssölä *et al.*, 2000). Instead, glycine betaine is most frequently produced through the oxidation of choline that typically must be imported by the cells prior to its conversion into glycine betaine. Various combinations of enzymes can be used to synthesize glycine betaine from choline.

In *B. subtilis*, a combination of an alcohol dehydrogenase (GbsB) and a glycine betaine aldehyde dehydrogenase (GbsA) is used to enzymatically convert choline to glycine betaine (Figure 11.2.5) (Boch *et al.*, 1996). Since *B. subtilis* cannot synthesize choline, it needs to import it from the environment. Two ABC transporters with high affinity for choline are used for

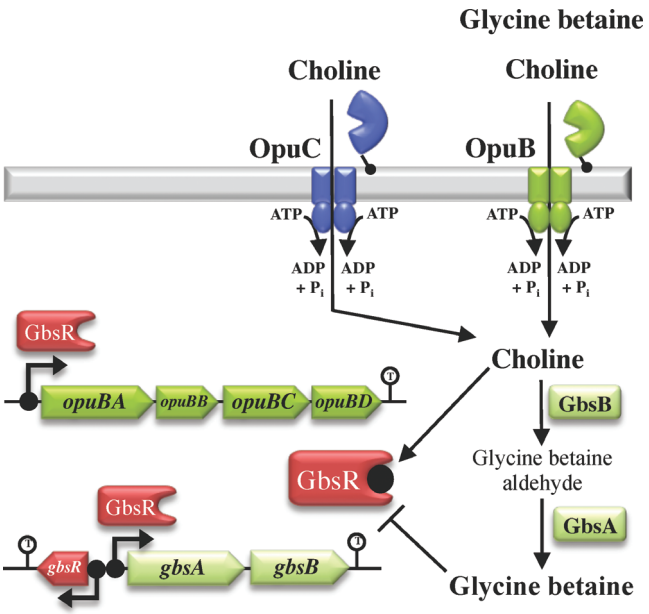

Figure 11.2.5 Synthesis of the osmostress protectant glycine betaine from the precursor choline. Shown are the OpuB and OpuC ABC transporters that are able to take up choline and/or glycine betaine and the GbsB–GbsA-catalyzed glycine betaine synthesis pathway from the precursor choline. Transcription of the corresponding operons (*opuB*, *gbsAB*) is under control of the choline-responsive glycine betaine synthesis repressor protein GbsR. This figure was crafted based on previously published data (Kappes *et al.*, 1999; Nau-Wagner *et al.*, 2012).

this purpose, OpuB and OpuC. The OpuB system only imports choline and the intermediate in glycine betaine synthesis, glycine betaine aldehyde, whereas OpuC cannot only import these two compounds but exhibits a very broad substrate specificity (for an overview of the types of compatible solutes imported via OpuC, see Hoffmann and Bremer (2011) and Broy *et al.* (2015)). The synthesis of glycine betaine from choline confers a considerable degree of osmotic stress resistance; it should be noted in this context that choline is no compatible solute per se, and its import therefore does not confer osmostress protection unless it is converted into glycine betaine (Boch *et al.*, 1994, 1996).

B. subtilis is a bacterium that frequently lives in association with plants or plant detritus (Belda *et al.*, 2013) and therefore can acquire choline from degraded phospholipids of eukaryotic cell membranes. Since neither choline nor glycine betaine can be used as nutrients by *B. subtilis* (Boch *et al.*, 1994), enhanced import of the glycine betaine precursor makes physiological sense only under conditions when the cells need to synthesize glycine betaine for osmoprotective purposes and when choline is present in the environment. The regulatory circuit that governs glycine betaine synthesis from choline reflects this fact. The GbsR repressor, a member of the MarR-type family of transcriptional regulators (Figure 11.2.5), controls both the *gbsAB* glycine betaine synthesis genes and the operon encoding the choline-specific OpuB transporter. Choline serves as the effector molecule for the GbsR repressor protein, and its binding prevents DNA binding by GbsR to its cognate operator sequences (Nau-Wagner *et al.*, 2012). A model has been proposed that envisions a structural rearrangement of the N-terminal DNA-reading head relative to the dimerization domain of GbsR once the effector molecule choline has been bound (Nau-Wagner *et al.*, 2012). Interestingly, glycine betaine aldehyde (Figure 11.2.5) also serves as an effector molecule for GbsR and thereby ensures that this toxic intermediate in glycine betaine synthesis (Boch *et al.*, 1996; Kappes *et al.*, 1999) never accumulates to significant intracellular levels.

B. subtilis sets its intracellular glycine betaine pool very precisely and in tune with the degree of the osmotic stress that is experienced by the cell (Hoffmann *et al.*, 2013). The GbsR-mediated regulatory circuit described in this chapter will lead to continuous glycine betaine synthesis as long as the inducer choline is present in the environment, regardless of the osmotic needs of the cell. This would be very wasteful, as the cell would be eventually forced to expel the newly synthesized glycine betaine to avoid physiologically inadequate values of turgor. Therefore, in the GbsR-mediated regulatory system, a negative feedback loop has been built in that re-establishes GbsR-mediated repression of the *gbsAB* and *opuB* operons once the cell has produced enough glycine betaine to balance the osmotic gradient across the cytoplasmic membrane. Not surprisingly, glycine betaine served as the cellular cue to accomplish this, and GbsR is the target of this genetic negative feedback loop. The GbsR regulatory protein thus records and integrates cellular and environmental signals for both the onset and the repression of the synthesis of the osmoprotectant glycine betaine in *B. subtilis* (Nau-Wagner *et al.*, 2012).

11.2.9 Osmotic control of gene expression

Due to the transient nature of the pulse in SigB-controlled gene expression (Young *et al.*, 2013), implementation of specific stress-adaptive pathways, such as the synthesis and uptake of osmoprotectants, is required for cellular adaptation to sustained environmental challenges (Bremer, 2002; Bremer and Krämer, 2000). However, there is an interesting interlink between the general stress response system and specific adaptation reactions of the *B. subtilis* cell exposed to sustained high-osmolarity environments. This interlink is manifested in the genetic control of the *opuE* and *opuD* genes.

OpuE mediates the import of proline as an osmostress protectant (von Blohn *et al.*, 1997). Genetic control of the *opuE* gene integrates general and specific stress responses via two intertwined and osmotically controlled promoters: One is controlled by the housekeeping sigma factor of *B. subtilis*, SigA, and the other is responsive to SigB (Figure 11.2.2b) (Spiegelhalter and Bremer, 1998). In the wild-type *opuE* gene, the level of transcription increases linearly in response to incremental, but lasting, increases in the external salinity (Figure 11.2.2c). The inactivation of either the SigA- or the SigB-type promoters revealed their individual contributions to the osmotic control of *opuE* expression in either salt-shocked or salt-adapted cells. The SigA-type promoter mediated a dose-dependent response in cells subjected to sustained salt stress, whereas the SigB-type promoter is completely inactive under these conditions (Figure 11.2.2c); however, it permitted a rapid but transient induction of *opuE* transcription subsequent to a salt shock. The SigA-type promoter was also responsive to a salt shock, but the kinetics of its activation was somewhat delayed in comparison with the SigB-type promoter (Figure 11.2.2d). Hence, the combined activities of these two promoters allow both a rapid and well-graded physiological response to salt challenges through the OpuE-mediated import of the osmoprotectant proline.

Interestingly, the *opuD* glycine betaine transporter gene (Kappes *et al.*, 1996) is also part of the SigB-controlled general stress response system (Nicolas *et al.*, 2012). As detailed for *opuE*, it is expressed from closely spaced SigA- and SigB-type promoters, whose activity is enhanced in response to osmotic stress (our unpublished data). As a consequence, the double osmotic control of *opuD* expression provides enhanced glycine betaine transport capacity both of osmotically upshocked cells and to *B. subtilis* cells continuously challenged by high osmolarity (Kappes *et al.*, 1996).

Genome-wide transcriptional analysis has been performed with both salt-shocked and continuously osmotically stressed *B. subtilis* cells (Hahne *et al.*, 2010; Kohlstedt *et al.*, 2014; Nicolas *et al.*, 2012; Steil *et al.*, 2003), and thereby provides a comprehensive overview of the transcriptional landscape of

osmotically stressed *B. subtilis* cells. It is beyond the scope of this overview to discuss in detail each of these changes in gene expression; instead, we focus on a few selected examples of genes where more detailed information is already available from targeted genetic and physiological studies.

In the transcriptome analysis reported by Steil *et al.* (2003), approximately 5% of the then annotated 4107 protein-coding genes differ significantly (at least threefold) in their expression levels between high- and low-salinity-grown cells. Transcription of 101 genes is downregulated, and most of these genes are functionally associated with either the synthesis of the flagellum, chemotaxis, or (unknown at that time) the production of an extracellular polysaccharide (EPS) matrix. Indeed, high-salinity-challenged *B. subtilis* cells can no longer swim (Steil *et al.*, 2003); the synthesis of the Hag protein, the major component of the flagellum, is drastically reduced (Hoffmann *et al.*, 2002); and the production of the sugar matrix involved in building the *B. subtilis* biofilm is downregulated (Rubinstein *et al.*, 2012). Expression of 123 genes is upregulated in the study conducted by Steil *et al.* (2003), and of these 21 are only indirectly affected by high salinity since such growth conditions triggered in the *B. subtilis* strain used a severe iron limitation (Hoffmann *et al.*, 2002). The group of 21 salt-inducible genes is functionally annotated either in connection with iron acquisition or with the synthesis of the iron chelator bacillibactin; a bioinformatics analysis revealed that they are all members of the Fur regulon (Hoffmann *et al.*, 2002; Steil *et al.*, 2003). Hence, the expression of 102 genes has been found to be truly induced by high salinity, and genes encoding either uptake or synthesis systems for compatible solutes are prominently represented in this group. A substantial number of genes involved in cell wall synthesis or modification are also represented among the salt-induced genes, indicating that *B. subtilis* modifies its cell wall when it is continuously exposed to high salinity (Steil *et al.*, 2003). Genes falling into this functional group have also been identified as osmotically controlled in other studies (Fischer and Bremer, 2012; Lopez *et al.*, 1998, 2000; Palomino *et al.*, 2009).

A substantial overlap (20 out of 102) of osmotically induced genes (Steil *et al.*, 2003) with members of the DegSU regulon (Mäder *et al.*, 2002) has been observed. This includes (i) the structural genes for the DegS sensor kinase and its cognate response regulator DegU, and (ii) an operon (*rapG–phrG*) that encodes an exported regulatory pro-peptide (PhrG) and a PhrG-responsive regulator (RapG) that controls the DNA-binding activity of DegU-P via protein–protein interactions (Ogura *et al.*, 2003). This implies that the level of DegSU is upregulated in high-salinity-grown cells and that a fine-tuning regulatory circuit exists that operates via the RapG–PhrG signaling system and sets the level and activity of DegU–DegU-P at high salinity. These findings are of special interest for two reasons: (i) The DegSU two-component regulatory system has been previously implicated in the salt stress response of *B. subtilis* through functional studies (Kunst and Rapoport, 1995; Ruzal and Sanchez-Rivas, 1998); and (ii) the DegS sensor kinase is one of only two sensor kinases out of 36 sensor kinases identified in *B. subtilis* that are localized in the *cytoplasm* (Fabret *et al.*, 1999). As we have discussed here in the context of the stressosome controlling the expression of the SigB general stress response system (Marles-Wright *et al.*, 2008), the cytoplasmic localization of DegS requires that the sensing of salt stress imposed by the environment has to rely on a derived cytoplasmic signal that then can be perceived by DegS. The nature of this signal and the way it might be recognized by the DegS sensor kinase are unknown.

The DegSU-regulated genes found by Steil *et al.* (2003) comprise only about one-fifth of the *B. subtilis* genes whose transcription is upregulated in response to sustained high osmolarity. Hence, the DegSU two-component system is definitely not the overarching osmotic stress-sensing and response system of *B. subtilis*, as has been suggested previously (Ruzal and Sanchez-Rivas, 1998). This conclusion is substantiated by detailed reporter studies and *degSU* mutant analysis in the framework of studies addressing the properties of the SigA-dependent *opuA* promoter, a promoter that is strongly inducible by high osmolarity but is not influenced by the DegSU two-component system (Hoffmann *et al.*, 2013). It also should be noted in this context that the DegSU system is involved in the regulation of a diverse set of cellular processes that probably generate different types of environmental and cellular signals to which the DegS sensor kinase will then respond (Cairns *et al.*, 2013). In no case have the precise type(s) of signal(s) and the molecular mechanism(s) of their perception by DegS been elucidated.

Here, we describe the osmotic control of the *opuA*, *proHJ*, and *yqiHIK* operons from *B. subtilis* since detailed transcriptional and mutational studies have been reported for their promoters. The *opuA* operon (*opuAA–opuAB–opuAC*) encodes the components of the OpuA ABC transport system for glycine betaine (Figure 11.2.1) (Hoffmann *et al.*, 2013; Kempf and Bremer, 1995), *proHJ* encodes the key enzymes for the osmostress adaptive proline biosynthetic route (Figure 11.2.4b,c) (Brill *et al.*, 2011a), and the *yqiHIK* operon encodes a lipoprotein exposed at the cell surface (YqiH), an extracellular *N*-acetyl-muramyl-*L*-alanine amidase (YqiI), and a cytoplasmic glycerophosphodiester phospodiesterase (YqiK) (Fischer and Bremer, 2012). In each of these three operons, the osmotically controlled promoter has been mapped by primer extension analysis (Figure 11.2.6a), and its activity has been studied genetically via reporter gene fusion analysis and site-directed mutagenesis studies. The sequences of the −10 and −35 regions of all three promoters resemble those of SigA-type housekeeping promoters from *B. subtilis* but deviate in key positions from the consensus sequence (Figure 11.2.6a). Mutations that improve the match of the *opuA*, *proHJ*, and *yqiHIK* promoters to the consensus sequence lead to enhanced gene expression both at low and at high salinity, indicating that the deviations from the SigA-type consensus sequence serve to keep the activity of these osmotically controlled promoters low in cells grown in low-salinity

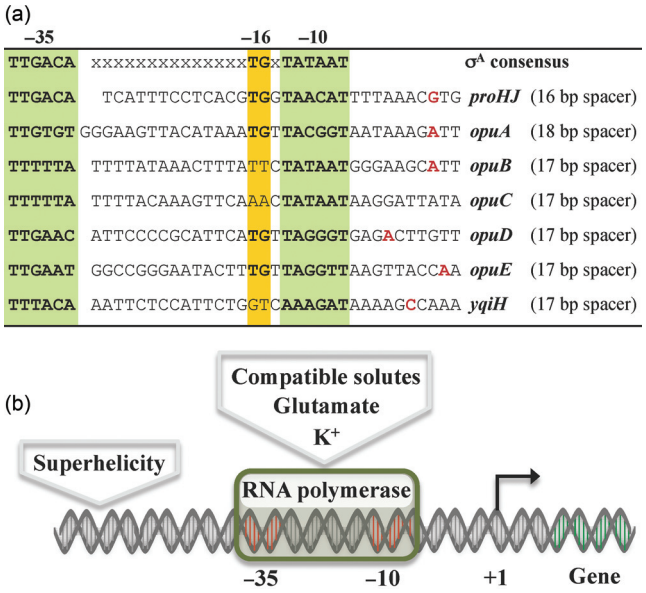

Figure 11.2.6 DNA sequences of osmotically regulated promoters of *B. subtilis* that have been studied experimentally. (a) Alignments of promoter sequences of *B. subtilis* that have been shown to be osmotically inducible. Green blocks mark the characteristic −10 and −35 regions of SigA-type promoters, and the yellow block marks a TG motif often found in *B. subtilis* promoters at position −16. The transcription initiation sites that have been mapped via primer extension analysis are marked in red. (b) Potential cytoplasmic cues that might trigger osmotically controlled gene expression.

minimal media. In none of the studied promoter variants is the osmotic control entirely lost, but it is often strongly reduced (Fischer and Bremer, 2012; Hoffmann *et al.*, 2013; T. Hoffmann, M. Bleisteiner, and E. Bremer, unpublished data).

Transcriptional studies with reporter gene fusions show that the activity of the promoters of the *opuA*, *proHJ*, and *yqiHIK* operons is finely tuned and sensitively linked to the degree of the osmotic stress imposed onto the cell. However, the pattern of gene expression is strikingly different among the three studied systems: (i) *opuA* transcription increases linearly with the environmental salinity up to a level of 0.6 M NaCl, and then levels off (Hoffmann *et al.*, 2013); (ii) *proHJ* transcription remains at a basal level up to medium salinity of 0.2 M NaCl, and then increases linearly up to the maximum tested salinity of 1.0 M NaCl (Brill *et al.*, 2011a); and (iii) *yqiHIK* transcription remains at a basal activity level up to the substantial salinity of 0.6 M NaCl, and thereafter increases linearly up to the maximum tested salinity of 1.2 M NaCl (Fischer and Bremer, 2012). These examples illustrate that the *B. subtilis* cell is able to transform information gleaned about environmentally imposed osmotic stress into an individual pattern of promoter activity.

The fact that the *B. subtilis* cell can really sense osmotic stress, rather than NaCl-elicited stress alone, has been established through studies with the *opuA* and *proHJ* promoters (Brill *et al.*, 2011a; Hoffmann *et al.*, 2013). Both promoters required for their osmotic activation the establishment of a trans-membrane osmotic gradient, since enhanced *opuA* and *proHJ* promoter activity could not be elicited by glycerol, an osmolyte that is readily membrane-permeable at high external concentrations. Conversely, both ionic (NaCl and KCl) and non-ionic (sucrose and lactose) osmolytes that are not membrane permeable triggered strongly enhanced levels of *opuA* and *proHJ* transcription. Curiously, the tested non-ionic osmolytes elicited a far stronger increase in gene expression in comparison with isoosmotic concentrations of ionic osmolytes, an observation that is so far not rationally explainable (Brill *et al.*, 2011a; Hoffmann *et al.*, 2013). Interestingly, the synthesis (e.g., proline or glycine betaine) or uptake (e.g., glycine betaine or carnitine) of compatible solutes downregulates the level of *opuA* and *proHJ* transcription. This fits with the idea that the degree of osmotic stress, as reflected by the steepness of the osmotic gradient between the outside and the inside of the cell, is sensed by the *B. subtilis* cell and is then equated into the transcriptional control of the *opuA* and *proHJ* promoters.

11.2.10 Biofilm formation and osmotic forces

B. subtilis has long been a favorite microorganism for bacterial geneticists and physiologists, and their work has made this bacterium *the* model system for Gram-positive bacteria. However, the long life in the laboratory led to the domestication of *B. subtilis*, and in the course of this process several mutations were acquired by the *B. subtilis* 168 lineage of laboratory strains that resulted in the loss of a key developmental trait, namely the ability to form robust biofilms on solid surfaces and at liquid–air interfaces (see Section 22). The ability of nondomesticated *B. subtilis* strains to form such biofilms (Branda *et al.*, 2001) is genetically determined by rather complex regulatory circuits, and we refer the reader to several excellent recent reviews on this topic (Lopez *et al.*, 2009b; Vlamakis *et al.*, 2013). It suffices here to state that the life of individual cells in liquid media is very different from that of cells encased in the biofilm that forms multicellular communities in which different cell types can coexist. This is particularly true when one considers the osmotic forces that exists within the biofilm and that shape its growth (Seminara *et al.*, 2012). The biofilms matrix formed by *B. subtilis* consists of a copious amount of EPS and an amyloid fiber-forming protein (TasA) (Vlamakis *et al.*, 2013). The surface of the biofilm is coated with a layer of the BslA protein, a hydrophobin that provides properties to the surface of these structures resembling those of Teflon; hence, water does not penetrate from above into the biofilm (Hobley *et al.*, 2013). The considerable osmotic potential of the EPS matrix results in the influx of water from the agar surface into the biofilm, and the thereby generated osmotic forces lead to the spreading of the biofilm (Seminara *et al.*, 2012).

Within the biofilm, microscopic water channels exist that transport nutrients and waste products.

The increase in osmotic pressure caused by the formation of the EPS is a cue that downregulates the gene cluster (*epsA-O*) required for the synthesis of the sugar component of the extracellular matrix and the operon (*tapA–sipW–tasA*) that directs the formation of the amyloid-like fibers (Nicolas *et al.*, 2012; Rubinstein *et al.*, 2012). These EPS-elicited nonspecific osmotic pressure effects activate the histidine kinase KinD, which in turn directs the phosphorylation of the master regulatory protein of sporulation and biofilm formation, Spo0A, which at high levels represses matrix gene expression (Rubinstein *et al.*, 2012). The physiological and genetic consequences of high osmotic pressure within the biofilm are not yet fully comprehended, but Rubinstein *et al.* (2012) suggested that the sensing of this physical cue might be a strategy to coordinate multicellular behavior in the confines of the biofilm (Rubinstein *et al.*, 2012).

An interesting connection between the genetic system that directs biofilm formation in *B. subtilis* and osmotically controlled expression of genes encoding uptake systems for compatible solutes has recently been uncovered in a study conducted by Kearns and coworkers (Winkelman *et al.*, 2013). A key regulator of biofilm formation, SinR, negatively controls expression of the *eps* and *tapA–sipW–tasA* operons. The RemA activator protein antagonizes the repressing effects of SinR on *eps* and *tapA–sipW–tasA* expression and thereby stimulates biofilm formation. In connection with a DNA-profiling study of a *remA* mutant strain, the expression of the *opuA*, *opuB*, and *opuC* operons in osmotically nonstressed cells was found to be affected (Winkelman *et al.*, 2013). DNA-footprinting studies with the purified RemA protein at the *opuA* promoter region revealed an extended RemA-binding region upstream of the *opuA* −35 sequence; it is composed of five distinct subsites (Winkelman *et al.*, 2013). The *opuA* regulatory region is thus a direct target of a transcription control protein that is essential for biofilm formation by *B. subtilis* (Winkelman *et al.*, 2009). Reporter gene studies showed that RemA, like its actions at the *eps* and *tapA–sipW–tasA* regulatory regions (Winkelman *et al.*, 2009, 2013), serves as an activator protein and is crucial for *opuA* expression under both low- and high-salinity growth conditions (T. Hoffmann and E. Bremer, unpublished data). However, it is not yet clear whether RemA transduces the osmotic signal to the *opuA* promoter, or whether it just serves a role of a general activator of *opuA* transcription, a function that would be independent from the signal transduction pathway that confers osmotic control of promoter activity.

We find the observation quite remarkable that the structural genes for three osmostress protectant uptake systems (OpuA, OpuB, and OpuC) are members of the RemA regulon (Winkelman *et al.*, 2013). It bears the question of how *B. subtilis* cells embedded in a biofilm might actually profit for their physiology and well-being from the import of compatible solutes.

11.2.11 The big challenge: sensing osmotic changes

Since the 1990s, the basic cellular adjustment strategies of *B. subtilis* to fluctuations in the external salinity have been worked out in quite some detail. However, many questions remain with respect to the precise physiological functions of those genes whose transcription is upregulated either under acute osmotic upshifts or under sustained salt stress (Hahne *et al.*, 2010; Höper *et al.*, 2005., 2006; Kohlstedt *et al.*, 2014; Nannapaneni *et al.*, 2012; Nicolas *et al.*, 2012; Steil *et al.*, 2003). The most pressing question that needs to be answered is how increases in the external osmotic pressure are perceived, genetically processed, and then transduced to the transcription apparatus so that altered gene expression will ensue. Players in such a process have been implicated in the case of the SigB-controlled stress response (via the stressosome), biofilm formation (via KinD), and the DegSU two-component regulatory system. KinD is a membrane-embedded sensor kinase, whereas the stressosome and the DegS sensor kinase are located in the cytoplasm. As discussed in this chapter in the context of these latter two systems, both the stressosome and DegS must perceive a cytoplasmic signal that is indirectly derived from changes in the external osmolarity. Although KinD, the stressosome, and DegS have all been implicated in the cellular osmostress response, it is unclear how the *B. subtilis* cell actually uses these devices to measure – either directly or indirectly – a change in an external physical parameter, environmental osmolarity. It should be noted here that the systems directly involved in ameliorating the dire consequences of high osmolarity on cellular physiology (e.g., systems for uptake and synthesis of compatible solutes) (Figure 11.2.1) are either fully independent of (e.g., *opuA* and *proHJ*), or are only partially dependent on (e.g., *opuE* and *opuD*), these rather general regulatory systems.

An overarching osmosensory and regulatory system that might operate in *B. subtilis* has not been detected. This might be due to the fact that such a system might be essential, that the screening procedures used to search for it might be unsuitable, or that it simply does not exist. If one just considers the last scenario to be correct, how might osmotic control of the SigA-type promoters of the *opuA*, *opuB*, *opuC*, *opuD*, *opuE*, *proHJ*, and *yqiHIK* genes (Figure 11.2.6a) be achieved in the absence of an osmostress-specific regulatory protein? High osmolarity alters the ionic composition and biophysical properties of the cytoplasm (Cayley and Record, 2003; Whatmore *et al.*, 1990; Wood, 2011). Such changes might be sensed directly by the RNA polymerase, and in combination with altered DNA-superhelicity (Higgins *et al.*, 1988; Krispin and Allmansberger, 1995), specific promoter sequences and their flanking regions might change the transcriptional activity of a given osmoregulated promoter (Figure 11.2.6b). It will be a challenge to experimentally verify or refute such a model. The *proHJ* promoter with its essentially linear dependence for its activity on the external osmolarity (Brill

et al., 2011a) might provide a suitable test system for the outlined hypothesis.

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