Review

Tamara Hoffmann and Erhard Bremer*

Guardians in a stressful world: the Opu family of compatible solute transporters from *Bacillus subtilis*

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Abstract: The development of a semi-permeable cytoplasmic membrane was a key event in the evolution of microbial proto-cells. As a result, changes in the external osmolarity will inevitably trigger water fluxes along the osmotic gradient. The ensuing osmotic stress has consequences for the magnitude of turgor and will negatively impact cell growth and integrity. No microorganism can actively pump water across the cytoplasmic membrane; hence, microorganisms have to actively adjust the osmotic potential of their cytoplasm to scale and direct water fluxes in order to prevent dehydration or rupture. They will accumulate ions and physiologically compliant organic osmolytes, the compatible solutes, when they face hyperosmotic conditions to retain cell water, and they rapidly expel these compounds through the transient opening of mechanosensitive channels to curb water efflux when exposed to hypo-osmotic circumstances. Here, we provide an overview on the salient features of the osmostress response systems of the ubiquitously distributed bacterium *Bacillus subtilis* with a special emphasis on the transport systems and channels mediating regulation of cellular hydration and turgor under fluctuating osmotic conditions. The uptake of osmostress protectants via the Opu family of transporters, systems of central importance for the management of osmotic stress by *B. subtilis*, will be particularly highlighted.

Keywords: glycine betaine; K^+ and Na^+ homeostasis; L-proline; osmoprotectants; osmotic stress.

***Corresponding author: Erhard Bremer,** Laboratory for Microbiology, Department of Biology, Philipps University Marburg, Karl-von-Frisch Str. 8, D-35043 Marburg, Germany; and LOEWE Center for Synthetic Microbiology, Philipps University Marburg, Hans-Meerwein-Str. 6, D-35043 Marburg, Germany, e-mail: bremer@staff.uni-marburg.de
Tamara Hoffmann: Laboratory for Microbiology, Department of Biology, Philipps University Marburg, Karl-von-Frisch Str. 8, D-35043 Marburg, Germany

Introduction

Bacillus subtilis, the model organism for Gram-positive bacteria, is ubiquitously distributed in the environment, and can be found in terrestrial, in plant-associated, and in marine ecosystems (Earl et al., 2008; Mandic-Mulec et al., 2015). One of its main habitats is the upper layer of the soil. The functional annotation of its 4.2 Mbp genome sequence (Barbe et al., 2009) shows that it is well adapted to life in this habitat through an abundance of uptake and catabolic systems allowing it to take advantage of many plant-derived compounds for growth (Belda et al., 2013). *B. subtilis* can exist in the soil as motile cells, actively seeking nutrients through chemotaxis (Yang et al., 2015), as complex-structured microbial communities (biofilms) established on roots and other plant material (Chen et al., 2012; Vlamakis et al., 2013), or as dormant endospores formed by vegetative cells when they were starving for nutrients (Higgins and Dworkin, 2012). The soil is a complex habitat for microorganisms (Carey, 2016) since both the nutrient supply and various abiotic factors (oxygen availability, pH, temperature, osmolarity) vary frequently (Earl et al., 2008; Mandic-Mulec et al., 2015). Hence, for life of *B. subtilis* in this challenging ecosystem there is only one constant: change.

One can readily envision how hyper- and hypo-osmotic conditions are created in the upper layers of the soil through desiccation and rainfall. This will affect the composition of the complex soil microbial community (Carey, 2016), and its metabolic activities (Warren, 2014; Bouskill et al., 2016). Here, we focus on those cellular events that ensue in *B. subtilis* cells when the external osmolarity fluctuates. Fluctuations in the environmental osmolarity will trigger in the stressed *B. subtilis* cell pronounced and varied osmostress-adaptive reactions (Steil et al., 2003; Hahne et al., 2010; Nannapaneni et al., 2012; Kohlstedt et al., 2014; Hoffmann and Bremer, 2016). We review here the salient features that allow this bacterium to cope with both increases and decreases in the environmental osmolarity of its varied habitats to achieve osmotic

homeostasis on a systems-wide level. We highlight the pivotal role played by transporters and channels in this complex cellular adjustment processes (Bremer and Krämer, 2000; Bremer, 2002).

Caused by the high concentration of nucleic acids, proteins and metabolites, the cytoplasm is a very crowded confined compartment with a considerable osmotic potential (Wood, 2011). Osmotically driven water influx will thus ensue (Record et al., 1998) and thereby generate an outward-directed hydrostatic pressure, the turgor (Wood, 2011; Booth, 2014). Turgor is considered as essential for cell viability and growth and is regarded as a driving force for cell expansion when the semi-elastic peptidoglycan sacculus is extended through biosynthetic processes during cell elongation (Typas et al., 2012). Notoriously difficult to determine experimentally (Deng et al., 2011; Wood, 2011; Booth, 2014), the magnitude of turgor in *B. subtilis* has been reported as 1.9 MPa (equals 18.75 atm) (Whatmore and Reed, 1990) (Figure 1), a value close to 10 times the pressure present in a standard car tire (2 atm). It should be noted however in this context, that the magnitude of the reported turgor in *Escherichia coli* (about 4 atm) might have been substantially overestimated (by more than 10-fold) (Deng et al., 2011; Booth, 2014), and it thus seems possible that turgor measurements in *B. subtilis* (Whatmore and Reed, 1990) might suffer from the same problem.

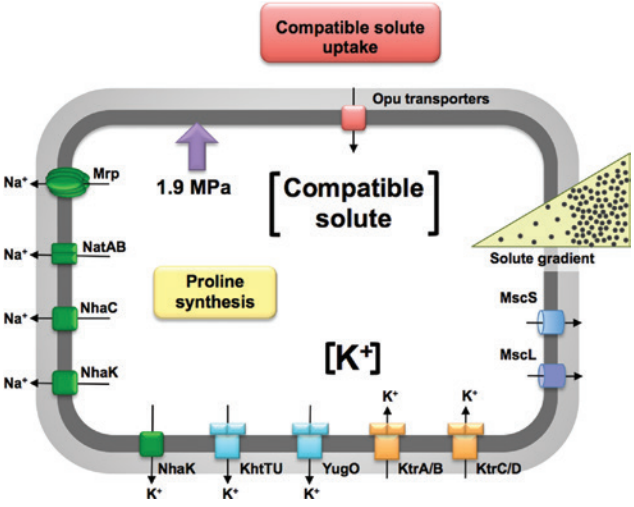

Figure 1: The core of the 'salt out' strategy of *B. subtilis*. Shown are the systems that mediate cellular K^+ and Na^+ homeostasis, synthesis of the osmostress protectant L-proline, the import of compatible solutes (Opu transporters), and MscS- and MscL-type mechanosensitive channels serving as safety valves for the rapid release of ions and organic solutes upon sudden osmotic down-shocks.

Many bacteria possess dedicated water-conducting channels, the AqpZ-type aquaporins, which mediate accelerated water fluxes across the cytoplasmic membrane (Delamarche et al., 1999; Calamita, 2000). However, their precise role during microbial osmostress adaptation is not comprehensively understood (Tanghe et al., 2006). They are certainly not essential for cellular osmotic homeostasis as a considerable number of bacteria lack them altogether; *B. subtilis* belongs to this latter group of microorganisms (Barbe et al., 2009). Regardless of the existence of aquaporins, no microorganism can actively (as an energy-dependent process) pump water in a directed fashion in or out of the cell to compensate for water fluxes triggered by changes in the external osmolarity. If these water fluxes would not be counterbalanced, dehydration and growth arrest (at high external osmolarity) or rupture (at low external osmolarity) of the cell would ensue (Bremer and Krämer, 2000; Booth, 2014). To avoid such catastrophic events, the cell actively adjusts the osmotic potential of its cytoplasm to indirectly scale and direct water fluxes across the cytoplasmic membrane in order to keep turgor within physiologically acceptable boundaries and to adjust the solvent properties of its cytoplasm (Bremer and Krämer, 2000; Wood, 2011). It increases the ion and organic solute pools to provide an osmotic driving force for water influx when it is exposed to hyperosmotic conditions (Kempf and Bremer, 1998; Bremer, 2002), and it expels these compounds again through the transient opening of mechanosensitive channels to curb water influx upon sudden exposure to hypo-osmotic surroundings (Booth and Blount, 2012; Booth, 2014).

The initial phase: managing potassium and sodium fluxes in response to an osmotic increase

In contrast to a selected group of *Bacteria* and *Archaea* that physiologically cope with permanently high-salinity habitats through the massively accumulation of ions (in particular Cl^- and K^+) (Oren, 2013), *B. subtilis* avoids the long-lasting amassing of inorganic solutes (Bremer and Krämer, 2000; Bremer, 2002). Instead, it prefers the energetically more expensive (Oren, 2011) accumulation of a particular class of physiologically compliant and highly water-soluble organic osmolytes, the compatible solutes (Kempf and Bremer, 1998; Ignatova and Gierasch, 2006; Street et al., 2006). This is commonly referred to as the 'salt out' strategy (Galinski and Trüper, 1994) and

provides microorganisms that use it with a considerable physiological flexibility to cope with environments in which the osmolarity fluctuates more frequently (Kempf and Bremer, 1998; Roesser and Müller, 2001). Nevertheless, the import and export of ions, in particular that of K^+ and Na^+, plays a pivotal role both in the initial and during the sustained cellular adjustment process of *B. subtilis* to high osmolarity surroundings (Whatmore and Reed, 1990; Whatmore et al., 1990; Bremer and Krämer, 2000; Gorecki et al., 2014).

As observed in many bacterial species (Csonka, 1989; Kempf and Bremer, 1998; Bremer and Krämer, 2000; Roesser and Müller, 2001; Wood et al., 2001), the rapid import of K^+ ions also constitutes the emergency stress reaction of *B. subtilis* upon a sudden osmotic upshift (Whatmore et al., 1990; Holtmann et al., 2003). *B. subtilis* cells grown in a chemically defined minimal medium at 25°C possess a K^+ pool of about 350 mM; this pool size increases to about 650–700 mM within 1 h when the cells are confronted with a moderate osmotic upshift elicited through the addition of 400 mM NaCl to the growth medium. Synthesis and uptake of compatible solutes (Hoffmann and Bremer, 2016) modulate over time the size of the initially build-up K^+ pool and can reduce it to levels lower that found in the pre-shocked cells (Whatmore et al., 1990).

The K^+ importers of *B. subtilis*

A considerable number of different types of potassium uptake systems operate in microorganisms (Hänelt et al., 2011; Diskowski et al., 2015). So far, only members of the Ktr family of K^+ importers have been identified in *B. subtilis* (Holtmann et al., 2003). These types of transporters belong to the monovalent cation (K^+ and Na^+) superfamily (Hänelt et al., 2011). Two closely related Ktr systems are present in the domesticated *B. subtilis* laboratory strains JH642 and 168: KtrAB and KtrCD (Holtmann et al., 2003) (Figure 1). The genes encoding the two subunits of the KtrAB transporter are organized in an operon, whereas those encoding the KtrC and KtrD proteins are found separately on the *B. subtilis* chromosome. Their transcription is not induced by K^+ limitation or by osmotic stress (Holtmann et al., 2003; Steil et al., 2003; Hahne et al., 2010). K^+ transport assays revealed that the KtrAB system has a moderate affinity for K^+ with a K_m value of approximately 1 mM, whereas KtrCD exhibit only a low affinity for this ion with a K_m value of approximately 10 mM. The V_{max} values of the two systems range between 40 and 100 nmol of K^+ taken up min^{-1} mg^{-1} dry weight of the cells (Holtmann et al., 2003). The K^+ demand of *B. subtilis* JH642 cells in which the KtrAB and KtrCD systems were simultaneously genetically disrupted increased drastically, and the remaining K^+ uptake activity [mediated by a genetically undefined transporter(s)] exhibits an apparent K_m value of approximately 110 mM (Holtmann et al., 2003). Mutant studies showed that cells lacking the higher affinity K^+ importer KtrAB cannot recover from an osmotic upshock elicited with 0.6 M NaCl, whereas wild-type cells recover with relative ease (Holtmann et al., 2003). The import of K^+ is therefore an integral part of the cellular efforts of *B. subtilis* to physiologically cope with the challenges imposed by imminent osmotic stress (Holtmann et al., 2003; Hoffmann and Bremer, 2016).

Each of the Ktr systems operating in *B. subtilis* (Figure 1) consists of two proteins: (i) a membrane-embedded component (KtrB and KtrD) that constitutes the K^+-conducting unit – this protein is evolutionarily related to K^+ channels (Durell and Guy, 1999); and (ii) a peripheral membrane-associated protein (KtrA and KtrC) that regulates the activity of the K^+-translocating protein in response to low-molecular-weight effector molecules such as NADH, ATP, and c-di-AMP. This latter protein contains a signature domain widely found in K^+ importers, K^+ exporters, and K^+ channels that is referred to in the literature either as KTN (K^+ transport nucleotide binding), or as RCK (regulating the conductance of K^+) (Hänelt et al., 2011).

The crystal structure of the *B. subtilis* KtrAB system has recently been determined (Vieira-Pires et al., 2013; Szollosi et al., 2016). It is composed of a homo-dimeric KtrB protein and each KtrB subunit has the characteristic architecture of a potassium channel pore domain (Vieira-Pires et al., 2013). The cytoplasmic KtrB-associated KtrA protein forms an octameric ring, resembling that of the RCK gating ring of the MthK K^+ channel from *Methanobacterium thermoautotrophicum* (Jiang et al., 2002; Ye et al., 2010; Vieira-Pires et al., 2013). The activity of the KtrAB K^+ transporter is regulated through the binding of nucleotides to the KtrA subunits; binding of ADP results in a low-activity state, whereas the binding of ATP results in a high-activity state (Vieira-Pires et al., 2013; Szollosi et al., 2016). A comparison of the crystal structure of the KtrAB complex with either ADP or ATP and further biochemical studies revealed structural changes that are propagated from the KtrA octameric ring to the KtrB membrane-embedded subunit. As a result of these studies it was possible to derive a molecular model for the nucleotide-dependent (ADP or ATP) activity regulation of the KtrAB K^+ transporter (Szollosi et al., 2016).

Exiting new data have recently emerged that link the control of the KtrAB-mediate K^+ import not only with

cellular potassium homeostasis, but also with cell wall synthesis and developmental programs through the activities of the second messenger cyclic diadenosine monophosphate (c-di-AMP) (Corrigan and Grundling, 2013; Commichau et al., 2015; Hengge et al., 2016). A systematic search for conserved bacterial c-di-AMP receptor proteins from *Staphylococcus aureus* revealed the KtrA/KtrC-related protein from the sole Ktr transporter of this human pathogen as a direct target for this second messenger-signaling molecule (Corrigan et al., 2013). The KtrA octameric ring possesses four-fold-symmetry and each subunit can be divided into RCK_N and RCK_C subdomains. The RCK_N lobe generates the ring structure, whereas the RCK_C domain is located at the periphery (Vieira-Pires et al., 2013; Szollosi et al., 2016). A binding site for ATP and other nucleotides has been identified in the RCK_N domain of the *B. subtilis* KtrA protein (Vieira-Pires et al., 2013; Szollosi et al., 2016), whereas c-di-AMP binds to the RCK_C domain of the corresponding *S. aureus* protein (Kim et al., 2015). Interaction of this sub-domain of the RCK-ring-forming KtrA protein with c-di-AMP triggers a conformational change that inactivates the Ktr system (Corrigan et al., 2013; Kim et al., 2015). Notably, *ktrA* mutants of *S. aureus* have growth defects under high osmolarity conditions (Corrigan et al., 2013). Since the Ktr systems of *S. aureus* and *B. subtilis* are closely related with respect to their amino acid sequences, the data reported on the activity control of the *S. aureus* Ktr system by the c-di-AMP signaling molecule will in all likelihood also apply to the corresponding systems of *B. subtilis*.

As mentioned above, the *ktrAB* genes from *B. subtilis* are genetically organized as an operon (Holtmann et al., 2003). Interestingly, this operon is controlled by a riboswitch, where the binding of the ligand to the riboswitch represents the 'off' state of the genetic regulatory circuit (Nelson et al., 2013; Ren and Patel, 2014). This type of riboswitch (generally known as the *ydaO* type) was originally thought to bind ATP (Watson and Fedor, 2012) but subsequent studies showed that it actually binds c-di-AMP with high affinity (Nelson et al., 2013; Gao and Serganov, 2014; Ren and Patel, 2014). It most likely functions through a transcription termination mechanism. Hence, cellular c-di-AMP levels act in the same direction to control both the activity of the Ktr transporter and the transcriptional activity of its structural genes. As pointed out by Commichau et al. in their insightful review, c-di-AMP is currently the only known second messenger that controls a biological process – Ktr-mediated K^+ uptake – by interaction both with a protein and the riboswitch that regulates its expression (Commichau et al., 2015). The cellular levels of c-di-AMP in *B. subtilis* are finely tuned through the opposing enzymes that make it and break it, as both c-di-AMP overproduction and depletion are highly detrimental to the cell. c-di-AMP has therefore be fashionably coined as an 'essential poison' (Gundlach et al., 2015). A c-di-AMP-responsive riboswitch resembling that of *ktrAB* is also present in front of the *ydaO* gene of *B. subtilis* (Barrick et al., 2004). The *ydaO* type of riboswitch is widely distributed in bacteria (Barrick et al., 2004; Block et al., 2010). According to the UniProt database, the *ydaO* gene from *B. subtilis* encodes a membrane protein predicted to function as an amino acid permease (http://www.uniprot.org/uniprot/P96589).

Growth and developmental phenotypes resulting from interference with the cellular levels of c-di-AMP in different bacteria strongly point to a central role of this signaling molecule in cellular physiology. Notably, a link between c-di-AMP levels, K^+ homeostasis, cell wall integrity, and osmostasis has recently emerged as a connecting theme among a plethora of other phenotypes associated with defects in c-di-AMP homeostasis (Corrigan and Grundling, 2013; Corrigan et al., 2013; Commichau et al., 2015; Gundlach et al., 2015, 2016; Hengge et al., 2016; Huynh et al., 2016; Moscoso et al., 2016; Zhu et al., 2016).

The Na^+ extrusion systems of *B. subtilis*

While the import and transient accumulation of K^+ ions are widely used as an emergency reaction in response to exposure to high salinity and osmotic stress (Csonka, 1989; Kempf and Bremer, 1998; Bremer and Krämer, 2000; Wood et al., 2001; Wood, 2011), sodium ions are highly cytotoxic for microbial cells. *B. subtilis* ensures through the operation of at least four Na^+ extrusion systems (Figure 1) a low sodium content of its cytoplasm (Gorecki et al., 2014). The major sodium extrusion system of *B. subtilis*, Mrp, is a Na^+/H^+ antiporter (Figure 1) consisting of seven subunits (MrpABCDEFG), and it is a member of the cation:proton antiporter-3 (CAP-3) family (Swartz et al., 2005). In contrast, the NhaC and NhaK Na^+ export systems are single-component transporters (Figure 1), function as monovalent cation/H^+ antiporters, and are members of the cation:proton antiporter-1 (CPA-1) family (Pragai et al., 2001; Fujisawa et al., 2005). A severe and sudden osmotic upshift triggers a rapid up-regulation in the transcription of the *mrp*, *nhaC*, and *nhaK* genes (Hahne et al., 2010), indicating that the corresponding Na^+ exporters play pivotal roles in maintaining low sodium content of the

cytoplasm. Indeed, as assessed by ^{23}Na-nuclear magnetic resonance spectroscopy, the intracellular Na^+ content of *B. subtilis* cells is so low that it cannot be reliably measured (Gorecki et al., 2014). However, the genetic inactivation of the Mrp system causes an increase in cellular Na^+ content up to 12 mM and the concomitant development of a severe Na^+-sensitive growth phenotype (Gorecki et al., 2014). The fourth Na^+ extrusion transporter (NatAB) operating in *B. subtilis* (Figure 1) belongs to the ABC transporter family (Cheng et al., 1997). Expression of its structural genes (*natAB*) is controlled through the NatK-NatR two-component regulatory system (Ogura et al., 2007). The NatAB system catalyzes ATP-dependent electrogenic Na^+ extrusion without being mechanistically coupled to H^+ or K^+ uptake; it primarily functions in Na^+ resistance at alkaline pH (Ogura et al., 2007). Consistent with this physiological trait, there is no increase in *natAB* expression at neutral pH under acute osmotic stress conditions (Hahne et al., 2010).

The osmotically induced glycine betaine transporter OpuD and the L-proline transporter OpuE (Kappes et al., 1996; von Blohn et al., 1997) (see below) belong to the sodium-solute-symporter (SSS) family (Jung et al., 2012) and are thus Na^+-coupled systems. Since OpuD- and OpuE-mediated import of glycine betaine and L-proline by osmotically stressed *B. subtilis* cells will generate substantial pool sizes of these compatible solutes (Hoffmann et al., 2013; Zaprasis et al., 2013, 2015), the cell must ensure that the concomitantly imported Na^+ ions are quickly removed to avoid cytotoxic effects. The Na^+ extrusion systems operating in *B. subtilis* (Figure 1) are thus an important and integral part of the cells overall efforts to cope with osmotic and sodium stress.

The second phase: cellular adjustment to sustained high osmolarity

Although often described as successive events in the complex cellular adjustment process to exposure to high osmolarity environments (Csonka, 1989; Kempf and Bremer, 1998; Bremer and Krämer, 2000; Wood et al., 2001), the response of microorganisms to imminent and then sustained osmotic stress is interwoven (Balaji et al., 2005). Soon after the *B. subtilis* cell responds to an increase in external osmolarity through the rapid import of K^+ ions (Whatmore et al., 1990; Holtmann et al., 2003), it begins to synthesize massive amounts of L-proline (Whatmore et al., 1990; Brill et al., 2011; Hoffmann et al., 2013; Zaprasis et al., 2013), a compatible solute and anti protein-aggregating compound (Ignatova and Gierasch, 2006), widely used as an osmostress protectant by both microorganisms and plants (Fichman et al., 2014). Genetic disruption of the osmostress-adaptive L-proline biosynthetic route causes a severe osmostress-sensitive growth phenotype (Figure 2A) (Brill et al., 2011). The size of the osmostress-adaptive L-proline pool in *B. subtilis* is sensitively correlated in an essential linear fashion to the degree of the osmotic stress imposed onto the cell (Figure 2C) (Brill et al., 2011). It reaches a pool size of about 500 mM in severely osmotically stressed *B. subtilis* cells (Hoffmann et al., 2013; Zaprasis et al., 2013). Production of L-proline up to pool sizes of approximately 500 mM in severely osmotically stressed cells (Hoffmann et al., 2013; Zaprasis et al., 2013) is an enormous physiological task if one considers that the synthesis of a single L-proline molecule requires the expenditure of about 20 high-energy phosphate bonds (Akashi and Gojobori, 2002). The energetic constraints imposed on already starving cells (Higgins and Dworkin, 2012) is probably also the reason why *B. subtilis* does not sporulate in high-salinity media (Widderich et al., 2016), despite the fact that spores are highly desiccation-resistant (Setlow, 2014) and could therefore ensure survival of the species under persistent highly unfavorable osmotic conditions in the soil and other ecosystems.

K^+ exporters

The onset of the osmostress-adaptive L-proline biosynthesis (Brill et al., 2011) and the import of osmoprotectants (see below), allow the cell to expel part of the initially imported K^+ (Whatmore et al., 1990). This will reduce the ionic strength of the cytoplasm without compromising its overall osmotic potential, a prerequisite for proper cellular hydration (Wood, 2011). Export of K^+ in *B. subtilis* is only incompletely understood but three systems with the ability to actively extrude K^+ ions have been identified: KhtTU and YugO (Figure 1). The two-component KhtTU system possesses K^+/H^+ antiporter activity and belongs to the cation/proton antiporter-2 (CPA-2) family (Fujisawa et al., 2007). KhtU is the membrane-embedded component of the antiporter and interacts with KhtT, a peripheral membrane-associated protein possessing an RCK domain that in all likelihood regulates the transport activity of KhtU through the binding of nucleotides (Fujisawa et al., 2007). The second K^+ extrusion system known to operate in *B. subtilis* is YugO (Figure 1), a protein related to the MthK potassium channel from *M. thermoautotrophicum* (Jiang et al., 2002; Ye et al., 2010).

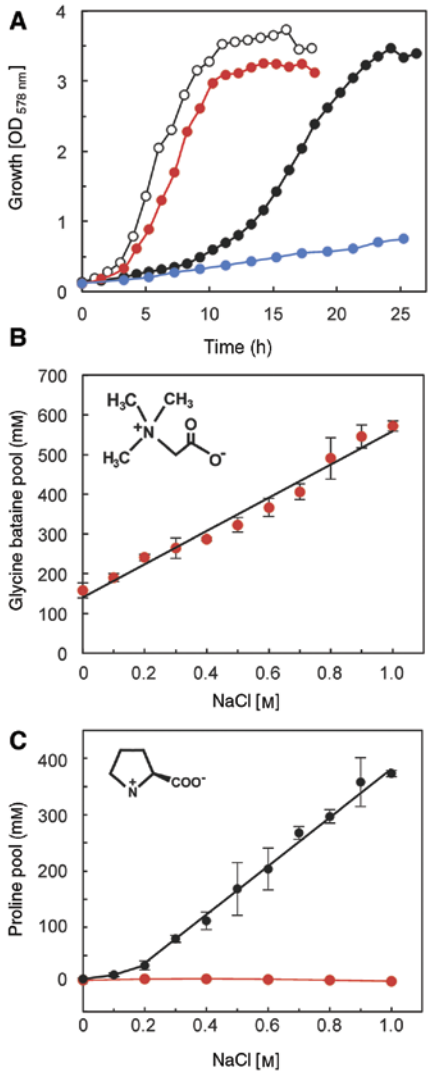

Figure 2: Osmostress protection of *B. subtilis* through synthesis of the compatible solutes L-proline and import of glycine betaine. (A) Growth of the *B. subtilis* wild-type strain JH642 in a minimal medium (SMM) without any additions (open circles), in SMM containing 1.2 M NaCl (black circles), or in SMM containing 1.2 M NaCl that was supplemented with 1 mM glycine betaine (red circles). Growth of a corresponding Δ*proHJ* mutant of JH642 with a defect in the osmoadaptive L-proline biosynthesis route was grown in SMM containing 1.2 M NaCl but lacking glycine betaine (blue circles). (B) Cytoplasmic glycine betaine pools in cells of the wild-type strain JH642 grown in SMM containing the indicated NaCl concentrations and 1 mM glycine betaine spiked with 0.64 μM radiolabeled [1-^{14}C] glycine betaine. The chemical structure of glycine betaine is shown in the insert. (C) Cellular L-proline pools in salt-stressed cells of the wild-type strain JH642 grown either in the absence (black circles) or the presence of 1 mM glycine betaine (red circles). The chemical structure of L-proline is shown in the insert. The values shown in this figure for compatible solute pools (B and C) of osmotically stressed *B. subtilis* cells were re-plotted from published data (Hoffmann et al., 2013); those for the shown data growth of osmotically stressed cells (A) are our unpublished results (T. Hoffmann and E. Bremer) and are representative for similar data reported in previous publications (Boch et al., 1994; Brill et al., 2011; Zaprasis et al., 2013).

YugO possesses a membrane-embedded segment (the K^+ channel proper) and, similar to the KtrA, KtrC and KhtT proteins, possesses a cytoplasmic RCK domain. In contrast to these proteins, however, the RCK domain is covalently fused to the channel-forming domain of YugO. Furthermore, there is an internal translational start site in the *yugO* gene leading to the formation of a separate RCK domain possessing the same amino acid sequence as the one fused to the channel-forming domain of YugO. Transcription of the *khtSTU* (also known as *yhaSTU*) gene cluster and that of the *yugO* gene is up-regulated in response to salt stress (Hahne et al., 2010). The transcriptional response of the *khtSTU* and *yugO* genes to a common environmental stimulus suggests that the KhtTU and YugO systems probably function in a concerted fashion (Figure 1) in the export of K^+ during the adaptation phase of the *B. subtilis* cell to sustained high-salinity surroundings. The third system with the ability to export potassium is the CAP-I-type monovalent cation transporter NhaK (Fujisawa et al., 2005).

The Opu osmostress protectant uptake systems: salient features

B. subtilis not only synthesizes osmostress-protective L-proline in large quantities (Whatmore et al., 1990; Brill et al., 2011) (Figure 2C), but it also imports a variety of compatible solutes from environmental sources to derive osmostress protection (von Blohn et al., 1997; Zaprasis

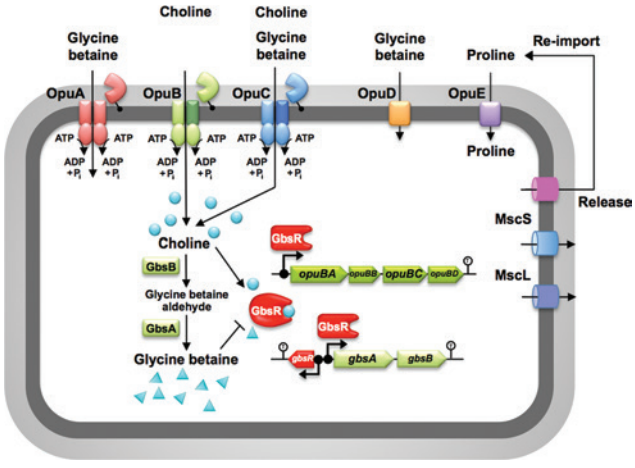

Figure 3: The Opu family of transporters, the synthesis route for glycine betaine from exogenously provided choline and the genetic regulation of the choline to glycine betaine synthesis pathway through the choline-sensing regulator GbsR.
See text for details.

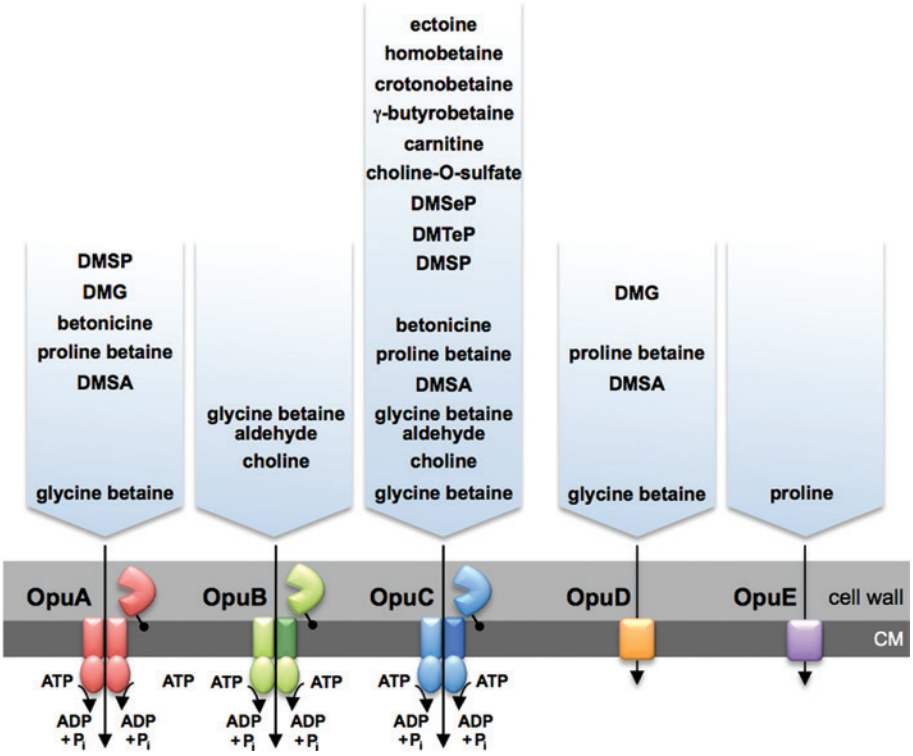

Figure 4: Substrate specificity of the Opu osmoprotectant uptake systems of *B. subtilis*.

et al., 2014; Hoffmann and Bremer, 2016) (Figures 3 and 4). The striking osmostress-protective effect conferred to *B. subtilis* by the uptake of glycine betaine (Boch et al., 1994), probably the most widely used compatible solute in nature (Yancey, 2005), is exemplified in Figure 2A.

The currently known 15 osmostress protectants for *B. subtilis* (Hoffmann and Bremer, 2011) (Figure 4) are essentially all derived from plants or stem from the biosynthetic activities of other microbes. As a consequence, these compounds are released into the soil habitat of *B. subtilis* through root exudates, rotting plant material, and decaying or osmotically down-shocked microbial cells (Welsh, 2000; Warren, 2014; Bouskill et al., 2016). They are typically present in very low concentrations (μM–nM) in natural ecological niches, and hence high-affinity uptake systems are required so that osmotically stressed *B. subtilis* cells can scavenge them (Figure 3). It is worth noting in this context that, with the exception of L-proline (Moses et al., 2012), none of the osmostress protectants listed in Figure 4 can be catabolized by *B. subtilis*. This allows a clean separation between the activities of the cell to acquire nutrients and its osmostress-adaptive efforts, but requires, in the case of L-proline complex genetic and physiological set-ups, to curb competition between these activities (Moses et al., 2012; Zaprasis et al., 2013, 2015).

Five osmotically regulated import systems for osmostress-protectants operate in *B. subtilis* (Figure 3); they were coined Opu, a nomenclature that stands for **o**smostress **p**rotectants **u**ptake (Kempf and Bremer, 1995; Kappes et al., 1996, 1999; von Blohn et al., 1997). OpuA, OpuB, and OpuC (Kempf and Bremer, 1995; Kappes et al., 1999) are members of the binding-protein-dependent ABC superfamily (Davidson et al., 2008; Berntsson et al., 2010; Eitinger et al., 2011). OpuD belongs to the betaine-choline-carnitine-transporter (BCCT) family (Kappes et al., 1996), a widely found group of microbial uptake systems for different types of compatible solutes (Ziegler et al., 2010). Finally, OpuE is a member of the sodium-solute-symporter (SSS) family of transporters (von Blohn et al., 1997; Jung et al., 2012). These transporters were characterized physiologically and molecularly even before the *B. subtilis* genome sequence was established. Now, the consultation of a database such as PFAM (http://pfam.xfam.org/) or UniProt (http://www.uniprot.org/) reveals thousands of transport systems in microbial genome sequences that are related to the *B. subtilis* Opu family of transporters. The vast majority of these transporters have not been functionally assessed; hence, much can be learned in terms of microbial physiology by considering the properties of the *B. subtilis* Opu transporters that have been studied in quite some detail over the last 20 years (Bremer and Krämer, 2000; Bremer, 2002; Hoffmann and Bremer, 2016).

As observed for the osmostress-adaptive *de novo* synthesis of L-proline (Figure 2C) (Brill et al., 2011; Zaprasis et al., 2013), the pool size of glycine betaine generated by *B. subtilis* through import is linearly dependent on the degree of the externally imposed osmotic stress. Its level reaches 0.5 M under severe osmotic stress conditions (Figure 2B) (Hoffmann et al., 2013). The linear relationship between the pool sizes of L-proline and glycine betaine and the external salinity (Figure 2B and C) indicates that *B. subtilis* can measure small increases in the osmolarity/salinity persisting in its environment. Apparently, the cell can then set either its biosynthetic (for L-proline) or transport (for glycine betaine) activities very precisely to meet the cell's need at a given time for the balancing of the osmotic gradient across its cytoplasmic membrane.

The accumulation of glycine betaine through transport not only confers strong osmostress protection for *B. subtilis* (Boch et al., 1994) (Figure 2A), but it also exerts a strong repressing effect on the size of the L-proline pool that is generated through *de novo* synthesis (Whatmore et al., 1990; Hoffmann et al., 2013) (Figure 2C). This effect is probably rooted both in the different physico-chemical properties of L-proline and glycine betaine that render them differently effective as osmostress protectants (Cayley et al., 1992; Street et al., 2006) and the different energetic requirements for the synthesis of L-proline and the import of glycine betaine. As mentioned above, the *de novo* synthesis of a single L-proline molecule requires the expenditure of 20 high-energy phosphate bonds (Akashi and Gojobori, 2002), whereas the hydrolysis of just two ATP molecules suffices to power the import of a glycine betaine molecule via ABC transporters such as OpuA or OpuC (Patzlaff et al., 2003).

The osmostress-protective effects exerted by glycine betaine and other compatible solutes are certainly strongly rooted in their ability to serve as physiologically compliant water-attracting osmolytes (Csonka, 1989; Cayley et al., 1992; Kempf and Bremer, 1998; Record et al., 1998; Bremer and Krämer, 2000; Wood, 2011). However, these compounds also confer beneficial effects on protein stability, solubility, and proper functioning under otherwise denaturing conditions (Bourot et al., 2000; Ignatova and Gierasch, 2006; Street et al., 2010; Wood, 2011). Their preferential exclusion from the protein backbone drives, for thermodynamic reasons, the protein into a compact and well-folded conformation, a process labeled the osmophobic effect (Bolen and Baskakov, 2001; Street et al., 2006). The function preserving attributes of compatible solutes led to their description in the literature as chemical chaperones (Diamant et al., 2001; Chattopadhyay et al., 2004).

Compatible solutes are not only excellent osmostress protectants, but they also serve as effective thermostress protectants at the very cutting upper (about 52°C) and lower (about 13°C) boundaries of growth of *B. subtilis* in a chemical defined medium (Holtmann and Bremer, 2004; Hoffmann and Bremer, 2011). We emphasize that at these critical temperature boundaries, the well-studied cold- and heat-stress response systems of *B. subtilis* that primarily rely on the function of proteins (Graumann and Marahiel, 1999; Schumann, 2003), are unable to secure growth, while import of low-molecular-weight stress protectants can do so efficiently (Holtmann and Bremer, 2004; Hoffmann and Bremer, 2011; Bashir et al., 2014a,b; Broy et al., 2015). We have previously proposed that the thermostabilizing properties of compatible solutes probably stem from their chemical chaperone activities (Holtmann and Bremer, 2004; Hoffmann and Bremer, 2016) but the molecular details of this process are not well explored in *B. subtilis*.

In general, compatible solutes that serve as osmostress protectants for *B. subtilis* also function as thermostress protectants, although some notable exceptions exist where a given osmostress protectant can serve as a heat- but not as a cold-stress protectant, or vice versa (Holtmann and Bremer, 2004; Hoffmann and Bremer, 2011; Bashir et al., 2014a,b; Broy et al., 2015). As found for osmostress protection, the import of compatible solutes serving as thermostress protectants depends on the *B. subtilis* Opu transporters. Hence, the members of the Opu transporter family (Figure 3) are true guardians of *B. subtilis* in a stressful world since desiccation (=increases in osmolarity and salinity) and flooding (=decreases in osmolarity) of the soil often go hand in hand with a rise or a drop in temperature, respectively.

When one views the complexity of the compatible solute uptake systems operating in *B. subtilis* as a whole, one wonders why this bacterium has so many osmostress protectant uptake systems (Figure 3). First of all, a certain degree of redundancy is build into the cellular osmostress response systems of many bacteria (Csonka, 1989; Bremer and Krämer, 2000; Wood et al., 2001; Morbach and Krämer, 2003; Wood, 2011). Second, the transport activities of some of the Opu systems are differently energized, which provides the cell with additional physiological flexibility in stressful environments. The OpuA, OpuB and OpuC ABC transporters are powered through the hydrolysis of ATP, while the BCCT-type OpuD and the SSS-type OpuE transporters harness the sodium gradient across the cytoplasmic membrane to energetically fuel their transporter function. Each of the Opu transporters is a high-affinity uptake system with K_m-values in the

low μM range but their transport capacities differ (Kempf and Bremer, 1995; Kappes et al., 1996, 1999; Zaprasis et al., 2014).

There are also other features that distinguish the OpuA, OpuC and OpuD transporters from each other. Most strikingly is their different substrate-specificity profile (Figures 3 and 4). With the exception of the compatible solute L-proline, OpuC can import every currently known osmostress protectant for *B. subtilis*, and disregarding ectoine with its K_i of about 1.5 mM (Jebbar et al., 1997), possesses a high affinity (in the low μM range) for each of its substrates. The OpuC system also serves as the uptake route for a synthetic toxic glycine betaine analog in which one of its methyl groups has been replaced by a nitro-benzol ring (Cosquer et al., 2004).

There are also differences in the transcriptional regulation of the *opuA* and *opuC* operons and that of the *opuD* and *opuE* genes. Expression of each of these operons/gene is induced in response to osmotic stress (Kempf and Bremer, 1995; Kappes et al., 1996, 1999; Spiegelhalter and Bremer, 1998; Steil et al., 2003; Hahne et al., 2010; Hoffmann et al., 2013). The expression of the *opuC* operon is also regulated by the OpcR repressor (Lee et al., 2013), a regulatory influence that is currently not understood with respect to its physiological ramifications. Furthermore, both the *opuD* and *opuE* genes possess, in addition to their osmotically inducible promoters recognized by the housekeeping sigma factor SigA, a second promoter that is responsive to the alternative sigma factor SigB, the key regulator of the general stress regulon of *B. subtilis* (Hecker et al., 2007). Since an acute salt-shock is a very strong inducer for the entire SigB-regulon (Nannapaneni et al., 2012; Young et al., 2013), the *B. subtilis* cell will be provided with a very rapid, albeit transient, increase in the transcription level of *opuD* and *opuE* (Spiegelhalter and Bremer, 1998; Hahne et al., 2010; Young et al., 2013) and thereby can respond immediately to this challenge through increased transport of the osmostress protectants glycine betaine and L-proline. Disruption of *sigB* causes sensitivity against severe salt shocks. A systematic inactivation of a large subset of SigB-controlled genes of *B. subtilis* (Nannapaneni et al., 2012) 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). Bioinformatics analysis of the predicted functions of the SigB-controlled 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.

Finally, the expression of the *opuA*, *opuB*, and *opuC* operons (but not that of the *opuD* and *opuE* genes) is activated by RemA, a major regulator of biofilm formation in *B. subtilis* (Vlamakis et al., 2013; Winkelman et al., 2013). This novel finding is really exiting from a physiological point of view since it provides a link between the osmostress responses of individual planktonic-living cells (Hoffmann and Bremer, 2016) with those living in a complex structured community of millions of cells held together by a matrix formed by sugars and cell wall binding proteins (Branda et al., 2001; Vlamakis et al., 2013). The sugar matrix of the biofilm exerts a considerable osmotic pressure onto the cells encased in it (Rubinstein et al., 2012) and osmotic pressure is also a key driving force for the growth and expansion of *B. subtilis* biofilms on solid surfaces (Seminara et al., 2012).

The binding-protein-dependent ABC transporters OpuA, OpuB, and OpuC

The *B. subtilis* OpuA, OpuB, and OpuC osmostress protectant uptake systems (Kempf and Bremer, 1995; Kappes et al., 1999) (Figure 3) are members of the binding-protein-dependent ABC superfamily that use the hydrolysis of ATP [two ATP molecules per imported molecule of substrate (Patzlaff et al., 2003)] to energetically fuel the overall transport process. As studied intensively through structural and biochemical analysis of the maltose uptake system, binding-protein dependent ABC transporters use an alternate-access mechanism to mediate substrate translocation across the cytoplasmic membrane (Khare et al., 2009; Carlson et al., 2016). The extracellular ligand-binding proteins that are associated with these types of transporters serve two important functions: (i) they capture the substrate from the environment with high-affinity (Berntsson et al., 2010), and (ii) their docking onto the dimeric inner membrane components sends a conformational signal to the overall transporter complex (Jacso et al., 2012; Alvarez et al., 2015). This elicits a power-stroke to hydrolyze ATP by the nucleotide-binding components of the transporter and thereby energizes the release of the substrate from the binding protein into the trans-membrane translocation pathway. A subsequent further conformational change then extrudes the substrate into the cytoplasm. These types of transporters can operate against steep concentration gradients (Davidson et al., 2008) and are therefore perfectly suited to scavenge osmostress protectants from scarce environmental sources (Welsh, 2000; Warren, 2014; Bouskill et al., 2016).

The substrate-binding proteins OpuAC, OpuBC, and OpuCC are the extracellular components of the OpuA,

OpuB, and OpuC ABC transporters (Figure 3) and are tethered to the external face of the cytoplasmic membrane via a lipid modification of a Cys residue present at their mature N-terminus (Kappes et al., 1999; Kempf and Bremer, 1995). Lipidation is not essential for their ligand-binding activities (Horn et al., 2006; Pittelkow et al., 2011), and the purified OpuAC and OpuBC binding proteins possess K_d-values in the low μM range (Horn et al., 2006; Smits et al., 2008; Pittelkow et al., 2011; Bashir et al., 2014a,b; Broy et al., 2015). No quantitative ligand-binding data are so far available for the purified OpuCC protein. The substrate specificities of the OpuA, OpuB, and OpuC transporters vary considerably, with OpuC being a highly promiscuous compatible solute import system (Figure 4). In contrast, its closely related counterpart OpuB is highly substrate specific and imports both the pre-cursor (choline) and the intermediate (glycine betaine aldehyde) in glycine betaine synthesis (Figures 3 and 4).

The expression of the *opuB* operon (Kappes et al., 1999) and that for the glycine betaine biosynthetic enzymes (*gbsAB*) (Boch et al., 1996) are jointly regulated through the choline-sensing repressor GbsR (Nau-Wagner et al., 2012) (Figure 3). This MarR-type regulator does not control the expression of the *opuC* operon despite the fact that it can, among other osmoprotectants, also import choline (Kappes et al., 1999). The intracellular accumulation of glycine betaine counteracts the choline-mediated release of GbsR from its *opuB* and *gbsAB* operator sequences, thereby establishing a homeostatic system that prevents a wasteful over-accumulation of the metabolically inert glycine betaine by osmotically stressed *B. subtilis* cells (Nau-Wagner et al., 2012).

The OpuA, OpuB, and OpuC transporters of *B. subtilis* are not only osmotically regulated at the transcriptional level of their corresponding structural genes (Kempf and Bremer, 1995; Kappes et al., 1999; Steil et al., 2003; Hahne et al., 2010; Hoffmann et al., 2013), but also osmoregulation at the level of their transport activity is expected (Biemans-Oldehinkel et al., 2006; Chen and Beattie, 2007). The mechanism of the high osmolarity-triggered increase in the activity of the OpuA, OpuB, and OpuC transporters has not been studied in any detail in *B. subtilis*, but in-depth analyses were conducted by Poolman and coworkers with the OpuA ortholog from *Lactococcus lactis*. This system differs from the *B. subtilis* OpuA transporter in that its ligand-binding binding protein is covalently fused to the trans-membrane component of the transporter (OpuAB-OpuAC) and that the two lobes of the *L. lactis* OpuAC protein are inverted relative to those of the *B. subtilis* system (Smits et al., 2008; Wolters et al., 2010). Nevertheless, as shown through structural studies, the glycine betaine-binding site of the *L. lactis* OpuAC protein closely resembles that of the corresponding *B. subtilis* protein (Horn et al., 2006; Wolters et al., 2010). The ATPases (OpuAA) of the *L. lactis* and *B. subtilis* OpuA transporters possess an unusually long carboxy terminus, which contain two cystathionine-beta-synthase (CBS) domains (Biemans-Oldehinkel et al., 2006). Through intensive *in vitro* reconstitution studies of the intact OpuA transporter of *L. lactis* either in membrane vesicles, or in nano-discs, and *in vivo* investigations, it was shown that the CBS domains function as sensors for the ionic strength of the cytoplasm. The thereby triggered conformational changes are transmitted to the nucleotide-binding domain of the OpuAA protein, thus affecting the overall substrate translocation activity of the OpuA transporter (Karasawa et al., 2011, 2013).

The findings on the osmotic control of the *L. lactis* OpuA transporter seem to be of general importance. Studies with the compatible solute uptake system OpuC from the plant pathogen *Pseudomonas syringae* demonstrated that tandem CBS domains in the ATP-binding component of this ABC transporter were correlated with osmoregulation of OpuC activity and transporter function (Chen and Beattie, 2007). Tandem CBS domains are also present in the C-terminal domain not only of the OpuAA protein, but also in those of the OpuBA and OpuCA ATP-binding component of the corresponding *B. subtilis* ABC transporters (Figure 3). The two levels of osmotic control conferred through increased transcription of the *opuA*, *opuB* and *opuC* operons (Steil et al., 2003; Hahne et al., 2010) and the presumed stimulation of transport activity, provide the *B. subtilis* cell with increased transport capacity for a broad spectrum of osmostress protectants (Figure 4) under both suddenly imposed and sustained increases in the external osmolarity.

In a striking new development, the second messenger c-di-AMP was shown to bind directly to the CBS domain of the OpuC osmolyte transporter from the human pathogens *Listeria monocytogenes* and *Staphylococcus aureus*. The intracellular c-di-AMP pool was inversely correlated with the OpuC-mediated import of the osmostress protectant L-carnitine (Huynh et al., 2016; Schuster et al., 2016). Furthermore, the overproduction of c-di-AMP was detrimental to the cell and resulted in a pronounced susceptibility of *L. monocytogenes* to high osmolarity-incurred stress (Huynh et al., 2016). Taken together, these data establish the second messenger c-di-AMP as a novel and probably key player in the osmostress response systems of bacteria (Commichau et al., 2015; Kim et al., 2015) and the activity control of Opu-type osmolyte ABC transporters (Huynh et al., 2016; Schuster et al., 2016). It will be most

interesting to see, if c-di-AMP is involved in controlling the transport activity of the OpuA, OpuB, and OpuC systems of *B. subtilis* as well (Figure 3).

Molecular determinants for the high-affinity binding of compatible solutes by the solute receptor proteins OpuAC, OpuBC and OpuCC

Crystal structures of the OpuAC, OpuBC, and OpuCC proteins showed that each of them adheres to the general fold observed in solute receptor proteins associated with ABC transporters (Berntsson et al., 2010). They possess two compact lobes that are connected via a hinge formed by two highly flexible regions of the binding protein (Horn et al., 2006; Du et al., 2011; Pittelkow et al., 2011). These types of ligand-binding proteins can sample different protein conformational spaces (Oswald et al., 2008, 2009; Tschapek et al., 2011; Gouridis et al., 2015). Upon encountering their specific substrate, they trap it via induced fit mechanisms in a deep cleft formed by a pivoting movement of the two lobes towards each other (Oswald et al., 2008, 2009; Gouridis et al., 2015). As an example, the overall crystal structure of the OpuAC protein in complex with its ligand glycine betaine is shown in Figure 5A (Horn et al., 2006). According to the structural classification scheme of ligand-binding proteins associated with ABC transporters, the OpuAC, OpuBC, and OpuCC proteins from *B. subtilis* belong to the sub-group F-III, to which other ligand-binding proteins for compatible solutes also belong (Berntsson et al., 2010).

The substrate-binding proteins of the OpuA, OpuB, and OpuC transporters have to achieve the high-affinity

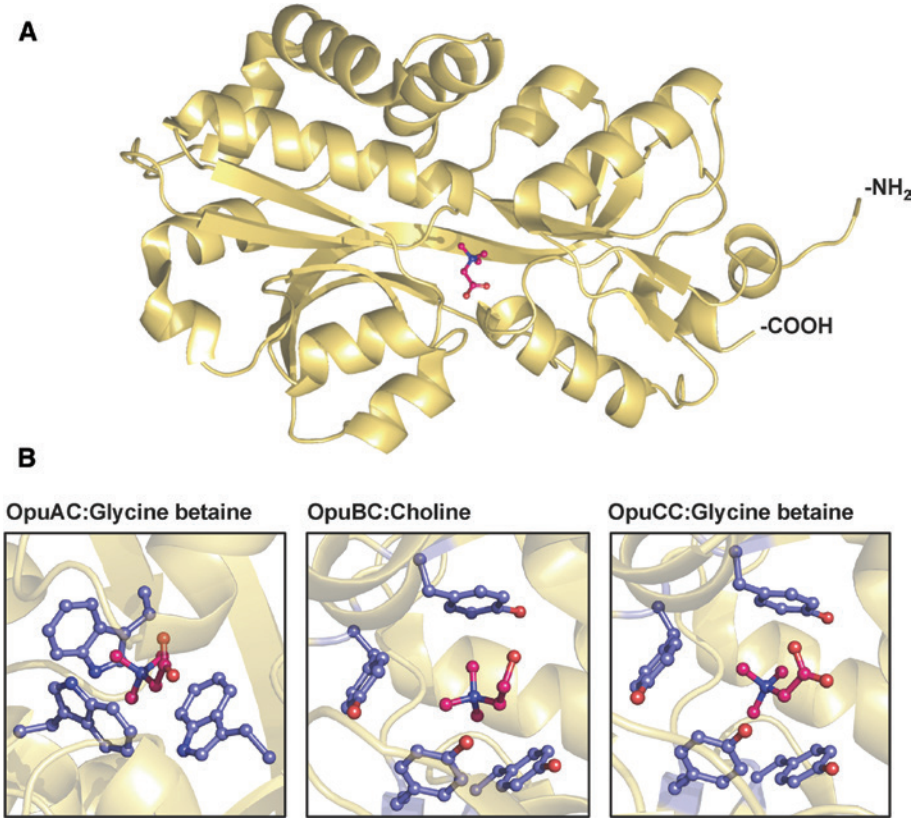

Figure 5: Crystal structure of the extracellular ligand-binding proteins OpuAC, OpuBC and OpuCC in complex with their substrates. (A) Overall structure of the OpuAC:glycine betaine protein complex (PDB code 2B4L). (B) Aromatic binding pockets of the OpuAC:glycine betaine (PDB code 2B4L) (Horn et al., 2006), the OpuBC:choline (PDB code 3R6U) (Pittelkow et al., 2011) and the OpuCC:glycine betaine (PDB code 3PPP) (Du et al., 2011) protein complexes. Aromatic amino acid residues coordinating the positively charged trimethylammonium head-groups group of glycine betaine and choline by cation-pi-interaction are highlighted. The graphs of the shown structures of the OpuAC, OpuBC and OpuCC protein:ligand complexes were prepared with PyMol (https://www.pymol.org/) using information deposited in the indicated protein database (PDB) (http://www.rcsb.org/pdb/home/home.do) files.

binding of a class of organic osmolytes that are otherwise preferentially excluded from protein surfaces (Bolen and Baskakov, 2001; Street et al., 2006; Capp et al., 2009). Crystal structures of the OpuAC, OpuBC, and OpuCC solute receptor proteins in complex with several of their ligands (Figure 4) revealed how this is achieved (Horn et al., 2006; Smits et al., 2008; Du et al., 2011; Pittelkow et al., 2011). Cation-π interactions (Mahadevi and Sastry, 2013) play a key role in each of these proteins for substrate recognition and high-affinity ligand binding. High-resolution crystal structures of the OpuAC protein in complex with glycine betaine (PDB code 2B4L), its sulfur analog dimethlysulfonioacetate (DMSA) (PDB code 3CHG) and proline betaine (PDB code 2B4M) have been reported (Horn et al., 2006; Smits et al., 2008). OpuBC was crystallized in the presence of choline (PDB code 3R6U) (Pittelkow et al., 2011), and OpuCC crystal structures were obtained in complex with choline, glycine betaine, carnitine and ectoine (PDB codes 3PPQ, 3PPP, 3PPO, 3PPR) (Du et al., 2011). The ligand-free form of this latter binding protein has also been resolved (PDB code 3PPN), and a comparison of the apo and substrate-bound OpuCC structures allows the tracing of the conformational changes that ensue upon ligand binding (Du et al., 2011).

As previously observed for the compatible-solute-binding proteins ProX from *E. coli* and the *Archaeon Archaeoglobus fulgidus* (Schiefner et al., 2004a,b), the OpuAC, OpuBC, and OpuCC proteins bind the positively charged head-groups of their ligands via cation-π interactions (Horn et al., 2006; Smits et al., 2008; Du et al., 2011; Pittelkow et al., 2011; Mahadevi and Sastry, 2013). Each of them possesses a ligand-binding site formed by the side chains of either three (OpuAC), or four (OpuBC and OpuCC) aromatic amino acid residues. This 'aromatic cage' allows a somewhat flexible positioning of the positively charged head-groups of the ligands within the binding site (Du et al., 2011; Broy et al., 2015), while the 'tails' of the various substrates protrude out of this cage and are held in place via salt-bridges, hydrogen bonds, or interactions with water molecules. A view into the aromatic cages of the OpuAC, OpuBC, and OpuCC proteins is provided in Figure 5B.

The consultation of a database such as PFAM (http://pfam.xfam.org/) or UniProt (http://www.uniprot.org/) reveals that the amino acids forming the aromatic cages in the OpuAC, OpuBC, and OpuCC substrate binding proteins are either strictly conserved in many of their orthologs, or have been conservatively substituted for by other aromatic amino acids. Indeed, extensive site-directed mutagenesis studies have shown that, as a rule of thumb, a given amino acid in the aromatic cage can be functionally substituted for by another aromatic amino acid with only modest effects on ligand-binding affinity. The introduction of non-aromatic or charged amino acids into the aromatic cage typically leads to a substantial reduction in the affinity of the corresponding binding protein, or it abrogates it altogether (Smits et al., 2008; Pittelkow et al., 2011). Finally, it is worth mentioning, that the aromatic cage can even accommodate non-natural compatible solutes as shown through physiological and modeling studies with a synthetic tellurium-derivative [dimethyltelluriopropionate (DMTeP)] of the naturally occurring sulfur- and selenium-containing compatible solutes dimethlysulfoniopopionate (DMSP) and dimethylseleniopropionate (DMSeP) (Figure 4) (Broy et al., 2015).

Small changes in the architecture of the ligand-binding sites of the OpuAC, OpuBC, and OpuCC proteins can have significant effects on the efficiency of ligand binding and substrate profile of the solute receptor protein, as has been shown through affinity assays, site-directed mutagenesis, and structural studies of OpuAC and its ligands glycine betaine and proline betaine (Smits et al., 2008) and a comparison of the OpuBC and OpuCC crystal structures (Du et al., 2011; Pittelkow et al., 2011). Particularly remarkable is the strikingly different substrate specificity of the OpuB and OpuC transporters (Figure 4), despite the fact that these ABC transporters have, in all likelihood, evolved through a gene-duplication event (Kappes et al., 1999). The amino acid sequence identity of their substrate-binding-proteins OpuBC and OpuCC is high (63%) (Kappes et al., 1999) and the root-mean-square deviation (r.m.s.d.) of the atomic positions of the superimposed OpuBC:choline and OpuCC:choline crystal structure complexes is only 0.6 Å (Du et al., 2011; Pittelkow et al., 2011). Hence, depending on their specific architecture (Figure 5B), aromatic cages are well suited to obtain ligand-binding proteins with either a very narrow or a rather broad substrate profile.

The BCCT-type transporter OpuD

The OpuD transporter from *B. subtilis* (Figure 3) is, together with BetP from *C. glutamicum*, the founding member (Kappes et al., 1996; Peter et al., 1996) of the BCCT family, a group of widely distributed microbial transporters, many of which are involved in compatible solute acquisition (Ziegler et al., 2010). Without doubt, BetP is the biochemically and structurally best-understood member of the BCCT family (Ott et al., 2008; Ressl et al., 2009; Perez et al., 2012, 2014; Maximov et al., 2014). Intensive crystallographic analysis revealed that BetP adheres to

the widely found LeuT-fold of transporters and functions through an alternate-access transport mechanism (Ressl et al., 2009; Perez et al., 2012, 2014). The transporter activity of BetP is Na^+-coupled (Schiller et al., 2004) and the reported structural data revealed important features for a functional understanding of the sequential formation of the substrate and Na^+ binding sites during the transport cycle (Perez et al., 2014). Most interestingly, these impressive crystallographic studies also revealed a conformation of BetP in which a glycine betaine molecule was captured in the centrally located ligand-binding site (Perez et al., 2012). A cage formed by the side-chains of three aromatic residues is key to coordinating and positioning the positively charged trimethlyammonium head-group of glycine betaine within the BetP binding site through cation-π interactions. One of the two Na^+ ions co-transported by BetP along with its substrate (Schiller et al., 2004) is involved in the structural coordination of the carboxylate of the glycine betaine molecule within the ligand-binding site (Ressl et al., 2009; Perez et al., 2012). Strikingly, the architecture of the glycine betaine-binding site of the membrane-embedded BetP transporter closely resembles that present in the soluble extracellular ligand-binding protein OpuAC from *B. subtilis* (Figures 3 and 5), with the exception that there is no Na^+ bound to the glycine betaine molecule sequestered by OpuAC (Horn et al., 2006). Hence, nature has found a common structural solution for the high-affinity binding of a ligand, glycine betaine, in both soluble proteins and membrane-incorporated proteins that are otherwise preferentially excluded from protein surfaces (Street et al., 2006; Capp et al., 2009). The three residues that form the aromatic cage in BetP are conserved in the *B. subtilis* OpuD protein (Kappes et al., 1996; Perez et al., 2012) and much what has been learned from the structural and functional analysis of BetP can probably also be extended to its homolog OpuD. These two transporters exhibit a degree of amino acid sequence identity of about 45%.

BetP is not only an efficient transporter but also serves as an osmosensor and osmostress responder since its glycine betaine importer activity increases substantially within milliseconds subsequent to a rise in the external osmolarity (Krämer and Morbach, 2004; Krämer, 2010). BetP tunes its transport activity to the degree of osmotic stress imposed onto the *C. glutamicum* cell by sensing changes in the cytoplasmic K^+ concentration via its extended carboxy terminus (Ott et al., 2008; Krämer, 2010; Perez et al., 2012, 2014; Maximov et al., 2014). The rise in the internal K^+ concentration is required for the osmosensory function of BetP but it is not sufficient since *in vivo* a transmembrane osmotic gradient is necessary as well (Maximov et al., 2014). The carboxy terminus also plays an important role for the communication of individual protomers in the trimeric BetP assembly during the alternate-access driven import of glycine betaine (Perez et al., 2012). It thereby communicates information on conditions in the cytoplasm of osmotically stressed cells to the transporter (Ott et al., 2008; Maximov et al., 2014). Like BetP, OpuD is also under dual osmostress control; the transcription of both corresponding genes is up-regulated in response to osmotic stress and their transporter activity is also stimulated by this cue (Kappes et al., 1996; Krämer and Morbach, 2004; Möker et al., 2004). However, in comparison with the K^+-sensing carboxy terminus of BetP, the carboxy-terminal tail of OpuD is substantially shorter (Kappes et al., 1996; Peter et al., 1996; Ziegler et al., 2010). It thus remains to be seen whether this OpuD extension also functions as a sensor for osmotically induced changes in the ion pool, in particular that of K^+, of the *B. subtilis* cytoplasm.

The SSS-type transporter OpuE

The OpuE transporter from *B. subtilis* (von Blohn et al., 1997; Zaprasis et al., 2014) is a member of the sodium solute symporter (SSS) transporter family (Figure 3) and a homolog of the well-studied PutP L-proline transporter that is used by *E. coli* to acquire L-proline as nutrient and energy source (Jung et al., 2012). L-proline is the only osmostress protectant that *B. subtilis* can both synthesize *de novo* (Brill et al., 2011) (Figure 2C) and also acquire from environmental sources (von Blohn et al., 1997; Zaprasis et al., 2014) (Figure 3). In comparison with an exogenous supply of glycine betaine, L-proline is not a particularly effective osmostress protectant (Zaprasis et al., 2015), a fact that is based both on the different physico-chemical properties of these two compatible solutes (Cayley et al., 1992; Street et al., 2006; Capp et al., 2009), and on the ability of *B. subtilis* to use exogenously provided L-proline as sole carbon and nitrogen source (Belitsky, 2011; Moses et al., 2012). Elimination of the PutBCP L-proline catabolic system (Moses et al., 2012) substantially improves the ability of *B. subtilis* to use exogenously provided L-proline as an osmostress protectant (Zaprasis et al., 2013, 2015). The transporter used for the acquisition of L-proline by *B. subtilis* as a nutrient is PutP, a member of the sodium solute symporter (SSS) transporter family as well, and a transporter protein closely related in its amino acid sequence (identity about 61%) to OpuE (von Blohn et al., 1997; Moses et al., 2012).

Residues that have been identified in the *E. coli* PutP protein as part of the ligand- and sodium-binding sites

(Jung et al., 2012) are conserved in the PutP and OpuE L-proline transporters of *B. subtilis*. However, both the different transcriptional regulation of the *B. subtilis putP* and *opuE* genes (Spiegelhalter and Bremer, 1998; Belitsky, 2011; Moses et al., 2012) and the different responses of the PutP and OpuE transporter proteins themselves to high-salinity surroundings allow a clear distinction between their respective physiological functions. The *putP* gene is part of the catabolic *putBCP* operon whose main promoter is subjected to positive control by the L-proline-responsive transcriptional activator PutR and negative control by the globally acting CodY protein (Belitsky, 2011; Moses et al., 2012), a regulatory protein involved in many catabolic process in *B. subtilis* (Belitsky and Sonenshein, 2013). Transcription of the *putBCP* operon is not induced by increases in the external osmolarity, whereas this environmental cue is a key determinant for the transcriptional profile of *opuE* (Spiegelhalter and Bremer, 1998; Young et al., 2013). Uptake assays with radiolabeled L-proline and the assessment of the response of appropriately constructed *putP* and *opuE* mutant strains to toxic proline analogs demonstrated that the transport activity of PutP is strongly inhibited in cells grown at high salinity, whereas the activity of OpuE can withstand such harsh growth conditions (Zaprasis et al., 2014). The molecular basis underlying these very different responses of the closely related PutP and OpuE transporter proteins to sustained high salinity remains to be determined. However, these different properties indicate that *B. subtilis* tries to curb the consumption of externally provided L-proline when it is exposed to high osmolarity environments in order to funnel this compatible solute more efficiently into its osmostress-adaptive stress reactions.

The OpuE transporter: an L-proline importer and recycling system

While the role of OpuE as an osmostress-relieving L-proline transport system had firmly been established (von Blohn et al., 1997; Spiegelhalter and Bremer, 1998; Zaprasis et al., 2014), it came as a great surprise that a *B. subtilis* mutant with a defective OpuE system had a clear growth disadvantage at high osmolarity (Hoffmann et al., 2012). Since the used chemically defined medium for these experiments did not contain L-proline as an osmostress protectant, the question arose why this was the case. An answer to this question was found when it was detected that the osmostress-adaptive L-proline pool in the *opuE* mutant was reduced by about 20% relative to the *opuE*$^+$ parent strain in cells that had been cultured in the presence of 1 M NaCl (Hoffmann et al., 2012). Where was the missing L-proline? Both by HPLC analysis and through a bioassay that relied on the cross feeding of an *E. coli* L-proline auxothrophic strain, it was found that the *opuE* mutant overproduced (by about 2.3-fold) L-proline and continuously released substantial amounts of it into the growth medium. In contrast, the supernatant of the growth medium of the *opuE*$^+$ parent strain contained very little L-proline due to the recapture of the released L-proline by the OpuE transporter (Figure 3) (Hoffmann et al., 2012). Is this phenomenon (synthesis – release – recapture) (Figure 3) a wasteful accident, or does it serve a physiologically relevant function for a wild-type *B. subtilis* cell? We believe that the latter is the case.

When a *B. subtilis* cell grows, it doubles its volume prior to cell division (Typas et al., 2012). We assume that temporary imbalances in turgor will occur during cell elongation. According to our hypothesis, the cell will then transiently release some of the newly synthesized L-proline to reduce turgor, and when it is needed as an osmostress protectant again, it will re-import the prior released L-proline (Figure 3). In this way, there is no waste in biosynthetic resources for the L-proline producing *B. subtilis* cell but there will be an energetic cost to bear for the operation of the OpuE L-proline import system. As an alternative to re-import, the secreted L-proline might also become a public good in natural settings, a phenomenon that has been observed for the sharing of newly synthesized and excreted glycine betaine by the marine pathogen *Vibrio cholera* under osmotic stress and biofilm growth conditions (Kapfhammer et al., 2005). In *Vibrio harveyi*, a connection between quorum-sensing and glycine betaine synthesis and uptake systems has been found that presumably enables this microorganism to coordinate its collective behavior with the operation of its osmostress response systems (van Kessel et al., 2015). The release and recapture of newly synthesized compatible solutes, as observed by us in *B. subtilis* for L-proline (Hoffmann et al., 2012), has also been observed for glycine betaine in *V. cholera*, *E. coli*, and *Actinopolyspora halophila* (Lamark et al., 1992; Nyyssölä and Leisola, 2001; Kapfhammer et al., 2005), for ectoine in *Halomonas elongata* (Grammann et al., 2002), and for glucosylglycerol in *Synechocystis* (Mikkat and Hagemann, 2000). This indicates that the observations we have made with the synthesis – release – recapture cycle for L-proline in *B. subtilis* is not some bizarre isolated incident.

What is known about the systems mediating the release of *de novo* synthesized compatible solutes from cells under osmotically steady state growth conditions?

Very little! The small multidrug resistance exporter protein EmrE from *E. coli* confers resistance to various antiseptic quaternary cationic compounds (Bay and Turner, 2012). Since both glycine betaine and choline possess a quaternary ammonium head-group, the role of EmrE in the export of these osmostress protectants was assessed in a system where plasmid-encoded copies of *emrE* were expressed under the control of an inducible synthetic promoter. Indeed, glycine betaine and choline were identified as substrates for EmrE. However, a chromosomal deletion of the *emrE* structural gene had no influence on the release of these compounds (Bay and Turner, 2012), leaving open the question as to whether EmrE is indeed a physiologically relevant glycine betaine and choline extrusion system in *E. coli*. In *C. glutamicum*, glycine betaine imported via the BetP transporter is released again via the MscS-type mechanosensitive channel MscCG, thereby contributing to the fine-tuning of the steady state concentration of this compatible solute accumulated in the cytoplasm under hyperosmotic stress. This process has been coined the 'pump and leak' mechanism (Börngen et al., 2010).

In the case of the release of L-proline by osmotically stressed *B. subtilis* cells, an involvement of MscS- and MscL-type channels operating in *B. subtilis* (see below) could firmly be ruled out (Hoffmann et al., 2012). This was also observed when the compatible solute 5-hydroxyectoine was recombinantly produced in synthetic *E. coli* cell factories (Czech et al., 2016). This leaves the following possibilities to explain the release of L-proline from *B. subtilis* cells subjected to sustained osmotic stress: (i) the existence of so-far not recognized types of mechanosensitive channels in *B. subtilis* (Zoratti et al., 1990; Hoffmann et al., 2008); (ii) the passive diffusion of L-proline out of the cell because of the L-proline concentration gradient across the cytoplasmic membrane is so steep that the cell cannot uphold it (Hoffmann et al., 2013; Zaprasis et al., 2013); (iii) the existence of so-far not genetically recognized dedicated compatible solute efflux systems.

It is well known that microorganisms possess specific efflux systems for various compounds, e.g. for amino acids (Eggeling and Sahm, 2003). Essentially all compatible solutes used by *B. subtilis* are either amino acids or chemical derivatives of them (Figure 4). Why should dedicated efflux systems for compatible solutes therefore not exist? MscS- and MscL-type mechanosensitive channels do not possess substrate specificity due to their very large channel diameters in their fully opened forms (Levina et al., 1999; Hoffmann et al., 2008; Booth and Blount, 2012; Booth, 2014). Hence, their transient opening will indiscriminately jettison low-molecular-weight compounds from the cell, including ions, amino acids, useful metabolites, compatible solutes, and ATP. In contrast, a dedicated excretion system for a given, or several, compatible solutes would allow the bacterial cell to specifically target the release of these compounds when the physiological need arises. It could do so in a very finely tuned manner and in the absence of an osmotic down-shock. It will be a challenge, but greatly rewarding in terms of cellular physiology, to genetically identify these putative compatible solute efflux systems.

Mechanosensitive channels: safety valves to smoothly manage the abrupt transition from high to low osmolarity environments

Increases in the osmolarity (salinity) of natural habitats of *B. subtilis* will most often only occur gradually, leaving the cell enough time to adjust to these adverse conditions through the import of potassium, and through the synthesis and uptake of compatible solutes (Bremer, 2002; Hoffmann and Bremer, 2016). In contrast, the sudden transition of a bacterial cell from seawater (about 1000 mOsm) to a freshwater habitat (10–100 mOsm) would trigger within a second an increase in turgor by approximately 22 atm (Booth and Blount, 2012). The mechanical properties of the peptidoglycan sacculus of neither Gram-negative nor that of Gram-positive bacteria (Typas et al., 2012) are able to withstand such dramatic increases in turgor; the cell will simply burst (Reuter et al., 2014). Hence, rainfall or flooding of the soil will decrease the external osmolarity very rapidly, thus requiring the almost instantaneous mounting of stress responses by the *B. subtilis* cell. In essentially all free-living microorganisms, this hypo-osmotic stress response entails the transient opening of mechanosensitive channels to rapidly jettison water-attracting ions and organic compounds from the cytoplasm in order to curb water influx and thereby reduce turgor (Booth and Blount, 2012; Booth, 2014). These safety valves open upon increases in the lateral pressure of the cytoplasmic membrane, a process triggered by water influx and the concomitant increase in turgor (Haswell et al., 2011; Booth and Blount, 2012; Naismith and Booth, 2012; Booth, 2014).

Microorganisms often contain multiple types of mechanosensitive channels that gate at different pressure set points and possess different channel diameters (Booth and

Blount, 2012; Booth, 2014). This allows a graded response to an increase in turgor and triggers the sequential opening of MscM (mini), MscS (small), and MscL (large) type channels (Edwards et al., 2012). Although MscS has a substantial pore size (about 13 Å) in its fully open form (Wang et al., 2008), the opening of the MscL channel is certainly the measure of last resort for osmotically down-shocked cells. It can release in essence all cytoplasmic ions and low-molecular weight metabolites (Haswell et al., 2011; Naismith and Booth, 2012). The calculated size of the open pore (about 30 Å) (Walton et al., 2015) is approximately three times as large as the diameter of the permanently open OmpC and OmpF porins responsible for non-specific passive diffusion of hydrophilic substances across the *E. coli* outer membrane (Kojima and Nikaido, 2014). While little is known about MscM (Edwards et al., 2012), *B. subtilis* possesses three representatives of the MscS-type channels (YhdY, YfkC, YkuT) and a single representative of the MscL-type channel (Figure 3) (Hoffmann et al., 2008; Wahome et al., 2009). MscL is certainly the dominant mechanosensitive channel operating in *B. subtilis*. In combination with the MscS-type channel YkuT, it provides efficient cellular protection against severe osmotic down-shifts. When these two channels are simultaneously genetically inactivated, *B. subtilis* cannot withstand a sudden drop in the external osmolarity, as evidenced by the death of most of the cells (Hoffmann et al., 2008). Hence, the gating activity of the turgor-controlled MscS- and MscL-type channels are indispensable for a smooth transition of *B. subtilis* from hyper- to hypo-osmotic environments. No physiological relevant function has so far been associated with the MscS-related YhdY and YfkC membrane proteins. As judged from electrophysiological studies, channels other than MscS (YkuT) and MscL might be present in *B. subtilis* (Zoratti et al., 1990).

Conclusions and perspectives

The core of the osmostress response systems of *B. subtilis* with its importers and exporters for ions, osmotically controlled synthesis pathways and transporters for compatible solutes, and turgor-controlled mechanosensitive channels (Figures 1 and 3) is now rather well understood (Hoffmann and Bremer, 2016). These systems can therefore be used as a point of reference when the physiology, biochemistry and genetics of the cellular adjustment processes of other microorganisms are studied. A gaping hole still exists, however, with respect to a molecular understanding of how changes in the osmolarity of the environment are perceived by *B. subtilis*, how the cell communicates the gleaned information to its transcriptional apparatus, and how it is converted into an activity control of the transporters and channels that are central for its osmostress adjustment process (Figures 1 and 3). Exciting new avenues for future studies have recently been opened by the findings that the second messenger c-di-AMP (Commichau et al., 2015) is involved in controlling the activity and level of transporters for K^+ (Corrigan et al., 2013; Nelson et al., 2013; Gao and Serganov, 2014; Kim et al., 2015; Moscoso et al., 2016) and compatible solutes (Huynh et al., 2016; Schuster et al., 2016). Equally thought-provoking is the report by Winkelman et al. (2013) that connects the life of *B. subtilis* in biofilms with the transcriptional control of the osmostress-relieving OpuA, OpuB, and OpuC transporter systems through the activator protein RemA.

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