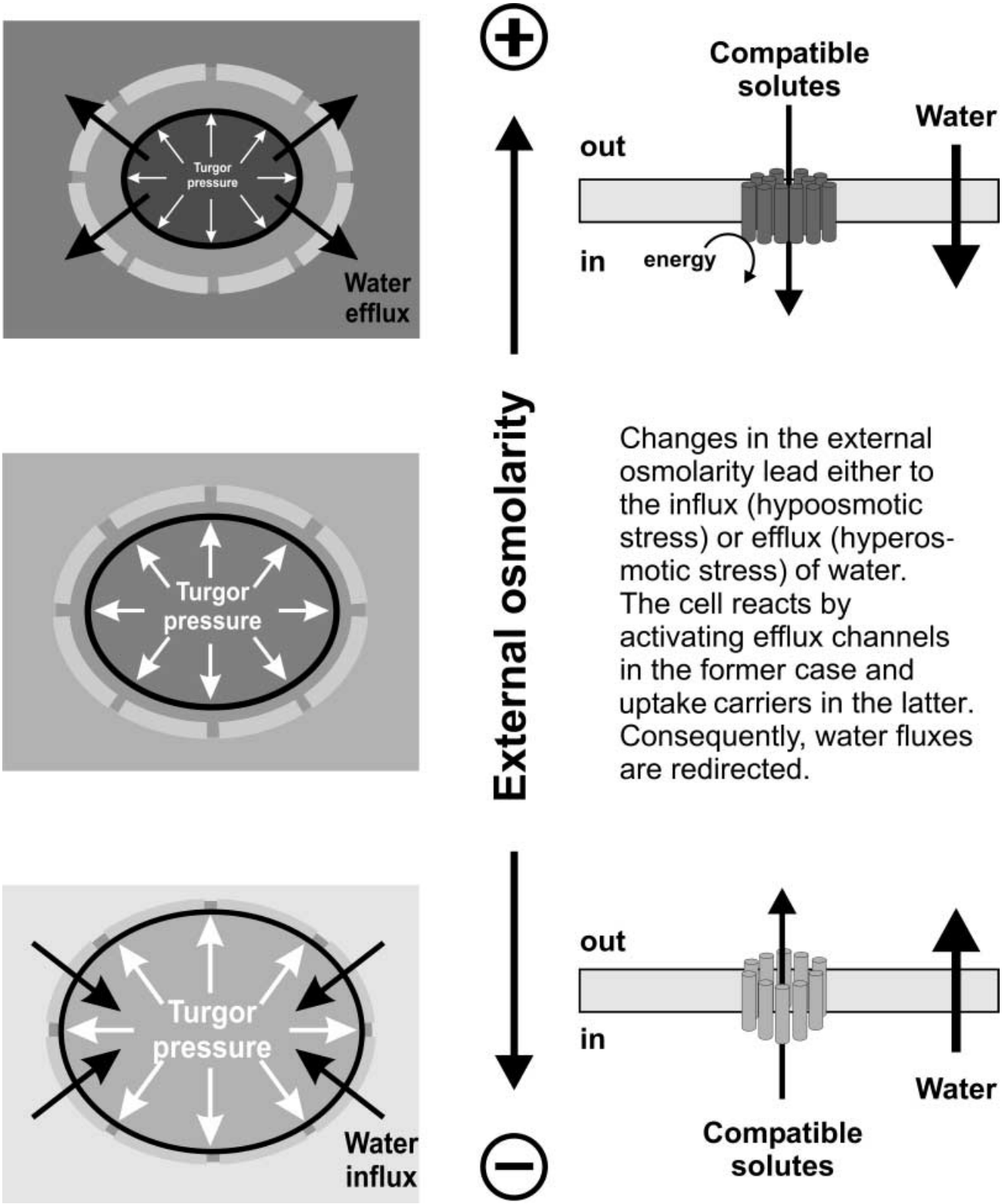


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# Body Shaping under Water Stress: Osmosensing and Osmoregulation of Solute Transport in Bacteria

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*Fluctuation of external osmolarity is one of the most common types of environmental stress factors for all kind of cells, both of prokaryotic and of eukaryotic origin. Cells try to keep their volume and/or turgor pressure constant; consequently, both a decrease (hypoosmotic stress) and an increase (hyperosmotic stress) of the solute concentration (correctly: increase or decrease in water activity) in the surrounding area, respectively, are challenges for cellular metabolism and survival. A common example from the prokaryotic world is the fate of a soil bacterium that, after a sunny day has dried out the soil (hyperosmotic stress), is suddenly exposed to a drop of distilled water from a rain cloud (hypoosmotic stress). The immediate and inevitable passive response to the sudden osmotic shift in the surroundings is fast water efflux out of the cell in the former situation and water influx in the latter. In the worst case, these responses may lead to either loss of cell turgor and plasmolysis or to cell burst. In order to overcome such drastic consequences cells have developed effective mechanisms, namely osmoadaptation, to cope with the two different types of osmotic stress. For a graded reaction to osmotic shifts, cells must be able (1) to sense stimuli related to osmotic stress, (2) to transduce corresponding signals to those systems that properly respond (3) by activating transport or enzymatic functions or (4) by changing gene expression profiles. In this review, membrane proteins involved in the cell's active response to osmotic stress are described. Molecular details of structure, function, and regulation of mechanosensitive efflux channels from various organisms, as well as of osmoregulated uptake systems are discussed.*

**KEYWORDS:**

membranes · osmoregulation · osmosensing · proteins · signal transduction

## 1. Introduction

The cytoplasmic membrane of bacteria is permeable to water but forms an effective barrier for solutes in the surrounding medium or for metabolites in the cytoplasm. In general, the total concentration of osmotically active solutes within a cell is higher than that in the environment, causing water to flow down its chemical potential into the cell. As a result, a hydrostatic pressure, the so-called turgor pressure, is exerted by the cytoplasmic membrane on to the cell wall. Consequently, turgor balances the difference in osmotic pressure between the cell interior and its surroundings. Turgor is maintained throughout the growth cycle as the cell elongates,[1] and is considered to be necessary for enlargement of the cell envelope and, thus, for growth and division.[2] Since the environment of a bacterial cell is permanently subjected to fluctuations in osmolarity, bacteria have been forced to develop efficient adaptation mechanisms to cope with conditions of hypo- and hyperosmotic stress.

In the case of soil bacteria, a common scenario of osmotic stress conditions is illustrated by a cell which, after adaptation to high external osmolarity as the result of a period of drought, is hit by a drop of rain. This situation of a drastic and sudden downward shift in the external solute concentration is actually life threatening. Water rushes in and immediately increases turgor pressure and membrane tension, consequently, the cell is faced with rupturing. Under these conditions emergency release valves, so-called mechanosensitive (MS) channels, are activated to prevent cell death. Cytoplasmic solutes are effectively jettisoned into the environment to reduce the force for water entry by lowering internal osmolarity. In bacteria, MS channels are characterized by an extremely high transport velocity[3] and conductance (permeability). Furthermore, they show little ion or solute specificity. As a result, MS channels allow an almost instant adaptation to a lowered external osmolarity. This function can only be mediated by channels and not by the activity of carrier systems which are generally slower and more specific.

If, on the other hand, bacteria are challenged by an increase in the osmolarity of their environment, water efflux occurs, the cell dehydrates and may plasmolyse, consequently growth stops. Bacterial cells respond to hyperosmotic stress in three overlapping phases:[4] 1) dehydration of the cytoplasm, 2) rehydration of the cytoplasm by adjustment of the cytoplasmic solvent composition due to accumulation of ions or compatible solutes, and 3) cellular remodeling changes as a consequence of gene expression profiles and exchange of ionic osmolytes against compatible solutes. As a result, growth starts again. It should be

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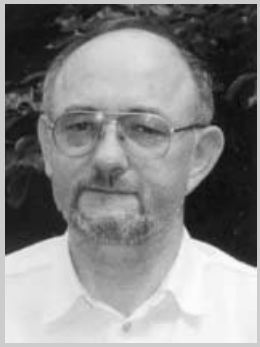
noted that some halophilic bacteria and archaea that are confined to environments of high osmolarity, for example, *Halobacterium* sp., have developed a different strategy, which is called the "salt-in strategy". These microorganisms maintain extremely high salt concentrations in the cytoplasm (molar concentrations of NaCl and KCl in halophilic bacteria, up to 7 M KCl in archaea), consequently, cellular proteins have to be adapted structurally to achieve high salt tolerance.[5]

Accumulation of compatible solutes is the major strategy of most bacteria under conditions of hyperosmotic stress (with the exception of those anaerobic bacteria employing the "salt-in" strategy). Compatible solutes combine two fundamental properties. On the one hand they cause rehydration of the cytoplasm by increasing the internal osmolarity. On the other hand, they are compatible with vital functions of the cell, even when accumulated to molar concentrations in the cytoplasm under conditions of hyperosmotic stress.[6, 7] Compatible solutes stabilize and protect enzymes mainly by being excluded from the protein surface, thus leading to a preferential hydration of the protein.[7] The spectrum of compatible solutes comprises amino acids or their derivatives, methylamines, sulfonium analogues, polyols, and sugars. In a particular bacterial species a selection of compatible solutes is normally found. Glycine betaine, ectoine, proline, and trehalose are probably the most widely used compatible solutes in the bacterial world.

As a common feature, bacterial species typically possess a multitude of different transport systems for the accumulation of compatible solutes. Uptake has the advantage of being energetically cheaper than synthesis. Additionally, uptake also leads to the recovery of solutes from the medium that had previously been synthesized by the cell.[8] In contrast to hypoosmotic shock, only transport systems and not channels can be used for coping with hyperosmotic stress, since internal accumulation of solutes needs some input of energy, in contrast to fast and relatively unspecific efflux. The high internal concentration of solutes reached as a consequence of uptake carrier activity requires high-substrate specificity in order to guarantee the accumulation of compatible solutes only.



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## 2. The Response of Bacteria to Hypoosmotic Stress: Mechanosensitive Channels

Bacteria avoid cell burst after a sudden downshift of external osmolarity by activating MS channels. These channels have been discovered in organisms of different phylogenetic origin, including archaea, bacteria, fungi, plants, and mammalian cells.[9–14] The presence of different types of MS channels in a diversity of bacteria has been detected by electrophysiological (patch clamp) and biochemical analyses.[3, 13, 15–19] In *Escherichia coli*, the best characterized organism with respect to MS channels, three different tension-gated (that is, mechanosensitive) pores were identified in patch-clamp analyses.[20, 21] Depending on their conductances, they were named MscL, MscS, and MscM (mechanosensitive channel of large, small, or mini conductance), and a direct correlation between permeabilities and activation thresholds of the different types of channels was found. In terms of physiology, this scenario allows a graded reaction to osmotic downshifts of varying extent.[21] In recent years the genes coding for MscL and MscS have been identified, whereas the *mscM* gene still remains elusive. The analysis of knock-out strains revealed a high redundancy of MS channels, since no phenotype could be determined if one gene only, either *mscL* or *yggB* (encoding MscS), were deleted in the genome of *E. coli*.[18, 22] Levina and co-workers[22] demonstrated that only the deletion of both genes caused cell death after a severe downshift of external osmolarity. This was actually the first indication for a physiological significance of MS channels and proved that they are designed to open at a pressure difference just below that which would cause cell disruption. Meanwhile three different gene families have been identified that encode MS channels. Besides the *mscL*[23] and the *kefA*/*yggB* families,[22] a new family was recently identified in the archeon *Methanococcus jannashii*.[14, 24] Interestingly, these archaeal channels show sequence and structural similarities to MscL and MscS (YggB), although they consist of 350 amino acids and are thus larger than MscL-type channels (approximately 136 residues) or the

YggB channels (286 residues). This was taken as an indication that prokaryotic MS channels originated from a common MscL-like anchestor gene through gene duplication and diversification.[14]

### 2.1. MscL: From function to structure

Most biochemical and structural knowledge of MS channels is based on work concerning MscL, which until recently was the only MS channel for which the encoding gene had been identified. In fact, identification of the *mscL* gene was a biochemical tour de force. First, the protein from *E. coli* membranes was enriched by chromatographic procedures, with application of reconstitution together with patch-clamp analyses for monitoring MscL activity in every single fraction. N-terminal sequencing of the isolated protein and database analysis then finally led to isolation of the *mscL* gene.[18] Functional reconstitution of MscL in liposomes showed that the MscL polypeptide alone was responsible for the channel activity and furthermore proved that neither an additional protein is involved in the gating process nor is MscL simply a regulator of the measured channel activity.

The MscL of *E. coli* consists of 136 amino acids with a molecular mass of approximately 15 kDa. The predicted membrane topology was verified by PhoA fusions leading to a model of MscL which consists of two transmembrane helices connected by a periplasmic loop. Both N- and C-terminal extensions are located in the cytoplasm (Figure 1 a).[25] Since one single monomer of MscL is not large enough to form a pore with the observed electrical conductance, it was concluded that the functional MscL channel consists of several subunits. The number of subunits necessary to form an active channel, however, was controversially discussed. The functional reconstitution of genetically fused MscL dimers and trimers suggested that the complex has a hexameric structure.[12, 25] This was further supported by the fact that hexamers were detectable after cross-linking experiments, as well as by electron microscopy studies on 2D crystals of MscL.[26] Finally, the X-ray analysis of the crystal structure of MscL from *Mycobacterium tuberculosis* (Figure 1 b)[27] showing a pentameric arrangement of MscL monomers with radial symmetry was a great surprise. Subsequently, the pentameric assembly of MscL was independently proven by a biochemical approach.[28] The 3D structure of MscL furthermore proved that each monomer consists of two transmembrane helices connected by a periplasmatic loop and possesses a cytoplasmically localized $\alpha$-helical structure at the C-terminal end. The first transmembrane domains (TM1) of each subunit form the pore-lining $\alpha$ helices, whereas the TM2 domains constitute an outer ring of the pentamer. The TM1 helices are tilted relative to the membrane normal so that they converge to form an "inverted teepee", similar to that of the TM2 helices of KcsA, a *Streptomyces lividans* potassium channel, the structure of which was solved just before that of MscL.[29] The constriction with an opening of 2 Å is thought to represent the closed conformation of MscL. Conductance measurements and molecular-sieving experiments suggested that the open conformation of MscL should have a pore diameter of 30 – 40 Å.[30] To achieve this, a tremendous change in the protein conformation is necessary. Two models are currently under discussion. Although the constriction of the closed channel is formed by TM1 only, Yoshimura and co-workers[31] propose that both TM1 and TM2 line the pore in the open conformation (Figure 2 a). Sukharev et al.,[32, 33] on the other hand, presented evidence that pore opening takes place through an iris-like movement of the TM1 helices sliding one along the other; this leads to their separation

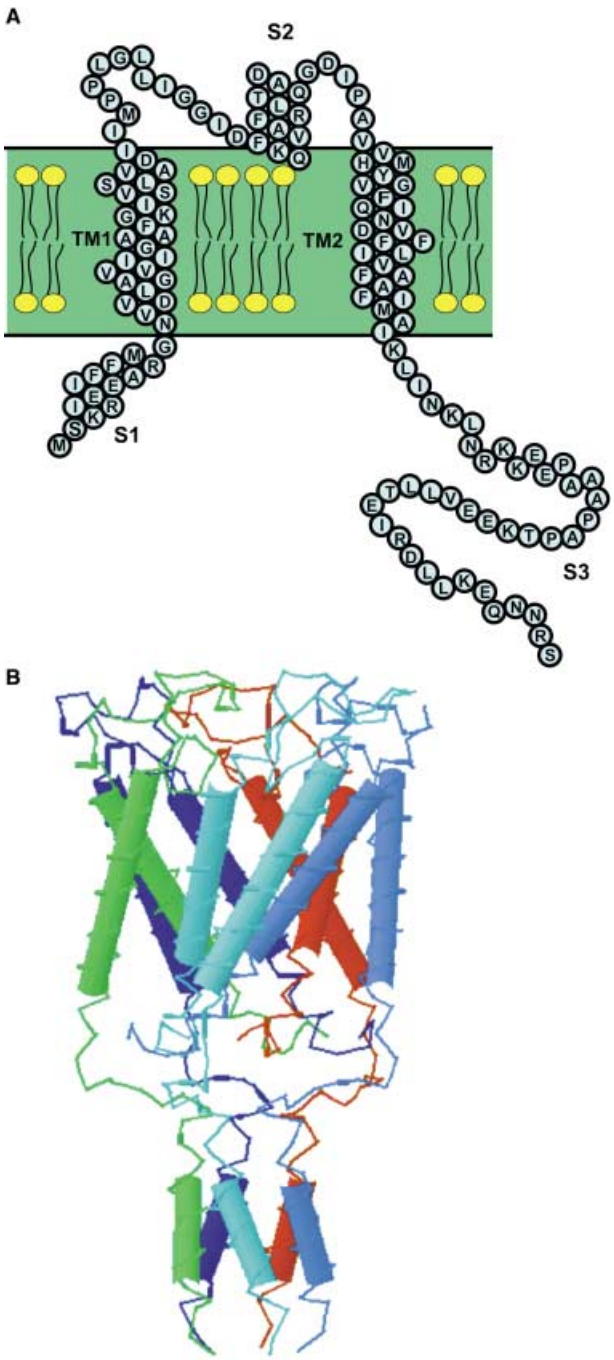


***Figure 1.*** *Secondary and 3D structure of MscL. A) Secondary structure prediction and membrane topology of one single MscL subunit of* E. coli. *The transmembrane segments are indicated by TM1 and TM2. Functionally important domains are designated S1, S2, and S3. B) 3D structure of the homopentameric MscL of* M. tuberculosis *(Protein Data Bank file no. 1MSL).*

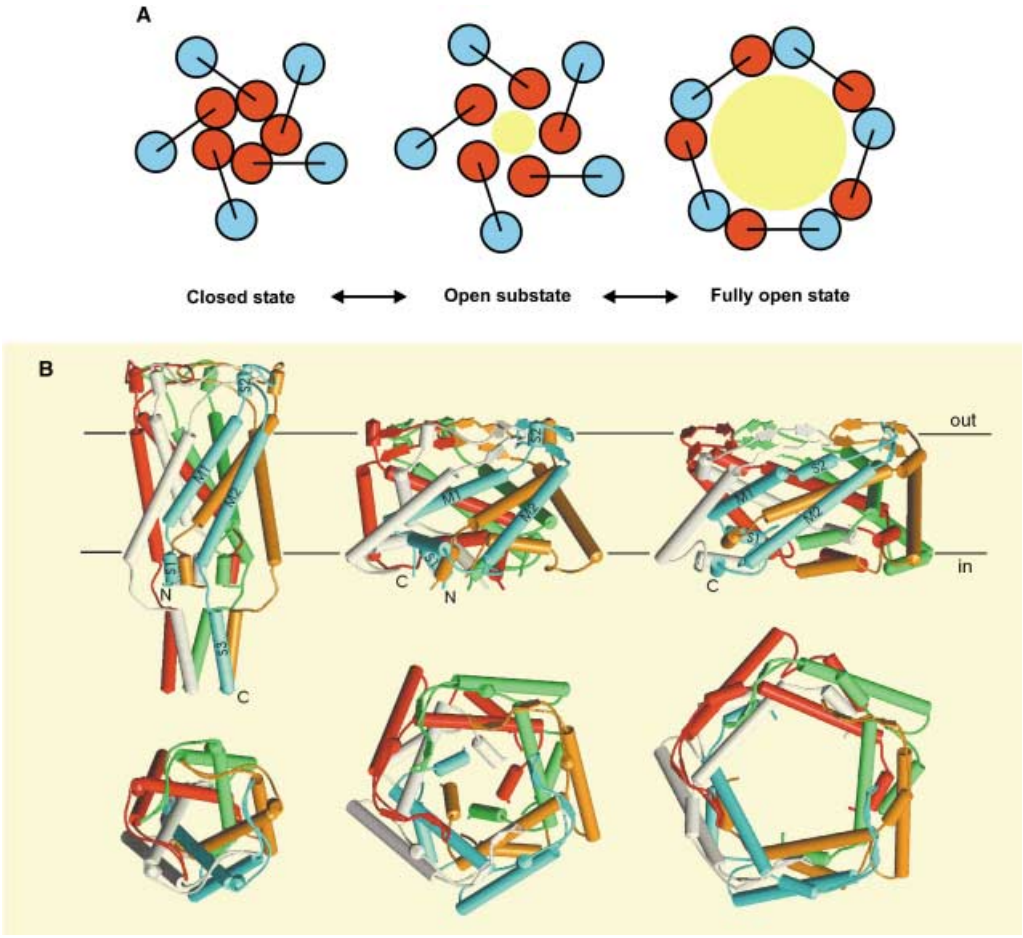


***Figure 2.*** *Two different mechanistic models of the channel opening of MscL. A) As illustrated by a view onto the plane of the membrane, the model of Yoshimura et al.[31] predicts that the channel constriction in the closed state is formed by the inner ring of TM1 helices (red circles) which is surrounded by an outer ring of TM2 segments (blue circles). During transformation of the closed conformation into the open conformation, the inner ring expands and together with TM2 forms the channel pore. B) The model of Sukharev et al.[32, 33] predicts that MscL possesses two independent gates. The first one is the constriction point at the cytoplasmic side of the membrane formed by the five TM1 helices. The opening of this gate occurs through an iris-like movement of the TM1 helices away from the central axis of the pore resulting in the closed expanded state. In this conformation the second gate, the S1 bundle, is still closed. Only if this α-helical bundle is disrupted, can gating occur. Unlike the model of Yoshimura et al., the overall architecture of the inner and outer ring is not disturbed during opening of the helices.*

at the constriction side. This movement involves flattening of the entire channel protein (Figure 2 b), a situation which has recently been suggested by molecular dynamics simulations to occur.[34] In contrast to the first model, the TM2 helices do not line the pore in the open conformation. Further details of MscL structure/function correlation are discussed in the following section.

Besides MscL, several 3D structures of α-helical channel proteins have been solved in the meantime, for example, the water channel AQP1[35, 36] and the glycerol facilitator GlpF,[37] as well as the previously elucidated potassium channel KcsA.[29] AQP1 and GlpF are members of the MIP (major intrinsic protein) family of transporters, which is divided into the subgroups of aquaporins, such as AQP1, and aquaglyceroporins, such as GlpF. Aquaporins are highly specific water channels accelerating the movement of water across the membrane.[38] They are found in animals and plants and also in Gram-negative bacteria; the only water channel in a Gram-positive bacteria identified to date was found in *Enterococcus faecalis* (reviewed in ref. [39]). Although aquaporins are involved in water transport across the membrane and thus contribute to water fluxes as a response of changes in the external osmolarity, their physiological importance in Gram-negative bacteria still remains elusive, since *aqpZ* (encoding for AqpZ) knock-out strains of *Eschericha coli* were found to be barely impaired.[40, 41] The high-resolution structures of AQP1 and GlpF (and also KcsA) helped the understanding of how a channel pore is able to discriminate between different very small substrates, for example, water and protons, or glycerol and water in the case of GlpF. The substrate is forced in single file through a narrow site within the so-called selectivity filter, and in this way identification of the correct ligand is possible.[29, 35–37] In the unspecific efflux channel MscL, on the other hand, a typical selectivity filter is absent. This may reflect the physiological need of MscL in transporting diverse solutes at a very high rate whereas the other systems are constructed to discriminate between chemically very similar ions or organic compounds.

One may ask whether there is a common principle in the design of membrane channels with α-helical transmembrane segments. A common feature seems to be a right-handed

packing of the pore-lining α helices.[42] Interestingly, right-handed helix packing is also observed in the only carrier protein which has been structurally elucidated at least to some extent, namely the bacterial 12 transmembrane $Na^+/H^+$ antiporter of *E. coli*.[43] Further work has to prove, however, whether these pore-lining helices are in fact directly involved in the transport process. In the future, this structural motif may be a helpful tool for identifying pore regions in density maps of channels and transporters for which high atomic resolution has not yet been achieved.[42]

### 2.2. Correlation of structure and function in MscL

Elucidating the structure of the mycobacterial MscL was extremely helpful for understanding the functional principles of mechanosensitivity of these channels. Unfortunately, at the time when its structure was determined, the *M. tuberculosis* homologue had not been shown to function as a MS channel. Hence, it was unclear how much of the knowledge gained about functionally relevant residues and domains of the *E. coli* model system was applicable to the structure derived from the putative orthologue. Moe et al.[44] demonstrated by three different experimental approaches that the mycobacterial MscL in fact forms a functional MS channel with typical conductances.[23] Since the sequence of the *E. coli* and *M. tuberculosis* MscL proteins share an identity of 37% and substitutions of highly conserved amino acids lead to similar changes in the gating properties, it appeared highly probable that the 3D structure of the *M. tuberculosis* McsL could be used as a basis for modeling the *E. coli* protein.

Consequently, after solving the 3D structure of the mycobacterial MscL channel, a series of results obtained earlier on the function of the *E. coli* protein could be reinterpreted. The most highly conserved parts within bacterial MscL proteins are the N-terminal extension and the first transmembrane helix. Analysis of mutants isolated after unspecific mutagenesis revealed that a particular gain of function (GOF) phenotype was correlated with substitutions of amino acid residues located at the cytoplasmic side of TM1.[45] In patch-clamp studies these MscL mutants showed a dramatically increased pressure sensitivity, that is, even small changes in the degree of membrane tension led to activation. In addition, after expression of the corresponding genes in *E. coli* the resulting growth inhibition was most severe when highly conserved hydrophobic residues (one valine and three glycine residues) were substituted by a hydrophilic or a charged amino acid.[31, 45] By using the 3D model of the mycobacterial MscL as a structural basis, it was possible to draw conclusions on closed-state stability and on channel gating. Blount and Moe[46] found that the "sensitive" hydrophobic residues are located either directly at the constriction point of the channel lumen or in close juxtaposition to a neighboring TM1 helix which forms a hydrophobic pocket serving as the channel gate. This hydrophobic "lock" is supposed to be broken in the wild-type protein only by a membrane tension just below a value at which the membrane tears, whereas in the GOF mutants this interaction is more easily disrupted due to the presence of more hydrophilic amino acids.[46] This model is supported by the fact that one of the glycine residues (G 22 in the *E. coli* MscL) is not found in actinomycetes containing mycolic acid or in *Synechocystis*, where an alanine residue is found at the corresponding position. Patch-clamp analyses of MscL channels of *M. tuberculosis* or *Synechocystis* synthesized in *E. coli* revealed that these channels have an activation threshold 2 – 3 times higher than that of other homologues.[23, 44] Consequently, the presence of the more hydrophobic alanine in this position in the *E. coli* sequence was found to result in a higher gating threshold.[44] The reverse substitution of an alanine to a glycine residue in the *M. tuberculosis* MscL, however, did not lead to the expected decrease in the activation threshold.[44] Since the actual opening thresholds of the wild-type *M. tuberculosis* MscL and of the mutant were measured near membrane tensions where disruption occurs (lytic limit of the membrane), this result should be interpreted with some caution. Further investigations are necessary to determine the significance of this particular residue for the different gating characteristics of the MscL homologues in *M. tuberculosis* and *Synechocystis*.

Recently, an interesting new suggestion for the gating mechanism of the MscL channel was put forward by Sukharev et al.,[32] as mentioned above. Besides a new model of an iris-like movement of TM1 during transition from the closed to the open state, a second gate formed by an α-helical bundle of the N-terminal extensions (S1) of the five subunits of MscL was predicted. As a matter of fact, this part of the channel did not show a distinct structure in the 3D structure of Chang et al.[27] The model of Sukharev et al. is based on the assumption that membrane tension first leads to an expanded closed confirmation. This was also indicated by thermodynamical analyses in which the channel was predicted to expand to two-thirds of its open size.[47] In this state the channel constriction formed by TM1 is open, but gating does not yet occur. Finally, the channel fully opens if the second gate is opened by disrupting the S1 bundle (Figure 2b). In this model the transmembrane helices act as an "elastic" barrel working as a tension sensor. Force is conveyed to the S1 gate only when expansion of the barrel fully extends the linker between TM1 and S1.[32] This model was experimentally supported by applying cross-linking experiments which showed that particular hydrophobic residues of the S1 bundle are in close contact in the inactive state, and that this interaction is broken after activating the channel. The authors argued that two gates in series might be required to make MscL absolutely leak-proof in a wide range of subthreshold tensions and therefore guarantee the sensitive balance between two important needs: avoiding cell disruption and maintaining electrical integrity of the membrane.

### 2.3. What kind of stimulus is sensed by MS channels?

In recent years a series of arguments and experimental indications has been presented to support the idea that MS channels act as tension sensors, that is, as devices able to sense changes in membrane strain which occur as a consequence of osmotic stress. Early observations using patch-clamp techniques in spheroplasts, for example, showed that the channels were closed at all membrane potentials unless suction was applied.

The fraction of channels which became activated immediately closed again upon release of suction.[21] In addition, the channels could be activated by inserting amphipathic molecules in the membrane, a fact which was taken as indication that the gating force is exerted through the surrounding lipid phase.[48] Meanwhile further concepts were developed to explain how the change in membrane tension could be coupled to a change in protein conformation. The addition of proteases in the patch-clamp set up revealed that limited proteolysis of the periplasmic loop resulted in a hypersensitive phenotype. Interestingly, the channel was still functional after proteolytic cleavage, since the gating still fully depended on the application of suction.[49] This was interpreted in terms of the periplasmic loop acting as a spring, resisting opening of the channel and promoting its closure when it is open. Also, limited proteolysis of the N- or C-terminal part of each subunit resulted in a hypersensitive, but still functional, MscL. Besides indicating that the N- and C-terminal extensions are also somehow involved in defining the sensitivity to membrane tension, this result proved that none of the different extramembranous domains are absolutely essential for mechanosensitivity.[49]

In principle, the conclusions drawn from limited proteolysis studies are in agreement with the model presented by Sukharev et al.[32] in which the membrane part functions as an elastic barrel reacting to pressure changes (see above). Taken together, these and further experimental details all argue for a concept in which the whole structure of the MscL protein acts as a tension sensor. The degree of pressure exerted by the surrounding membrane is transformed into a graded movement of the transmembrane helices of MscL, which leads to gating only if the threshold is reached. Additional data, however, seem to be necessary for understanding the modulating influence of the extramembranous domains on the channel sensitivity. A further interesting question still to be answered addresses the structural reasons which cause the particular transmembrane helix arrangement of the MscL channel to be extraordinarily sensitive to membrane tension, in contrast to other, related solute channel structures.

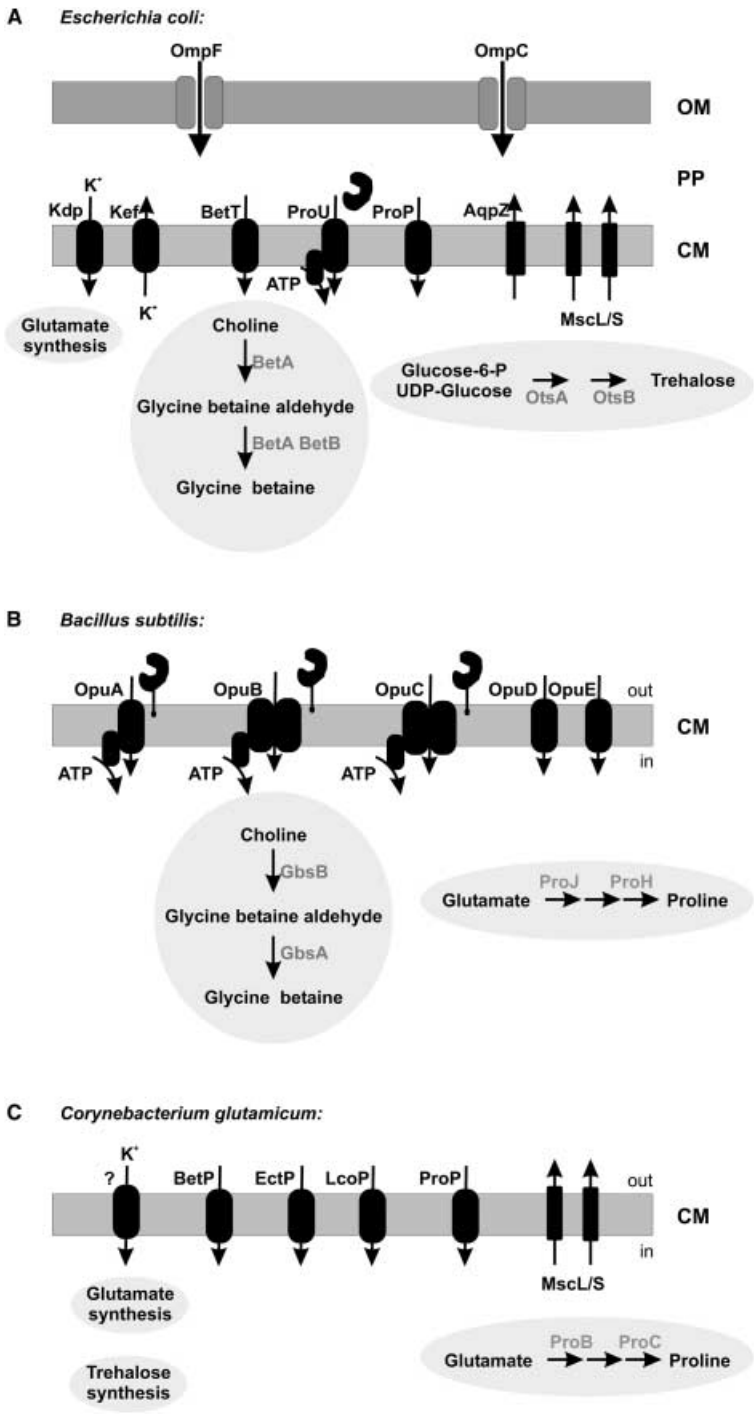


***Figure 3.*** *Systems involved in the osmotic response by biosynthesis and solute uptake of A)* E. coli, *B)* B. subtilis, *and C)* C. glutamicum. *OM = outer membrane, PP = periplasm, CM = cytoplasmic membrane.*

## 3. Means to Stay under Pressure: Adaptation to Hyperosmotic Stress

As far as studied in detail, the first reaction of bacterial cells, including *E. coli*, *Bacillus subtilis*, and *Corynebacterium glutamicum*, to the passive efflux of water after an osmotic upshift is uptake of $K^+$,[50–54] which is accompanied by the synthesis of glutamate in *E. coli* and *C. glutamicum* or of proline in *B. subtilis* (Figure 3). With this event the process of rehydration of the cytoplasm and restoration of cell turgor starts. The initial fast response of $K^+$ accumulation leads to unfavorably high ion concentrations in the cytoplasm which may induce aggregation of macromolecules by enhancing hydrophobic interactions.[4] Therefore, bacteria (with the exception of halophilic organisms of the "salt-in strategy") exchange initially accumulated potassium for compatible solutes (see the Introduction). For being effective in situations of hyperosmotic stress, compatible solutes have to be accumulated to high intracellular concentrations, either by synthesis or by uptake from the medium. A variety of compatible solutes is used by microorganisms,[55–57] but only a few of the biosynthetic pathways involved have been elucidated in detail so far. Examples are the

disaccharide trehalose, the trimethylammonium compound glycine betaine, the tetrahydropyrimidine ectoine, and the amino acid proline. In the absence of any compatible solute in the medium, trehalose was found to be the preferred compatible solute synthesized in *E. coli*,[50] whereas in *B. subtilis* or *C. glutamicum* proline plays an equally prominent role.[52, 58, 59] Osmoprotection through synthesis of compatible solutes is not the focus of this article and the reader is referred to other reviews on this topic.[4, 58, 60]

### 3.1. Transport of compatible solutes

In addition to the de novo synthesis of compatible solutes, bacteria are able to acquire them from exogenous sources if present. In the ecosystem these compounds originate from decaying microbial, plant, and animal cells, as well as from root exudates; this leads to to locally varying concentrations of osmoprotectants.[55, 61] This is the reason for the particular properties of most uptake systems for compatible solutes: (1) high affinity for their substrates, (2) the capacity for high internal accumulation of the transported solute against its concentration gradient (energetic coupling), and (3) high activity under conditions of increased osmolarity and ionic strength, where transporters for nutrients are generally found to be inhibited.[62] The significance of compatible solute uptake for microbial cells is further emphasized by the fact that almost all bacteria possess a multitude of uptake systems, which in general show different substrate specificities and affinities and thus provide an optimal adaptation to varying environmental conditions (Figure 3). Up to now osmotically regulated uptake systems for compatible solutes were identified among all different classes of transport systems, namely binding protein dependent ABC transporters (for example, ProU of *E. coli* or OpuA of *B. subtilis*), binding protein dependent secondary transporters (ectoine uptake in *Halomonas elongata*; J. Kunte, personal communication), or $Na^+$ or $H^+$ dependent secondary transporters (for example, BetP and EctP of *C. glutamicum* or ProP of *E. coli*).

In most cases studied so far these transporters are strictly regulated at the level of activity and very often also at expression level. Regulation of activity, which is the focus of this review, means an instant change of transport activity after an increase in external osmolarity. This also requires the ability to optimally adapt the catalytic activity (solute transport) to the extent of osmotic stress (osmoregulation). For this purpose, carrier systems have to perceive a certain stimulus related to hyperosmotic stress (osmosensing) and to transmit the perceived signal from sensory to catalytic units (signal transduction). Two principle ways can be thought of as to how this signal transduction network may be organized to result in activation of compatible solute uptake. Either separate membrane-integrated sensor proteins or cytoplasmically located receptors perceive the stimulus and transmit the signal to the respective carrier systems, or the transporter itself combines the two functions of sensing and regulation. For three osmoreactive carrier systems, namely ProP of *E. coli*,[63] BetP of *C. glutamicum*,[64] and OpuA of *Lactococcus lactis*,[65] it was recently demonstrated by functional reconstitution of the respective purified proteins in proteoliposomes that they indeed comprise both the functions of an osmosensor and an osmoregulator. Other well-known examples for proteins with osmosensory functions are the regulatory modules of the two-component systems EnvZ and KdpD.[6, 66–68] They are not directly involved in transport processes, but regulate gene expression of the outer membrane porins OmpC/OpmF or of the $K^+$ uptake system Kdp of *E. coli*, respectively, with dependence on the external osmolarity.

### 3.2. What is known about the structure of compatible solute transporters?

In contrast to the expanding knowledge of 3D structures of channel proteins like MscL, no high resolution 3D structure of the membrane part of any ABC-type or secondary transporter is available. In particular, secondary transport proteins with a typical mass of 45 – 60 kDa have resisted all attempts for high resolution X-ray studies so far. The reason for this is supposed to be the extremely high flexibility and dynamic properties of these membrane proteins which makes it difficult to obtain crystals of high quality. Recently, the structure of the $Na^+/H^+$ antiporter NhaA from *E. coli* was analyzed by 2D electron crystallography to 7 Å resolution,[43] which provided an impression of the overall arrangement of the 12 transmembrane helices within the membrane part of the carrier. Unfortunately, this example is also still far from atomic resolution, which would be necessary for combining functional data with knowledge on structure, as in the case of MscL. Also for the osmosensory proteins KdpD, ProP, BetP, and OpuA, which will be discussed in more detail below, only secondary structure predictions and/or data have been obtained up to now.[69–76] Nevertheless, protein domains involved in the sensing process have been determined for BetP, ProP, and KdpD (see below and Figures 4 – 7).

### 3.3. What are the possible stimuli for osmosensors?

Direct response as well as long-term adaptation to osmotic stress have been studied in several bacterial species in great detail, for example, *E. coli*, *Salmonella typhimurium*, and *B. subtilis* (reviewed in refs. [4, 56, 58]), but the mechanisms related to sensing hyperosmotic stress are not well understood. This refers in particular to the physical parameters which are supposed to be osmorelevant stimuli. Over the course of the years, many possible stimuli have been discussed as putative parameters triggering activation of osmoresponsive membrane-embedded proteins after an osmotic upshift.[4, 56, 77, 78] Possible candidates are: (1) turgor pressure, (2) membrane strain or shrinkage, which affects the bilayer in the plane of the membrane (curvature stress) in response to a change in cell volume or turgor pressure, (3) external osmolarity, ion concentration or ionic strength, (4) cytoplasmic osmolarity, ion concentration or ionic strength, (5) change of the concentration of specific solutes in the cytoplasm or in the surrounding of the cell, (6) the transmembrane osmotic gradient, or (7) macromolecular crowding as a consequence of the change of the cytoplasmic volume. Since all

these parameters (besides the external conditions) are consequences of the water efflux from the cell following a hyperosmotic shift, that is, of a process which occurs within a range of milliseconds, it is very likely that these changes also take place more or less simultaneously. That is one of the reasons why it is impossible to discriminate in vivo, which of the different putative stimuli is actually perceived by an osmosensory protein. Consequently, to successfully dissect this scenario of events, it is necessary to reduce the complexity of the system. In the examples discussed below, this goal was achieved by using functional reconstitution of osmoregulated proteins in proteoliposomes. This experimental approach has two major advantages. First, putative factors contributing to osmoregulation can be defined more easily in a simple system consisting of a purified protein and a defined lipid bilayer only. Second, in proteoliposomes the three different phases surrounding the inserted protein, that is, both the external and internal solvent as well as the hydrophobic membrane, are freely accessible to experimental variation.

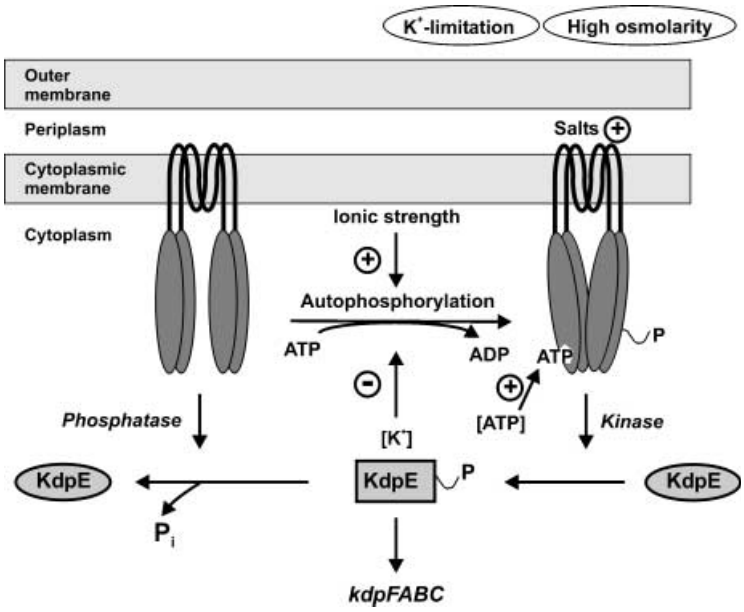


***Figure 4.*** *Model of the regulation of the KdpD activity of* E. coli *from Jung et al.*[90] *The autophosphorylation activity of KdpD is increased by $K^+$ limitation or high external osmolarity resulting in the phosphorylation of KdpE. In this activated state KdpE enhances the transcription of the* kdpFABC *operon. In vitro assays further showed that high internal $K^+$ concentrations are inhibitory for KdpD, but high ionic strength or binding of ATP to the N-terminal domain increases the kinase to phosphatase ratio of KdpD.*

### 3.4. Bacterial osmosensors

In spite of increasing knowledge in recent years on physiological and genetic responses of bacteria to osmotic stress, much less is known concerning the type of stimulus that is perceived by osmosensory mechanisms and osmosensing systems, and no unifying factor or trigger mechanism has been identified as being responsible for osmoreactive activation of solute transport systems up to now. In order to provide an overview of the best-studied examples of such kind of systems, four membrane proteins involved in bacterial osmosensing and osmoregulation, namely KdpD and ProP of *E. coli*, BetP of *C. glutamicum*, and OpuA of *L. lactis* will be described in more detail in the following sections.

#### *3.4.1. KdpD of Escherichia coli*

The first physiological response of *E. coli* cells to a hyperosmotic shift is the fast uptake of $K^+$ through the low-affinity Trk and the high-affinity Kdp transport systems.[79] Increase in external osmolarity results in the activation of both transport systems, Trk and Kdp, within seconds after upshift. Whereas Trk is constitutively expressed, osmostress and $K^+$ availability control, through the KdpD/KdpE two-component regulatory module, the expression of the *kdpFABC* operon which encodes Kdp, a P-type ATPase. For this regulation the following model was proposed (see also Figure 4). Signal perception induces autophosphorylation of the sensor kinase KdpD, the phosphate group is transferred to the soluble response regulator KdpE, which then binds to the *kdpFABC* promoter and stimulates transcription. KdpD is also responsible for the repression of the *kdpFABC* operon, since it also includes a phosphatase activity resulting in deactivation of KdpE.[80–84]

The kind of stimulus which is perceived by KdpD is controversially discussed. Analysis of *kdpFABC* expression in response to osmotic stress, to low external $K^+$, and after modulation of internal $K^+$ concentrations suggested that the Kdp system is synthesized in response to low turgor, summarized in the so-called turgor model.[68] This is in agreement with the observation that *kdp* transcription is only transiently induced after osmotic upshock. This regulatory pattern is also consistent with the observed restoration of turgor by an increase in intracellular $K^+$ levels. Subsequent studies, however, challenged this model. Expression was found to depend on the nature of the solute, ionic solutes being more effective than isoosmotic amounts of neutral solutes.[85, 86] Furthermore, mutant KdpD versions were generated, which proved to be either sensitive to osmotic upshifts and no longer to $K^+$ limitation or vice versa, thus indicating that KdpD can be activated by different stimuli.[69, 70]

Two regions within the KdpD protein have been identified as being important for activity regulation (kinase/phosphatase ratio) and thus for its sensing properties. KdpD (894 amino acids) functions as a homodimer[71] and consists of a central domain of four membrane-spanning segments and a long cytoplasmatic N- and C-terminal domain.[87] The occurrence of the 400 amino acid long N-terminal domain is confined to KdpD proteins in various microorganisms and is not found in any other known sensor kinase.[88] This extension includes two ATP binding motifs, which are important for the kinase activity, since deletion of the whole domain or inactivation of the ATP-binding sites resulted in a drastically reduced expression of the *kdpFABC* operon.[88] In addition, amino acid substitutions leading to a constitutive *kdpFABC* expression because of the loss of phosphatase activity are clustered in the last transmembrane segments of KdpD or in

parts adjacent to them.[69, 89] The availability of simplified experimental systems, that is, proteoliposomes and membrane vesicles, allowed direct study of the triggers possibly influencing KdpD kinase and/or phosphatase activity. By use of right-side-out membrane vesicles Jung and co-workers[90] recently showed that by increasing the external osmolarity KdpD became activated, whereby salts were more effective in activation than neutral solutes. In this kind of experiment, however, vesicle shrinkage and the resulting increase in internal solute concentration occur simultaneously and one can thus not distinguish whether changes in membrane strain or in the concentration of specific solutes are responsible for the effect on autophosphorylation. In experiments directly varying the solute composition, internal $K^+$ concentration and/or ionic strength were identified as signals influencing KdpD activity. Interestingly, potassium concentrations higher than 1 mM were inhibitory whereas an increase in ionic strength by other ions inside the vesicles led to stimulation of KdpD autophosphorylation.[90] The results are in agreement with the idea that under $K^+$-limited growth the intracellular $K^+$ concentration falls below a certain threshold, thereby releasing inhibition of KdpD autophosphorylation. One has to keep in mind, however, that the in vivo cytoplasmic $K^+$ concentrations are at least about 200 times higher than those found to be inhibitory in the vesicle system. On the other hand, Roe et al.[91] determined high expression levels of the *kdpFABC* operon in a recombinant *E. coli* strain (*kdp*$^+$, *kdp-lacZ*, *trk*$^-$) only in growth media with limiting $K^+$ concentrations. Under these conditions, the authors found high internal $K^+$ concentrations of 477 mM, and therefore suggested that *kdpFABC* expression, besides by turgor, is regulated by the external $K^+$ concentration.

#### 3.4.2. ProP of Escherichia coli

Two osmoregulated uptake systems, namely the ABC-type transporter ProU and the secondary carrier ProP, mediate the uptake of most compatible solutes in *E. coli* (reviewed in ref. [6]). In contrast to ProP, the ProU system responds to osmotic stress mainly on the level of expression.[77, 92] ProP of *E. coli* or of *S. typhimurium* is a single-component $H^+$ symport carrier accepting a broad variety of compatible solutes structurally related to glycine betaine or proline.[93] After an osmotic upshift ProP is activated with a half time of about 1 min both in vivo and in vitro, with a higher extent of osmotic stress being required to reach maximum activity in vesicles (0.8 Osmol $kg^{-1}$; Osmol = a measure of osmolarity) than in cells (0.2 Osmol $kg^{-1}$).[94, 95] In addition, exogenous $K^+$ is necessary for maximum activation of the carrier in *E. coli* and *S. typhimurium*.[93, 96] ProP was the first carrier protein which has been shown to function both as osmosensor and osmoregulator by functional analysis in proteoliposomes.[63]

ProP (500 amino acids), a member of the major facilitator superfamily, is predicted to possess 12 transmembrane segments as well as N- and C-terminal hydrophilic domains (Figure 5). Based on its similarity to the $\alpha$-ketoglutarate transporter KgtP of *E. coli*, the topology of which had been investigated by PhoA fusions,[97] it was concluded, that the terminal domains face the cytoplasm. This was confirmed by

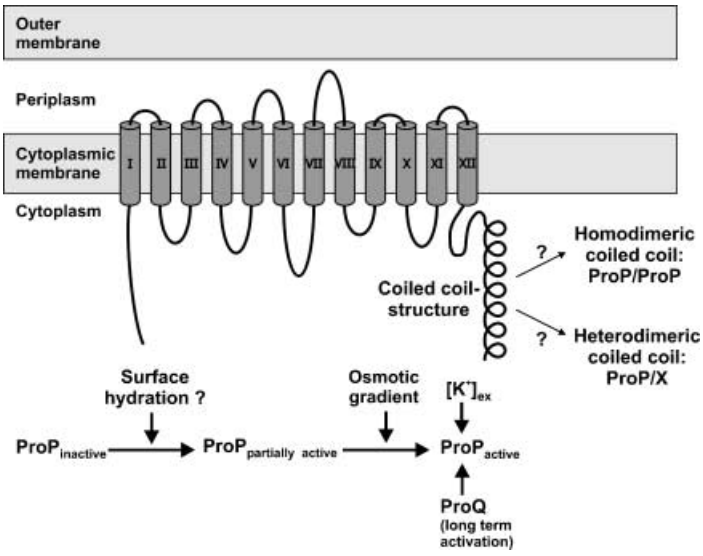


***Figure 5.*** *Model of the activation process of ProP in* E. coli *under hyperosmotic stress. ProP is fully activated after an osmotic upshift by membrane impermeable solutes causing an osmotic gradient. Partial activation is also detectable with small permeable solutes (such as PEG), which indicates a direct influence of solutes on the surface hydration of ProP. In addition, the activation of ProP depends on the availability of external potassium. The C-terminal domain of ProP is suggested to have a regulatory function and maybe involved in the formation of a homodimeric or heterodimeric coiled-coil structure.*

studies in membrane vesicles with antibodies against the C-terminal domain (Wood, personal communication). The osmoregulated carriers ProP of *E. coli* and OusA of *Erwina chrysanthemi*[98] differ from related transporters like KgtP by possessing a C-terminal extension with six to seven so-called heptad repeats, a characteristic of $\alpha$-helical coiled-coil forming proteins.[72, 73] Experimental evidence has been accumulated indicating that this structure has regulatory functions in ProP: (1) a derivative of ProP lacking 26 amino acids at the C-terminal end was found to be inactive although integrated into the membrane, (2) a synthetic polypeptide corresponding to the C-terminal domain of ProP was shown to form a dimeric coiled-coil, and (3) stabilization of the coiled-coil structure by substitution of strategic amino acids led to higher activation thresholds in vivo.[73] If the propensity of the C-terminal domain to form coiled-coil structures in fact plays a central role in osmosensing by ProP, further studies are necessary to identify the possible interaction partner with respect to the formation of a homodimeric (ProP/ProP) or heterodimeric coiled coil (ProP/X). In any case, coiled-coil formation seems to be a particular feature of the *E. coli* ProP, since other osmosensors, at least those described here, lack this structure. Interestingly, the homologue of ProP in *C. glutamicum* is not predicted to contain a coiled-coil structure, although being osmoregulated.

The osmoresponsive action of ProP in *E. coli* is more complicated by the fact that ProQ, a soluble cytoplasmic protein, is known to influence ProP activity. In a ProQ-defective strain, ProP activity is fivefold lower, and ProQ was found to be necessary to maintain ProP activation for longer periods of time.[99] Since ProP alone is able to act as osmosensor, ProQ is supposed to function in fine tuning the osmotic response.[4]

Besides being an interesting object for defining domains related to osmosensing, ProP is also an example where likely candidates for physico-chemical stimuli have been dissected. In intact cells, the triggering signals for *E. coli* ProP have not been fully elucidated so far. Indirect evidence for $K^+$ being the relevant signal has been provided,[4, 96] but no unequivocal correlation was possible because of the multiplicity of consequences on cellular functions when altering cytoplasmic $K^+$. Turgor pressure as a trigger is unlikely, since osmosensing has been shown to function in proteoliposomes where a cell wall is missing.[63] In recent studies in the reconstituted system it was observed that ProP, although depending on liposome shrinkage for full activity, could be activated to a small but significant extent by addition of membrane-permeable solutes of low molecular mass, like polyoxyethylene glycol (PEG), which do not lead to vesicle shrinkage.[100] The authors conclude that ProP activation could also be triggered, at least to some extent, by direct influence of solutes (in this case PEG) on surface hydration of the carrier protein. This reflects a common hypothesis, in which preferential hydration, due to exclusion of added solutes from the macromolecular surface, is thought to be the basis for conformational effects of solutes on macromolecules.[7]

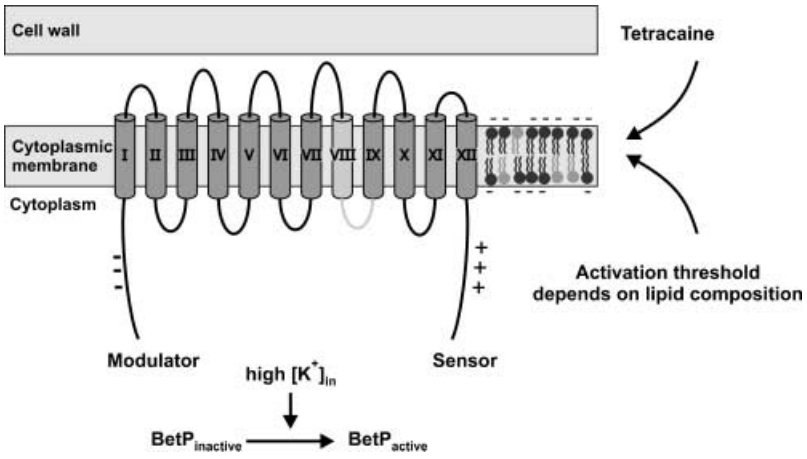


***Figure 6.*** *Model of the activation of BetP in* C. glutamicum *under hyperosmotic conditions. An increasing internal $K^+$ concentration is the stimulus for BetP activation. In proteoliposomes membrane shrinkage caused by an osmotic gradient does not lead to the activaton of BetP. The phospholipid composition of the lipid bilayer defines the threshold of activation. The amphipathic tetracaine also influences BetP activation. Furthermore, the N- and C-terminal domains of BetP are involved in the sensing processes.*

### *3.4.3. BetP of Corynebacterium glutamicum*

The Gram-positive soil bacterium and biotechnologically important amino acid producer *C. glutamicum* is equipped altogether with four secondary uptake systems for compatible solutes.[74, 101] Among these, BetP, a member of the so-called BCCT family of transporters, is tightly regulated in response to osmotic challenge both at the level of expression and activity. BetP is specific for glycine betaine, the uptake of which is coupled to the cotransport of $2\,Na^+$ ions and thus leads to extremely high accumulation ratios.[102] BetP has been isolated and reconstituted in proteoliposomes where it was shown to have retained its catalytic (kinetic properties), regulatory (response to osmotic stress), and osmosensory functions,[64] thereby indicating that all these aspects are mediated by one single polypeptide.

BetP has a size of 595 amino acids, consists of 12 transmembrane segments, and carries two hydrophilic domains of 55 – 60 amino acids at both the N- and C-terminal end which are oriented towards the cytoplasm (Figure 6).[103] In intact cells, BetP is inactive in the absence of osmotic stress and becomes activated on a subsecond time scale in response to an increase of external osmolarity. BetP is regulated at the level of activity not only in *C. glutamicum*, but also when expressed in *E. coli*, although in the heterologous host it shows an optimum of stimulation at lower values of osmolarity.

By functional analysis of mutant proteins expressed in intact cells, the terminal domains of BetP were found to be directly involved in osmosensing. Truncation of the N-terminal domain of BetP led to a decrease in osmosensitivity, that is, much higher osmotic stress is necessary to activate a N-terminally truncated BetP. On the other hand, a truncation of the C-terminal domain by 12 or 23 amino acids led to a complete deregulation of BetP. Consequently, this mutant form of BetP is constantly catalytically active, that is, with respect to betaine transport, but does not any more respond to osmotic stress.[75] It has also been shown that in the case of EctP, another osmoregulated secondary carrier in *C. glutamicum* belonging to the same carrier family as BetP, truncations of the corresponding C-terminal domain lead to uncoupling of catalytic and regulatory function.[104] Although the C-terminal domain of EctP is not at all structurally related to that of BetP, truncations led to a very similar effect, that is, to constant activity which does not respond to osmotic stress. Osmosensory signal input directly related to hydrophilic terminal domains thus seems to be a more general mechanism, at least in *C. glutamicum*.

Detailed studies have been carried out to define the kind of physico-chemical stimulus involved in osmosensing by BetP. As in the case of ProP of *E. coli*, turgor is not a likely candidate in view of the fact that the osmoregulatory function in proteoliposomes is retained. Membrane strain as the result of osmotic stress is generally thought to be an important stimulus. At least in the case of reconstituted BetP, vesicle shrinkage as a response to hyperosmotic conditions was per se not able to induce activation of transport.[103] From all kinds of possible stimuli, including internal and external osmolarity as well as solute composition, the increase of $K^+$ concentration at the cytoplasmic side of BetP was exclusively found to be responsible for osmoreactive activation.[103] It is interesting to note that on the basis of these results, a putative mechanosensor, formerly suspected of perceiving membrane strain or related stimuli, has been reinterpreted to be a chemosensor.

The identification of $K^+$ being a direct stimulus for BetP in osmosensation, however, has to be corroborated by detailed

investigations in intact cells to exclude other factors as putative additional stimuli. This is true in particular for possible direct effects of surrounding phospholipids on BetP in view of the facts (1) that the optimum of hyperosmotic stimulation has been shown to be modulated by the kind of phospholipid species used for preparing the proteoliposomes,[64] and (2) that BetP activity was found to be influenced by the presence of the membrane-active amphipathic compound tetracaine both in intact cells[75] and in proteoliposomes.[64] These amphiphiles are predicted to alter curvature stress in phospholipid membranes.[105, 106]

#### 3.4.4. OpuA of Lactococcus lactis

Lactic acid bacteria are known for their limited capacity to synthesize compatible solutes, and most of these organisms are multiple amino acid auxothrophs. They reside in environments where these amino acids are present, as well as the compatible solutes glycine betaine or carnitine. The only way by which they are able to recover from hyperosmotic stress is uptake of compatible solutes.[78] In contrast to other bacteria, lactic acid bacteria seem to be quite limited in the number of uptake systems for osmoprotectants. Recently, OpuA (also named BusA), an ABC-type transporter of *Lactococcus lactis* was isolated and characterized.[65, 76, 107] Sequence analysis revealed that this uptake system belongs to a new type of ABC transporters, where the binding protein is fused to the membrane-embedded part of the carrier (Figure 7). In typical ABC transporters of Gram-positive organisms the binding protein is membrane-anchored

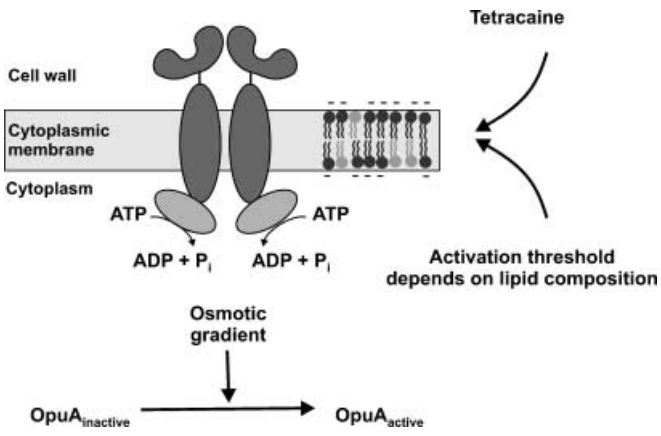


***Figure 7.*** *Model of the activation of OpuA in* L. lactis *under hyperosmotic conditions. OpuA is fully active after an osmotic upshock by membrane-impermeable solutes causes an osmotic gradient. The phospholipid composition of the membrane is critical for the set point of activation of OpuA. Furthermore, the amphipathic tetracaine influences OpuA activity.*

by a fatty acid covalently bound to a cysteine residue. In contrast to the other osmosensors discussed here, it has not been investigated so far which parts of this multidomain transporter are responsible for or related to the sensing function. Functional reconstitution of OpuA in proteoliposomes allowed several possible physico-chemical stimuli,[65] such as turgor pressure, to be excluded. Based on the observation that OpuA was activated after an osmotic upshift with either ionic or nonionic membrane impermeable solutes and not by glycerol, which is membrane permeable in *L. lactis*, it was concluded that this carrier does not sense the absolute external or internal osmolarity, but rather the transmembrane osmotic gradient. The authors suggest that the most likely stimulus would be a change of the physical properties of the phospholipid membrane. In accordance with this interpretation, addition of the amphipathic tetracaine led to activation of OpuA in the absence of osmotic stress, an observation which was also made in the case of BetP from *C. glutamicum*. Given the role of the membrane in transducing osmotic signals to OpuA, it is likely that different physical properties associated with different lipid compositions will affect the transport system. This hypothesis was supported by the finding that OpuA, when reconstituted in *E. coli* lipids, showed a higher sensitivity to increasing osmolarities than under in vivo conditions.[65, 76, 107] A similar observation was also made for BetP of *C. glutamicum*.[64] Further studies will reveal whether the head group composition, the acyl chain length, or the degree of saturation are responsible for modulation of carrier activity.

## 4. Summary and Outlook

An effective and adapted response to osmotic stress is a fundamental prerequisite for survival of all prokaryotic and many eukaryotic cells. Besides the fact that osmoresponsive mechanisms are abundantly found in archaea, bacteria, and eukarya, their importance is further emphasized by the observation that a high redundancy of systems is generally found in these cells for coping with both hypoosmotic and hyperosmotic shock. The correlation between permeability and activation pressure threshold within the family of mechanosensitive channels, namely MscL, MscS, and MscM, which are perfectly tuned to the extent of osmotic downshift with the smallest channel being activated first, suggests that the sum of these channels is in fact able to provide a graded response to hypoosmotic shock. On the other hand, the observed high redundancy of uptake systems may be the basis for a well-adapted response to hyperosmotic conditions of varying extent in the presence of various compatible solutes of different suitability for intracellular accumulation available in the surrounding.

In terms of evolutionary origin, the situation is quite different for the efflux versus the uptake systems. As known so far, at least two types of gene families encoding MS channels, namely those of the MscL and MscS types, have evolved in bacteria. The high similarity within these families may be interpreted by an origin from a common ancestral gene as suggested by Kloda and Martinac.[14] On the other hand, osmoregulated uptake systems show an extreme diversity in terms of structure and function. All kinds of mechanisms are found, ABC transporters, $H^+$-and $Na^+$-coupled secondary carriers, and potential-driven binding protein systems, which indicates convergent evolution to osmoresponsive systems from different origins.

The complete signal transduction pathway of the cell's response to osmotic challenge is still far from being understood. The sequence of events starts with an osmotic shift in the surrounding medium leading to a physico-chemical stimulus

which is detected by the osmosensory receptor systems of the cell. The signal is then transduced to transport systems and biosynthetic enzymes leading to an immediate response of the cell on the level of protein activity. Furthermore, signal transduction takes place in similarly unknown ways through transcription factors to osmoregulated promoters resulting in osmoresponsive changes of gene expression and finally in adapted equipment within the cell, with carriers, enzymes, and structural proteins.

In terms of osmosensing, the situation has emerged to be not as complex as for the efflux systems. Mechanosensitive channels seem to respond by a common mechanism directly to the alteration of membrane strain as the result of hyperosmotic stress. Investigations in this direction were greatly stimulated by the progress in structure elucidation of these channel proteins. Unfortunately, this is not the case with uptake carrier proteins, where no 3D structures are available, as is true for membrane transport systems in general. Whereas significant knowledge is available with respect to kinetic mechanisms and to regulation at the level of both activity and expression, the kind of the osmosensory stimulus is still under debate. In principle, common mechanisms of osmosensing could be expected in view of identical physico-chemical stimuli. Data available so far, however, seem to indicate a multiplicity of sensing mechanisms, which is different to the tension-regulated mechanosensitive channels. The reason for this multiplicity may be that osmoreactive uptake systems have evolved from very different structural origins, which thus possibly involved different lines of invention with respect to stimulus perception. The most urgently needed basis for a better understanding of these aspects is certainly the 3D structure of one of these membrane proteins, together with a detailed analysis of protein dynamics under conditions of osmotic response.

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