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Bacterial osmosensing: roles of membrane structure and electrostatics in lipid-protein and protein-protein interactions

Poolman, B.; Spitzer, J.J.; Wood, J.A.; Wood, Janet M.

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Review

Bacterial osmosensing: roles of membrane structure and electrostatics in lipid–protein and protein–protein interactions

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Abstract

Bacteria act to maintain their hydration when the osmotic pressure of their environment changes. When the external osmolality decreases (osmotic downshift), mechanosensitive channels are activated to release low molecular weight osmolytes (and hence water) from the cytoplasm. Upon osmotic upshift, osmoregulatory transporters are activated to import osmolytes (and hence water). Osmoregulatory channels and transporters sense and respond to osmotic stress via different mechanisms. Mechanosensitive channel MscL senses the increasing tension in the membrane and appears to gate when the lateral pressure in the acyl chain region of the lipids drops below a threshold value. Transporters OpuA, BetP and ProP are activated when increasing external osmolality causes threshold ionic concentrations in excess of about 0.05 M to be reached in the proteoliposome lumen. The threshold activation concentrations for the OpuA transporter are strongly dependent on the fraction of anionic lipids that surround the cytoplasmic face of the protein. The higher the fraction of anionic lipids, the higher the threshold ionic concentrations. A similar trend is observed for the BetP transporter. The lipid dependence of osmotic activation of OpuA and BetP suggests that osmotic signals are transmitted to the protein via interactions between charged osmosensor domains and the ionic headgroups of the lipids in the membrane.

The charged, C-terminal domains of BetP and ProP are important for osmosensing. The C-terminal domain of ProP participates in homodimeric coiled-coil formation and it may interact with the membrane lipids and soluble protein ProQ. The activation of ProP by lumenal, macromolecular solutes at constant ionic strength indicates that its structure and activity may also respond to macromolecular crowding. This excluded volume effect may restrict the range over which the osmosensing domain can electrostatically interact. A simplified version of the dissociative double layer theory is used to explain the activation of the transporters by showing how changes in ion concentration could modulate interactions between charged osmosensor domains and charged lipid or protein surfaces. Importantly, the relatively high ionic concentrations at which osmosensors become activated at different surface charge densities compare well with the predicted dependence of 'critical' ion concentrations on surface charge density. The critical ion concentrations represent transitions in Maxwellian ionic distributions at which the surface potential reaches 25.7 mV for monovalent ions. The osmosensing mechanism is qualitatively described as an "ON/OFF switch" representing thermally relaxed and electrostatically locked protein conformations.
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Keywords: Lipid–protein interaction; Protein–protein interaction; Osmosensing; Membrane transport; Mechanosensitive channel; Signal transduction; Electrostatic force; Debye–Hückel; Maxwellian electrostatics; Dissociative electrical double layer

Abbreviations: ABC, ATP-binding cassette; BCCT, betaine-carnitine-choline transporter family; CBS, cystathionine-β-synthase; CX, co-ion exclusion; DEDL, dissociative electrical double layer; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; LMO, Lubtekin, Middleton, Ottewill; LP, low potential; MFS, major facilitator superfamily; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; $\Delta\Pi$, osmotic pressure difference; $\Delta\mu^{+}_{Na}$, electrochemical sodium ion gradient; $\Delta\mu^{+}_{H}$, electrochemical proton gradient; ΔpNa, sodium gradient; ΔpH, proton gradient; $\Delta\Psi$, membrane potential; $1/\kappa$, Debye-length; b, co-ion exclusion boundary; σ_0, surface charge density; ψ_0, surface potential

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1. Introduction

Osmosensing and osmoregulation are critical survival mechanisms for any living cell. The cytoplasm of a bacterial cell is typically composed of 300–400 g/l of macromolecules (proteins, DNA, RNA) which occupy a significant fraction of the cellular volume (20–30%) [1]. The primary contributors to cytoplasmic osmolality (100–200 g/l) are low molecular weight solutes (or osmolytes), most of them ionic. The osmolyte concentration gradient across the bacterial cell envelope results in an osmotic pressure difference ($\Delta\Pi$) that can be a few atmospheres in Gram-negative and up to 20–30 atm in Gram-positive bacteria. An increase (upshift) or a decrease (downshift) in the external osmolality causes water to flow across the membrane, thereby concentrating or diluting the cytoplasm. In response, many critical enzymatic activities are affected, such as the regulation of metabolic pathways and expression of proteins. From a physicochemical standpoint, the cell undergoes changes in volume, turgor, viscosity, and membrane tension and possibly in membrane potential and ion gradients. Changes of the thermodynamic activity of cytoplasmic water alter interactions among ions, signalling molecules, and macromolecules, e.g., electrostatic interactions, hydrogen bonding, hydration and 'macromolecular crowding'. The changes in transmembrane ion gradients and in membrane potential also alter force fields at the membrane–cytosol interface where osmosensing interactions take place. Consequently, transmembrane proteins are expected to assume new conformations and associations, and to change their interactions with membrane lipids.

When the medium osmolality drops, water will flow into the cell, the $\Delta\Pi$ will increase and ultimately the cell will leak or may lyse. Conversely, when the medium osmolality increases, water will leave the cell, the $\Delta\Pi$ will decrease, and plasmolysis will occur after $\Delta\Pi$ has dropped to zero. To prevent a cell from lysing or plasmolysing, cells respond to osmotic down- or upshifts by rapidly releasing or accumulating low molecular weight osmolytes [2–4]. The response to osmotic shifts involves the activation of channels and transporters, which monitor the changing medium osmolality and regulate the rapid adjustment of the intracellular osmolyte pools in proportion to the osmotic stress. It is now clear that the responses of channels to osmotic downshifts are fundamentally different in mechanism from the responses of transporters to osmotic upshifts, and these will be treated separately in the sections on osmosensing mechanisms. Osmotic swelling appears to be detected by membrane-tension activated mechanosensitive channels, whereas the osmotic shrinkage is detected by osmoregulatory transporters that respond to the increasing concentrations of cytoplasmic constituents (see Table 1 for a summary).

In addition to the direct activation of channels and transporters, cells can have osmosensing devices that control the rate of expression of genes encoding these channels and transporters, and determine their concentration in the membrane. Other genes encode enzymes that biosynthesise compatible solutes, adjust membrane lipid compositions and modify the strength and elasticity of the cell wall. These involve relatively slow responses that allow cells to adapt to long-lasting osmotic stresses, but the underlying mechanisms will not be discussed in this paper.

2. Role of lipids in membrane protein function

How could lipid–protein interactions have a role in osmosensing and regulation of channels, transporters and membrane-embedded signal transduction components? For cells, the immediate consequence of a change of external osmolality is water influx or efflux. Even though archaeal,

Table 1
Consequences of osmotic down- and upshifts and properties of osmosensors

Property/type of system/factor	Downshift	Upshift
External osmolality	Down	Up
Water flow	Into the cell	Out of the cell
Cytoplasmic volume	Increases	Decreases
Cytoplasmic solutes	Diluted	Concentrated
Type of translocation system activated	MS channels	ATP-driven and ion-linked transporters
Type of transport	Diffusion down the concentration gradient	Metabolic energy-driven accumulation
Examples	MscL, MscS, MscK	OpuA, BetP, ProP
Signals sensed	Membrane tension	Cytoplasmic/lumenal concentration of ions and macromolecules
Proposed controlling physicochemical mechanism	Interactions between protein residues and membrane acyl chain region control channel gating	Transporter activity is controlled by electrostatic interactions (lipid–protein and/or protein–protein) and macromolecular crowding

bacterial, algal and fungal cells are surrounded by a wall that provides mechanical stability, the wall components (e.g., peptidoglycan in bacteria) are elastic and allow limited cell swelling and shrinkage in response to osmotic downshifts and upshifts. Thus, water influx initially causes stretching of the cell wall and the cytoplasmic membrane, and the increase in tension in the membrane could well provide the trigger for activation of a protein, as will be illustrated for the mechano*s*ensitive (MS) channels. On the other hand, water efflux will change all solute concentrations, the ionic strength, the water activity, the hydration of macromolecules and the crowding of macromolecules in the cytoplasm, and this could affect interactions between membrane-embedded proteins and lipids, either directly or indirectly. This strategy in some form seems to have been employed by transporters and signal transduction proteins that are activated by osmotic upshifts.

Proteins embedded in a membrane are surrounded by a shell of lipid molecules that interact with protein residues. Assuming a lipid surface area of 60 $Å^2$ [5], a lipid molecule occupies ~8.7 Å of the circumference of the hydrophobic surface of a membrane protein. The circumference of a protein composed of 12 transmembrane α-helical segments is about 160 Å [6], which implies that ~37 lipid molecules are required to form a bilayer shell around such a protein. These lipids constitute the annular shell. Although these lipids are less mobile than those in the bulk phase, the differences are relatively small and annular lipids are able to exchange with those in the bulk phase on the time scale of (sub)microsecond [7]. Besides the annular and bulk lipids, the presence of non-annular lipids, located between the transmembrane α-helices or at protein–protein interfaces, has been suggested for a number of membrane proteins. These lipids can be tightly bound and hard to remove, even in the presence of excess of detergent during the solubilisation and purification of the protein. The non-annular lipids can be critical for the activity of the protein, but, based on the available data, they do not seem to play a direct role in the gating of the MS channels or the activation of the transporters and will therefore not be treated here. In some cases (described below), changes in the bulk lipid composition or the addition of amphipaths are known to affect the osmotic regulation of channels and transporters. These effects are most likely exerted via exchange with the annular lipids, resulting in altered lipid–protein interactions. Lipid–protein interactions within the annular shell show little structural specificity. However, membrane protein function is often lipid-dependent. Preferences for a specific (usually anionic) lipid, for a particular membrane thickness or for a particular membrane phase are often observed [7].

The different local intermolecular forces between lipid molecules in a fluid membrane originate from steric hindrance, hydration, electrostatic charge and/or hydrogen bonding in the headgroup region, interfacial tension, and acyl chain pressure. The differences in the components of the interactions as a function of membrane depth are believed to lead to enormous local transverse pressures that correspond to bulk pressures of several hundreds of atmospheres [8,9]. The local pressure as a function of membrane depth is thus nonuniform; this parameter is referred to as the lateral pressure profile. An osmotic downshift may transiently offset the lateral pressure but not necessarily change the pressure profile. On the other hand, changes in the lipid composition of the membrane or lipid asymmetry are expected to change the lateral pressure profile, and, depending on where the change in lateral pressure is sensed, this might influence the structure and function of osmosensors. Statistical thermodynamic calculations of the equilibrium pressure profiles of membranes predict large redistributions of lateral pressure when the acyl chain length, the degree, position and configuration of unsaturation, and headgroup repulsion are varied [9]. For instance, non-bilayer lipids lower the lateral pressure in the headgroup region which is compensated by a higher lateral pressure in the acyl chain region. On the contrary, the incorporation into a lipid membrane of cholesterol or interfacial active solutes such as anaesthetics is predicted to increase the lateral pressure selectively near the aqueous interfaces, resulting in a compensating decrease in lateral pressure near the center of the bilayer. Such changes in the lateral pressure profile have been postulated to influence protein conformation and activity [9]. It is also possible that changes in the composition, ionic strength or osmolality of the solutions bathing the membrane surfaces may modulate lipid–lipid and lipid–protein interactions in a way that changes the lateral pressure profile.

Although cell membranes are thought to work best when the lipid bilayer is in the liquid crystalline state, there is increasing evidence that lipids are not homogeneously distributed but rather segregated into domains with different physical properties [10]. A well-studied lipid domain, proposed to be present in eukaryotic membranes, is that formed when membranes are enriched in sphingolipids and cholesterol. These so-called membrane rafts are formed when cholesterol becomes intercalated with long and saturated acyl chains such as those present in sphingolipids [11]. The physical properties such as membrane fluidity, bilayer thickness, interfacial polarity, charge of lipid headgroups and lateral pressure in- and outside the raft lipid domain will be different and this may present a means to regulate the folding and/or activity of membrane proteins [12]. A different lipid domain formation has been reported for bacteria, in which, on the basis of the partitioning of fluorescent lipid analogues, phosphatidylethanolamine and phosphatidylglycerol are proposed to be segregated [13]. Although there is no experimental evidence that osmotic down- or upshifts lead to instantaneous changes in lipid domain formation, it is well possible that osmotic stress-induced changes in bilayer composition influence the activity of osmoregulated channels and transporters through formation or alterations of lipid domains. The relevance of this adaptation mechanism will be discussed.

3. Mechanosensitive channels: activation by osmotic downshift

To excrete osmolytes, that is, in the event the $\Delta\Pi$ becomes too high, organisms activate mechanosensitive channels. Several proteins that contribute to these channel activities in *E. coli* have been identified, i.e., MscL, MscS and MscK (formerly KefA) [14–16]. Inspecting published genome sequences shows that homologues of one or more of these molecules are present in many diverse bacterial species. By far, the best studied MS channel is the protein responsible for the largest conducting activity, MscL, where a large number of mutants from *E. coli* and a crystal structure of the protein from *Mycobacterium tuberculosis* are available [17,18]. Upon gating, MscL jettisons solutes with little discrimination, except for size. The transport takes place down the concentration gradient and does not require the input of metabolic energy in the form of ATP or electrochemical ion gradients across the membrane. It has been well established that MscL responds to tension within the membrane plane rather than pressure normal to the membrane. The combination of computational, patch clamp, mutational (disulfide trapping), structural and spectroscopic data has led to models for channel gating, in which lipid–protein interactions play a critical role [19–23]. The structural rearrangements in going from the closed to the open state involve a substantial increase in helical tilt, which allows a pore with an estimated diameter of ~30 Å and a conductance of ~3 nS to be formed. This pore appears to be a final resort to release high pressures resulting from acute external osmotic downshifts, as demonstrated best for *E. coli*. The reduction of osmotic pressure of the cytoplasm at less severe hypo-osmotic stresses seems to be mediated by other mechanosensitive channels, e.g., MscS and MscK in *E. coli*, which are more sensitive to membrane tension and only 1 nS in conductance [15,16]. The crystal structure at 3.9-Å resolution of MscS from *E. coli* has recently been determined [24], and, although this protein is structurally different from MscL and sensitive to the electrical potential across the membrane, the mechanosensitive gating mechanism of both MS channels may be similar.

What is the role of membrane lipids or lipid protein interactions in the gating of these mechanosensitive channels? It is evident that an osmotic downshift, and the accompanying increase in cell volume, leads to an increase in tension and a decrease in lateral pressure within the lipid bilayer. What are the consequences of the altered tension or lateral pressure for the lipid–lipid and lipid–protein interactions in the membrane? A number of experimental studies have confirmed the role of membrane lipids and lateral pressure in the gating mechanism of MscL. First, asymmetric bending of a membrane by introducing charged amphiphiles or lyso-phospholipids in one of the leaflets clearly lowers the activation threshold of MS channels [25]. Second, it has been shown that an increase in the fraction of the non-bilayer lipid DOPE relative to the bilayer lipid DOPC results in higher gating tensions [26]. The effects of the amphiphiles, lysolipids and non-bilayer lipids thus point in the same direction, that is, a higher tension is needed for gating when the lateral pressure in the acyl chain region increases relative to the pressure in the headgroup region. Molecular dynamics simulations with POPC/POPE membranes showed that the number of lipid–protein hydrogen bonds increased when the phosphatidylcholine was replaced by phosphatidylethanolamine [27], and this could also contribute to a higher gating tension. Third, by reconstituting MscL in lipid bilayers with different acyl chain length, it could be shown that hydrophobic mismatch alone was unable to open the channel, but decreasing bilayer thickness lowered the activation energy for opening [21]. MD simulations with lipid acyl chain shortening showed that hydrophobic matching leads the lipids bordering the channel to thin less than the lipids in the bulk of the membrane, and causes the MscL protein to diminish its hydrophobic length [27]. Clearly, each of these examples demonstrates that changes in the physicochemical states of the bilayer can induce conformational changes in the MscL channel protein. The different lipid–protein interactions are likely to act in concert and constitute a significant part of the energy barrier for pore opening which, in vivo, can be overcome by the osmotic downshift-triggered increase in

bilayer tension. Although changes in the lateral pressure profile and specific lipid–protein interactions can never be studied in isolation, we feel that the lateral pressure in the acyl chain region is the most important determinant for MscL gating.

4. Transporters: activation by osmotic upshift

Solute flux via MS channels takes place down the solute electrochemical gradient and does not require the input of metabolic energy. Transporters that are activated by osmotic upshift, on other hand, move solutes across the membrane against their electrochemical gradients in processes which require metabolic energy input in the form of ATP (ATP-binding cassette transporters) or an independently generated electrochemical ion gradient (ion-linked transporters). The input of metabolic energy allows the accumulation of solutes to near molar levels. The turnover numbers of transporters are in the range of 0.1 to 10 s^{-1}, which is many orders of magnitude lower than the ion conductance of MscL in the fully open state (10^8–10^9 s^{-1}). Although a bacterial cell may have more copies of osmotic upshift-activated transporters than downshift-activated MS channels [28], the relatively low turnover number of the transporters determines that it takes several minutes to accumulate a solute to submolar levels whereas the same amount can be jettisoned via the MS channels in a fraction of a second (Refs. [29,30] and references cited in Ref. [4]). In the transport systems, the osmotic stress-triggered changes in lipid bilayer properties do not provide the energy for creating a large pore, rather the alterations in lipid–protein interactions may overcome a kinetic barrier for translocation catalysis and involve only a relatively small conformational change. Below we will present evidence for the idea that electrostatic interactions in the transporters or between anionic lipids and protein residues serve as switches to activate the systems. The conformational changes associated with translocation are driven by ATP or the electrochemical ion gradient ($\Delta\mu_X^+$, X^+ refers to proton or sodium ion).

To compensate for loss of water, cells accumulate so-called compatible solutes upon osmotic upshifts. A compatible solute is a cytoplasmic co-solvent whose level can be modulated over a broad range without disrupting cellular functions [2]. Commonly used compatible solutes include ectoine, glycine betaine and proline. Bacterial cells accumulate these compatible solutes in proportion to the osmotic upshift or the osmolality of their external medium, and in case of glycine betaine intracellular concentrations in the (sub)molar range have been observed [29,30]. Accumulation of compatible solutes can be effected by de novo synthesis or uptake of osmoprotectants from the medium [2]. The most rapid response to an osmotic upshift involves the immediate activation of solute transporters that are already present in the membrane and mediate accumulation of available osmoprotectants. Although several transporters and sensor kinases have been studied with regard to regulation by osmotic stress, the best-understood systems include the ABC transporter OpuA from *Lactococcus lactis*, and the ion-linked transporters BetP from *Corynebacterium glutamicum* and ProP from *E. coli*. The in vivo properties of these systems are retained when analysed in proteoliposomal systems, and the in vitro analyses have made it possible to determine the parameters that are relevant for osmosensing. Before describing the osmosensing and osmoregulatory mechanisms of these systems, some of their relevant structural and functional properties are summarised.

4.1. ABC transporter OpuA

The ATP-binding casette transporter OpuA is composed of two chimeric substrate-binding/translocator and two ATP-binding cassette subunits [31,32]. The ATP-hydrolysing subunits are somewhat unusual for ABC transporters because of the presence of two domains in tandem that belong to the CBS family [33]. The CBS domains are found in proteins with various functions (channels, transporters, enzymes, regulatory proteins) and predicted to have a regulatory role, but their actual function(s) have not been elucidated. The second, C-terminal, CBS of the ATP-binding cassette subunit has the highly charged extension DIPDEDEVEEIEKEEENK, but its possible role in osmosensing is not proven. OpuA facilitates the transport of glycine betaine at the expense of two molecules of ATP [34]. When incorporated in proteoliposomes (Fig. 1), the orientation of the OpuA molecules is random and half of the molecules has the substrate-binding domains on the outside and the ATP-hydrolysing subunits on the inside (‘in vivo or right-side-out orientation’); the other half has the ‘inside-out orientation’ [35]. Because the transport of glycine betaine is unidirectional and dependent on access of the ABC subunits to the membrane-impermeant co-substrate, ATP, the molecules with the right-side-out and inside-out orientation can be studied separately. This unidirectionality offers an experimental advantage in analysing the translocation and osmosensing mechanism (see Fig. 1), which is not possible for the ion-linked transporters where the directionality of transport is not determined by the orientation of the molecules (see below). The initial rates of solute uptake via OpuA vary as a function of medium osmolality and can be described by a sigmoid function from which the ‘iso-osmotic’ activity, the activation threshold and the maximal activity can be derived (Fig. 2A).

4.2. BCCT transporter BetP

A member of the betaine-carnitine-choline transporter family (BCCT) [36], BetP is a 595-residue integral membrane protein [37] that catalyzes symport of glycine

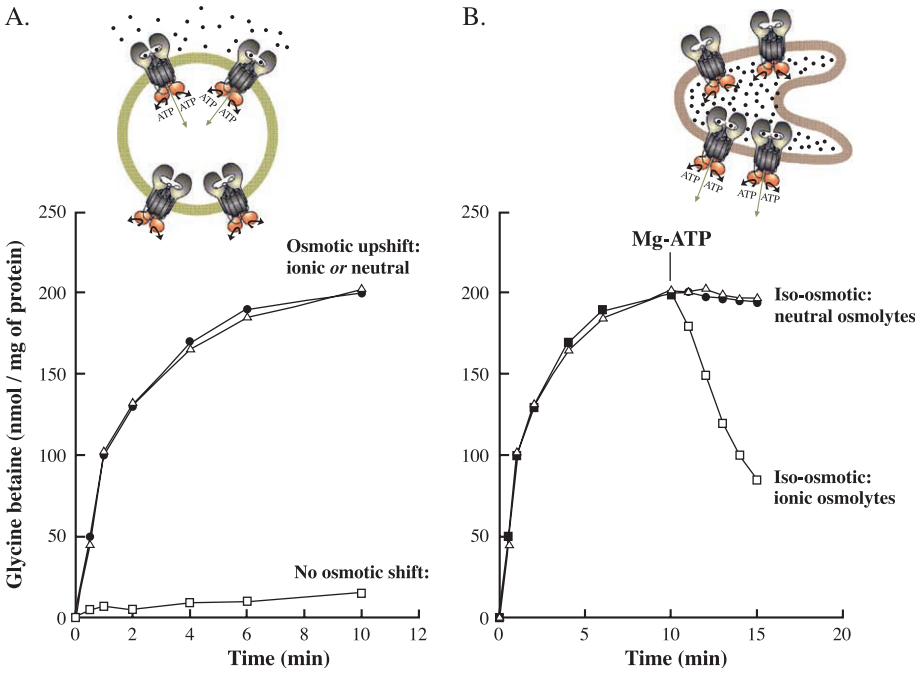


Fig. 1. Glycine betaine uptake and efflux in proteoliposomes containing OpuA. (A) Uptake of glycine betaine in proteoliposomes preloaded with ATP-regenerating system and resuspended in 50 mM KPi, 100 mM KCl, pH 7.0. Uptake assays were performed under iso-osmotic (50 mM KPi, 100 mM KCl, pH 7.0, open squares) or hyperosmotic conditions (50 mM KPi plus 400 mM KCl, pH 7.0, closed circles; 50 mM KPi plus 430 mM sucrose, pH 7.0, open triangles). (B) After 10 min of uptake, efflux of glycine betaine was stimulated by addition of 50 mM KPi, pH 7.0, containing 9 mM ATP/Mg^{2+} in the presence of 430 mM sucrose (open triangles) or 400 mM KCl (open squares). Modified after Ref. [34].

betaine with two sodium ions [38]. The accumulation of glycine betaine is driven by $\Delta\mu_{Na^+}$, which consists of a sodium gradient (ΔpNa) and a membrane potential ($\Delta\Psi$). As for other ion-linked transporters (including ProP, described below), glycine betaine transport via BetP is most likely bidirectional and determined by the imposed electrochemical gradient rather than by the orientation of the protein in the membrane. BetP activity is a function of medium osmolality in intact cells (*C. glutamicum* or *E. coli*) [37] and after purification and reconstitution in

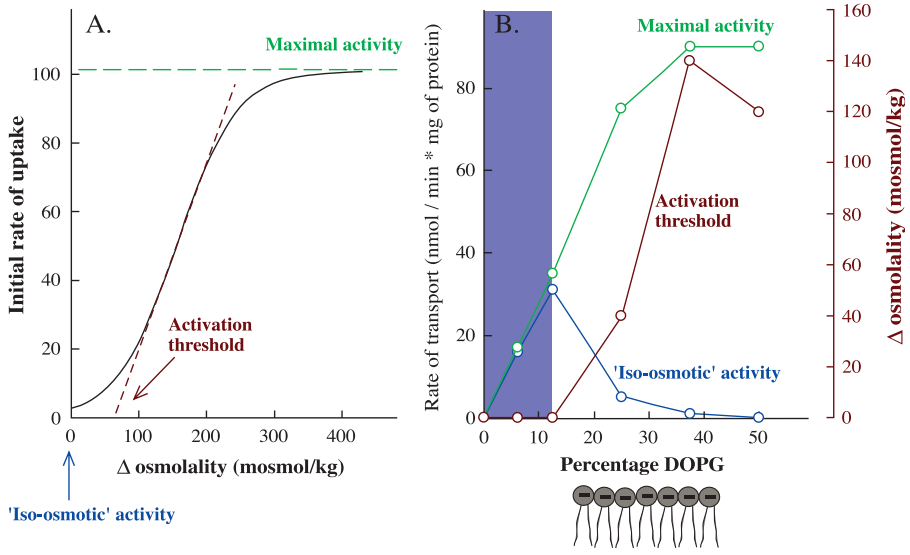


Fig. 2. Osmotic activation profile (A) and DOPG-dependence of activity of OpuA (B). (A) ΔOsmolality of zero corresponds to equal internal and external osmolality; (B) the maximal activity, iso-osmotic activity and activation threshold (as defined in A) of OpuA in proteoliposomes as a function of mol% DOPG. The proteoliposomes were composed of DOPG, DOPC and DOPE (25 mol%); mol% DOPC decreases with increasing mol% of DOPG (modified after Ref. [55]).

proteoliposomes [39]. After full osmotic activation in *C. glutamicum* or *E. coli*, BetP has a high specificity and affinity for glycine betaine (apparent K_M of about 10 μM) and a lower affinity for Na^+ (apparent K_M of about 5 mM) [37]. The apparent K_M's for glycine betaine and Na^+ are 3.6 μM and 15 mM, respectively, after full osmotic activation in proteoliposomes prepared with a polar lipid extract from *E. coli* [39]. BetP has cytoplasmic amino- and carboxyl-termini and is predicted to span the membrane 12 times [40]. The basic C-terminal domain plays a role in osmosensing (Section 7).

4.3. MFS transporter ProP

A member of the major facilitator superfamily, ProP is a 500-residue polypeptide integral to the cytoplasmic membrane of *E. coli* [41]. ProP catalyzes H^+-osmoprotectant symport in response to the proton motive force ($\Delta\mu_{H^+}$) which consists of a proton gradient (ΔpH) and a membrane potential ($\Delta\Psi$) [42–44]. The substrates for ProP, a broad specificity transporter, include proline, glycine betaine and ectoine [42]. Purification and reconstitution of ProP-$(His)_6$ in proteoliposomes provided the first evidence that a single protein can serve as both an osmosensor and an osmoregulator [45]. In cells or proteoliposomes, the initial rate of proline uptake via ProP is a sigmoid function of medium osmolality (not osmotic shift) that can be fit to an empirical model. In this way, transporter function can be characterized in terms of the activity approached at high osmolality (A_{max}), the osmolality required to attain half that maximal activity ($\Pi_{1/2}/RT$) and the gradient of the osmolality response (B) [46,47] (Fig. 3, top). Since the K_M for proline is osmolality-dependent (Fig. 3, middle), the osmotic activation of ProP is best viewed in terms of the osmolality dependence of V_{max} (Fig. 3, bottom) [46]. Although ProP can undergo osmotic activation in the absence of other proteins, soluble protein ProQ is required for full osmotic activation of ProP in vivo [48,49]. Loci *proP* and *proQ* are distant from one another, though both are components of the core genome based on their uniform co-occurrence within all sequenced *E. coli* genomes. Lesions in *proQ* affect neither *proP* transcription nor ProP protein levels [48,49]. Elimination of ProQ attenuates A_{max} approximately fivefold and retards the rate at which ProP responds to osmotic upshifts without dramatically affecting $\Pi_{1/2}/RT$ [48].

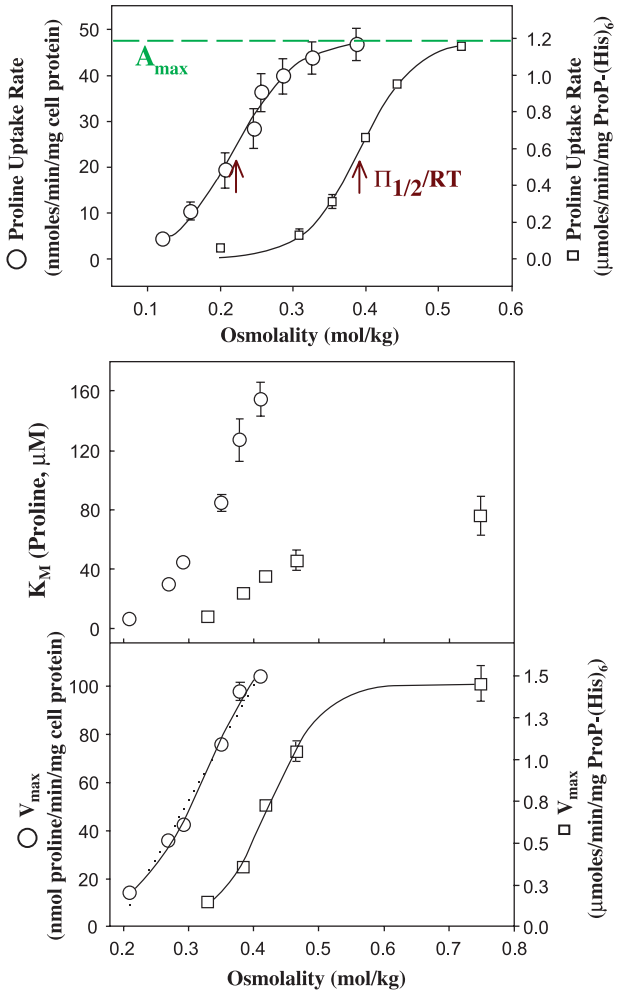


Fig. 3. Osmotic activation of ProP. Top: The initial rate of proline uptake via ProP-$(His)_6$ is a sigmoid function of external osmolality in bacteria (circles) and in proteoliposomes prepared with *E. coli* lipid and the pure protein (squares). Transporter function can be characterized in terms of the activity approached at high osmolality (A_{max}), the osmolality required to attain half that maximal activity ($\Pi_{1/2}$/RT) and the gradient of the osmolality response (B). Middle: The apparent K_M of ProP for proline is a direct function of osmolality in both bacteria (circles) and proteoliposomes (squares). The absolute values of K_M for these two systems differ, however. Bottom: ProP is activated at different osmolalities in bacteria and in proteoliposomes even if the osmolality dependence of K_M is taken into account. Reprinted with permission from Culham et al. [46]. Osmosensor ProP of *Escherichia* coli responds to the concentration, chemistry and molecular size of osmolytes in the proteoliposome lumen [46]. Copyright 2003 American Chemical Society.

ProP can be modeled on the structures of MFS members GlpT [50] and LacY [6] (R.A.B. Keates and J.M. Wood, unpublished data), suggesting that it shares an emerging, common fold [51]. Experimental analysis, to date, validates the 12 transmembrane helix model (Ref. [47], Wood et al., unpublished data). The cytoplasmic C-terminus of ProP is approximately 50 residues longer than those of its closest *E. coli* paralogues (KgtP and ShiA, neither of which is an osmosensor or osmoregulator) [41] (Fig. 4). NMR [52], EPR [53] and CD spectroscopies and analytical ultracentrifugation [54] have been used to show that a peptide corresponding to the C-terminal extension of ProP forms a homodimeric, antiparallel, α-helical coiled-coil structure stabilized by electrostatic interactions between ProP–R488 (in a core, coiled-coil heptad a position) and apposed aspartate residues. This structure may mediate dimerization of intact ProP in vivo. ProQ is a basic, hydrophilic protein which has no strong sequence homologues of known function. Although ProQ can be overexpressed and purified, further studies of its function

Group	Accession Number	Organism	Similarity to *E. coli* ProP		Function
			% ID	Score	
A	Q_47421	*Erwinia chrysanthemi*	80	759	Osmosensing/Osmoregulation
	NP_745058	*Pseudomonas putida*	80	793	Unknown
	NP_356328	*Agrobacterium tumefaciens*	65	636	Unknown
B	NP_635478	*Xanthomonas campestris*	51	434	Unknown
	NP_00033242	*Burkholderia fungorum*	49	454	Unknown
	NP_626583	*Streptomyces coelicolor*	49	394	Unknown
	CAA_73136	*Corynebacterium glutamicum*	37	308	Osmosensing/Osmoregulation
C	NP_416488	*Escherichia coli*	30	202	Shikimate Transport
	AAC_75640	*Escherichia coli*	28	182	α-Ketoglutarate Transport

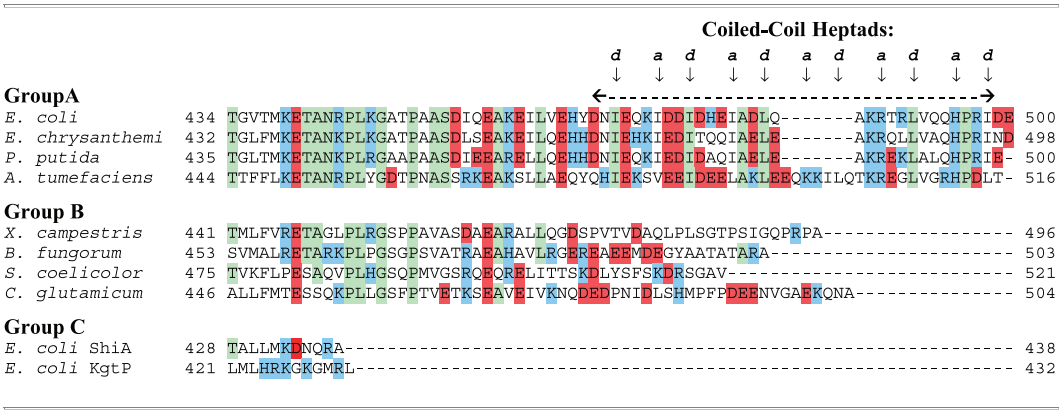


Fig. 4. Incidence and distribution of ionizable residues within the C-terminal domains of known and putative osmosensors. Top: The sequences listed (by accession number) are those most similar to *E. coli* ProP within the genomes of the listed organisms (Groups A and B) or those most similar to *E. coli* ProP with known functions (Group C). Bottom: The aligned sequences C-terminal to putative transmembrane helix 12. Blue: basic residues; red: acidic residues; green: residues conserved among all four ProP orthologues with a coiled-coil domain (and some others). Arrows denote the bipolar nature of sequence in *E. coli* ProP whose structure is known to form an antiparallel α-helical coiled-coil [52].

in osmoregulation are hampered by its instability in solution (R.A. Crane, M. Smith, S. Bourgeois and J.M. Wood, unpublished data).

5. Roles of electrolytes and ionic strength in osmosensing

Osmotic upshifts imposed with membrane-impermeant osmolytes modulate many cellular properties, including turgor pressure, membrane lateral pressure, cytoplasmic osmolality, ionic strength and viscosity, cytoplasmic solute concentrations, and the hydration and crowding of cellular macromolecules. Thus, the semi-permeable, cytoplasmic membrane transduces extracellular osmotic changes, creating an array of secondary signals that could be detected by osmosensors. Extensive studies, particularly studies of systems OpuA, BetP and ProP performed with proteoliposomes, have narrowed the list of signals to which these systems could respond. Most notably, they do not detect changes in turgor pressure or mechanical deformation of the membrane [40,46,55].

When OpuA, BetP or ProP is incorporated into artificial lipid vesicles, the transporter activity is osmotically regulated in a manner that is qualitatively similar to the regulation in vivo. The osmolality threshold for transporter activation in cells can differ from that in proteoliposomes. For OpuA, this relates to differences in lipid composition; the osmolality threshold increases with increasing fraction of anionic lipids (see Section 6). Membrane lipid composition is also a major determinant of the osmoregulation of BetP activity since the osmolality for activation in *E. coli* or after incorporation in proteoliposomes prepared with *E. coli* lipid is much lower than that required in *C. glutamicum* cells [37,39]. Plasma membranes of exponentially growing *E. coli* consist of 70–80% phosphatidyl ethanolamine, approximately 20% phosphatidylglycerol and some diphosphatidylglycerol (cardiolipin) [56], whereas phosphatidylglycerol is the predominant lipid in the cytoplasmic membrane of *C. glutamicum* (87%) [57]. The osmolality required to activate ProP in proteoliposomes made with *E. coli* lipid exceeds that required in *E. coli* cells (Fig. 3) [46]. While this may reflect some difference in lipid composition between the cells and proteoliposomes, it could also reflect the absence of macromolecular solutes and/or of ProQ from the proteoliposome lumen [46] (see Section 8).

The activities of all three systems are low under isoosmotic conditions at low osmolality, that is, with 100

to 200 mosM/kg of ionic equivalents on the inside (see Section 6 for a detailed description of the role of lipids in the ion dependence of OpuA). When the osmolality of the outside medium is increased by the addition of membrane-impermeant, ionic or neutral osmolytes, and the appropriate driving force for transport is available (ATP or an ion motive force), the transporters are rapidly activated (e.g., Figs. 1B and 3). The membrane of proteoliposomes is not supported by a cyto- and/or exoskeleton and the volume of the lipid vesicles decreases in proportion to the osmotic upshift. Consequently, the osmolytes in the proteoliposome lumen are concentrated. The activities of OpuA, BetP and ProP in electrolyte-loaded proteoliposomes correlate with the resulting concentration of lumenal ionic osmolytes. However, there are also noticeable differences among these systems. For instance, OpuA and ProP seem to be indifferent to the nature of the concentrated ions. OpuA and ProP respond similarly to lumenal concentrations of Na^+, K^+, Li^+ or Cs^+ (tested for ProP only) with phosphate, sulfate (tested for OpuA only) or phosphate and Cl^- as anions [46,55]. BetP can be activated with K^+, Cs^+ or Rb^+ but not choline in the proteoliposome lumen, and is not anion-specific [40]. It is technically challenging to assess the role of Na^+ (and discriminate it from K^+) in BetP activation because Na^+ is the coupling ion for transport [37,39,57]. Nevertheless, their observations led Rübenhagen et al. [40] and Schiller et al. [58] to propose that BetP is a K^+ sensor (a chemosensor rather than an osmosensor), implying the existence of a binding site with a K^+ affinity of approximately 0.2 M.

The lumenal ion concentration required for OpuA activation has been shown to depend on the fraction of anionic lipids (Ref. [55], see Section 7) and preliminary experiments with BetP point in the same direction [37,39]. Half maximal BetP activity is attained at a lumenal K^+ concentration of 0.22 M [40], but the osmolality (and hence the lumenal K^+ concentration) required to activate BetP increased and the activity attained decreased dramatically as the anionic lipid content was increased by incorporating phosphatidylglycerol in proteoliposomes prepared with *E. coli* lipid [39].

Since transport via OpuA is unidirectional, the two orientations of the system in proteoliposomes can be studied separately. With pre-accumulated glycine betaine on the inside, efflux via inside-out oriented OpuA can be triggered by the addition of Mg-ATP (Fig. 1B). Consistent with a mechanism involving ionic regulation, efflux catalysed by OpuA, reconstituted in liposomes with 50% anionic lipids, was observed when the proteoliposomes were suspended in media with 400 mM of K-phosphate or KCl and not in media of the same osmolality with only 50 mM of K-phosphate or KCl and other osmolytes being nonionic. Moreover, the same activation pattern was observed when the ATPase rather the transport activity of OpuA was measured [34,55]. These experiments have provided strong evidence for the idea that electrostatic interactions in the protein or between lipids and protein residues are influenced by the ionic strength that is sensed by the cytoplasmic 'inward-facing' domains of OpuA. Although the observed regulation is consistent with the in vivo data, it is intriguing that OpuA can be switched from 'inactive' to fully active by increasing the salt concentration from 100 to 200 mM (Fig. 2A; see Section 6 for factors such as lipids that determine the activation threshold). Intuitively, and in line with classical Debye–Hückel theory, one would expect that the majority of Coulombic interactions is already screened at 100 mM salt, and that the presence of another 100 mM of ionic osmolytes has little or no additional effect on the electrostatic interactions (see Section 9 for an alternative view on the changes in electrostatic forces over a narrow range of ionic strength).

Concentrating low molecular weight organic solutes has no effect on the activation of OpuA (sucrose, glucose or lactose) [55], or BetP (proline, ectoine, carnitine or glucose) [40] and little effect on the activation of ProP (low molecular weight poly(ethylene)glycols, glucose) [44,46]. In the case of ProP, effects of larger organic solutes have also been tested. When polyethylene glycols (PEGs) with defined molecular sizes (radii of gyration 8 Å through 18 Å) were incorporated in potassium phosphate-loaded proteoliposomes, PEG size-dependent effects on ProP activation were observed [46]. The largest PEGs activated ProP in the absence of any osmotic upshift. When osmotic upshifts were imposed, the osmolality (and hence lumenal K^+ concentration) required to attain half maximal ProP activity ($\Pi_{1/2}/RT$ or $K^+_{1/2}$) decreased systematically as PEG size increased. Bovine serum albumin had parallel effects (D.E. Culham and J.M. Wood, unpublished data). For systems without ion specificity, the dependence of transporter activity on lumenal electrolyte concentration implies an osmosensory mechanism based on electrostatic interactions, perhaps one governed by ionic strength. It is difficult to experimentally distinguish ion concentration from ionic strength using proteoliposomes because they are structurally sensitive to multivalent cations. However, it is clear that, for ProP in PEG-loaded proteoliposomes, transport activity is not simply correlated with either ion concentration or ionic strength [46]. Rather, ProP likely responds to osmotically induced concentration of both electrolytes and macromolecules in the bacterial cytoplasm. Concentration of macromolecules could alter membrane or ProP structure by competing for water of hydration, via steric exclusion from protein- and/or membrane-associated water pools and/or via macromolecular crowding. Such effects might modulate the ion (physiologically, K^+) affinity of a regulatory binding site on ProP or modulate conformational changes in the transporter that are otherwise electrostatic in origin, involving the transporter alone or its interaction with membrane lipid.

6. Lipid dependence of ionic regulation of OpuA

The ionic regulation of OpuA has been studied in proteoliposomes with different lipid composition [55]. By systematically varying the acyl chain lengths, the number and position of the *cis* or *trans* double bond, and the lipid headgroups, it could be shown that only the charge of the lipid headgroups influences the osmotic activation profile of the transporter. The 'maximal activity' is highest when at least 50% of the non-bilayer lipid phosphatidylethanolamine and 35% of anionic lipid are present, the acyl chain length is 16–18 carbon atoms, and the lipids are mono-unsaturated. Under these conditions, the 'iso-osmotic activity (with 100 mM of salt at the cytoplasmic face of the transporter) is close to zero and the activation threshold is 150–200 mM of salt (Δosmolality of ~140 mosM/kg in Fig. 2B). The activation threshold, indicator of osmosensor activity, is highly dependent on the fraction of anionic lipids (phosphatidylglycerol or phosphatidylserine) as illustrated in Fig. 2B. Below 12–13 mol% DOPG (or DOPS), the 'iso-osmotic' and maximal activity are the same and there is no activation threshold (blue zone in Fig. 2B). The maximal activity increases when the mol% of DOPG is increased from 0 to 35–40, which, to our opinion, reflects a general requirement for anionic lipids that can be observed for many bacterial transporters, and irrespective of whether they are osmoregulated or not (unpublished results). Importantly, above 12–13 mol% DOPG (or DOPS), the iso-osmotic activity decreases and the activation threshold increases, and this (white zone in Fig. 2B) corresponds to the osmoregulated activity of OpuA. It has been proposed that at relatively low ionic strength (≤100 mM of salt), electrostatic interactions between the headgroups of anionic lipids and cationic residues in the protein keep the system in the inactive state [4,55]. Since at least 35–40 mol% of DOPG (or DOPS) are needed to inactivate the system, we propose that multiple anionic lipid–protein interactions play a role. By raising the ionic strength at the cytoplasmic face, the OpuA system can be switched within a second from inactive to fully active. The higher the fraction of anionic lipids (DOPG or DOPS), the more salt is needed to reach the maximal activity, supporting the view that more and more electrolytes are needed to screen the multiple electrostatic interactions between membrane surface and protein for activation of the system.

Support for the idea that bulk anionic lipid–protein interactions determine the activity of OpuA also comes from experiments with charged amphipaths (Refs. [31,34,55], J. Patzlaff and B. Poolman, unpublished results). Within limits the activation threshold can be shifted to lower values by adding cationic amphipaths such as chlorpromazine, tetracaine, and verapamil, whereas anionic amphipaths mimic anionic lipids and shift the threshold to higher values. Although the ion sensor of OpuA has not been specified in structural terms, the sensor must be located on the cytoplasmic face. Evidence for this notion is twofold: (i) osmotic activation of OpuA is only observed when the ionic strength at the cytoplasmic face is increased, that is, at the site where ATP is hydrolyzed; (ii) cationic amphipaths activate OpuA instantaneously when the cytoplasmic face is on the outside of the proteoliposomes (inside-out-oriented OpuA; unpublished), whereas it takes hours (most likely corresponding to the time required for the amphipaths to flip from the outer to the inner leaflet of the membrane) when the cytoplasmic face is on the inner surface of the proteoliposomes.

7. Lipid dependence and the role of the C-terminal domain in ionic regulation of BetP

Lipid–protein interactions may play a similar role for BetP, since the activation threshold of BetP shifts to higher osmolalities when the fraction of phosphatidylglycerol in the membrane is increased [37,39]. The roles of the hydrophilic, N- and C-terminal extensions of BetP in osmosensing were explored by measuring the activities of BetP deletion variants in *C. glutamicum* [59]. Truncation of the acidic N-terminal domain (up to 60 of approximately 60 residues) raised the extracellular osmolality required for maximal BetP activity approximately twofold but did not alter the threshold for osmotic activation. In contrast, truncation of the basic C-terminal domain (up to 52 of approximately 55 residues) rendered the transporter insensitive to extracellular osmolality. Regardless of the osmolality, variants lacking 12 or 23 C-terminal residues were comparable in V_{max} to the fully activated wild type (the expression levels of these BetP variants could not be tested). These observations led Rübenhagen et al. [40] to conclude that a K^+ sensor resides in the C-terminal domain of BetP, but no sequence characteristic of a known K^+ binding site has been identified. Although the higher order structure of the BetP C-terminus is not known, the C-terminus of BetP (last 23 amino acids) is expected to have a net charge of approximately +10, rendering it a good candidate to interact directly with an anionic membrane or protein surface.

8. Roles of protein–protein and lipid–protein interactions in osmosensing by ProP

The dependence of ProP activity on membrane lipid composition has not yet been defined, but other lines of evidence implicate protein–protein and/or protein–lipid interactions in osmosensing and the osmoregulation of ProP activity. In response to osmotic upshifts, the ProP activity of cells and cytoplasmic membrane vesicles increases with a half time on the order of 1 min,

approaching a maximum that is sustained indefinitely [43]. Amino acid replacement R488I dramatically elevated the osmolality required to attain half maximal ProP activity and reduced the activity attained approximately twofold. Moreover, the same replacement disrupted α-helical coiled-coil formation by a peptide corresponding to the ProP C-terminal domain and rendered the osmotic activation of ProP transient. In this mutant, ProP activity rose to a maximum approximately 2 min after an osmotic upshift, then rapidly decayed. As a result, bacteria cultivated in high osmolality media showed very low ProP activity [54]. These and other experiments support the view that dimerization of ProP mediated by this homodimeric, antiparallel coiled-coil is essential for sustained but not for transient ProP activation.

The C-terminal domains of *E. coli* ProP and its most similar putative orthologues (Fig. 4, Group A) are marked by a terminal, basic region (residues 486–497 of the *E. coli* protein) preceded by an acidic region (residues 468–485). In the coiled-coil formed by a peptide corresponding to residues 468–497 of the *E. coli* protein, charged residues are clustered on the *c*/*g* surface and the two helices are aligned antiparallel so that these acidic and basic regions form stabilizing, intermolecular salt bridges [52]. At low osmolality, this coiled-coil domain of each ProP monomer may interact with lipid or other protein surfaces, only to be released to mediate ProP dimerization as cytoplasmic ionic strength rises in response to osmotic upshifts. For this group of proteins, osmotic activation would occur in two stages. An initial conformational change, coinciding with the onset of ProP activation, could involve release of the C-terminal domain from association with the membrane, another part of ProP itself, or another protein. This would be followed by a conformational change, depending on the coiled-coil structure, essential to sustain transporter activity in the steady state.

Putative ProP orthologues that do not possess C-terminal coiled-coil domains have also been identified (Fig. 4, Group B). Among the Group B orthologues, ProP of *C. glutamicum* is known to function as an osmosensor and an osmoregulator [45]. Among these ProP orthologues the C-terminus is truncated and terminates in an acidic region. Organisms containing Group B orthologues must either not require sustained transporter activation or attain it in a way that does not require coiled-coil formation.

Although *E. coli* ProP acts as an osmosensor after purification and reconstitution in proteoliposomes, the osmotic activation of ProP is attenuated approximately fivefold in bacteria lacking cytoplasmic protein ProQ [49,60]. Thus, the ProP activity observed in proteoliposomes seems to represent the attenuated activity of the transporter observed in ProQ-deficient bacteria [46]. ProQ includes a highly basic N-terminal domain which could readily interact with acidic regions of ProP (including those within the C-terminus), interactions that could be modulated by cytoplasmic ionic strength. Taken together, these observations suggest that increasing extracellular osmolality leads to a structural change in the transporter due to the combined impact of increasing cytoplasmic solute concentrations (electrolytes and non-electrolytes, including macromolecules). This yields a new transporter conformation that is active but unstable. In response to the conformational change triggered by increasing osmolality, the C-terminus may form homodimeric coiled-coils, mediating (tighter) ProP dimerization and in turn being stabilized by an interaction with ProQ [3]. This ternary complex would then constitute the most active form of *E. coli* ProP.

9. Maxwellian double layer models of upshift osmosensing

The experimental data discussed in the preceding sections, which involve three species of bacteria (*L. lactis*, *E. coli*, *C. glutamicum*) and three different transport mechanisms (ATP-driven and Na^+- and H^+-linked transporters), suggests that electrostatic interactions may play a decisive role in osmosensing. The common denominator appears to be the activation of all three transporters by increased ionic content of the proteoliposome lumen. Low molecular weight electroneutral osmolytes do not activate the transporters. Two additional factors that change ionic interactions have been identified: (i) the ionic lipid/amphiphile compositions of the bilayer (OpuA, BetP; Sections 6 and 7) which determine membrane charge densities, and (ii) 'macromolecular crowding', or excluded volume effect (ProP; Section 8), which would reduce the distance over which electrostatic forces act to activate the transporters.

In general, ionic strength dependencies suggest a role of electrostatic interactions according to the classical electrolyte and double layer theories [61–66]. These theories predict that electrostatic interactions between charged surfaces are screened by a thermal distribution of small ions (ionic cloud), which reduce the range of Coulombic forces as measured by the Debye's length, usually designated by $1/\kappa$. Because the activation of the osmoregulating proteins takes place over a narrow range of ionic strengths (e.g. from 0.10 M, $1/\kappa$=9.62 Å, to 0.30 M, $1/\kappa$=5.55 Å), the difference of the range of electrostatic forces is only 4.1Å. Although this difference in the range of electrostatic forces between the "activated" and "non-activated" osmosensor seems small, it is still about the double of the diameter of a water molecule. Thus, the electrostatic forces cannot be considered as being 'completely screened out' in respect to other short-range forces already reviewed [2,4], such as 'macromolecular crowding' [67–69] or changes of hydration of proteins, phospholipids, and small ions [70–74].

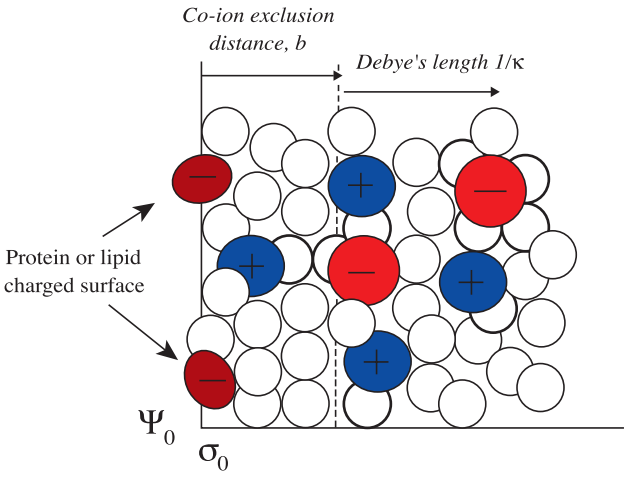


Fig. 5. A snapshot (possibly on a 0.1–0.01 μs 'exposure') of the Maxwellian double layer. The new feature is the appearance of the co-ion exclusion distance b, which separates the Debye–Hückel ionic cloud (characterized by the Debye's length $1/\kappa$) from the surface charge σ_0. The range of electrostatic forces is the sum of the co-ion exclusion distance and the Debye's length.

The case for the role of electrostatic forces is strengthened further by the considerations derived from the recently reformulated Maxwellian linear Poisson–Boltzmann equation for high electrostatic potentials [75–78], which defines the Dissociative Electrical Double Layer (DEDL) theory. This new approach derives from the linear (Maxwellian) distribution of ions rather than from the usual Boltzmann exponential distributions, which are known to lead to electrostatic inconsistencies. The new feature of the DEDL model is a co-ion exclusion boundary, b, the thickness of which is added to the Debye-length, $1/\kappa$, to define the range of electrostatic forces [76,77]. This boundary, calculated from the theory, defines a region from which co-ions are on average excluded. A simplified DEDL model, possibly applicable to biological surfaces at high ionic strength, is shown in Fig. 5. The protein (or phospholipid) charges define a charged surface with surface charge density σ_0 and surface potential ψ_0. The model is simplified by assuming that counter-ions can 'penetrate' the surface and be in the same plane as the surface charges. The distance of closest approach of counter-ions and surface charges is thus zero, reflecting the 'soft' or 'thermally mobile' nature of the charged surfaces. The second simplification is to take advantage of the LMO (Lubtekin, Middleton, Ottewill) law [76]:

$$\alpha = \frac{1}{1 + p\kappa} \tag{1}$$

where α is the degree of counter-ion association to the charged surface, $1/\kappa$ is Debye's length, and p is an empirical parameter that determines the ease of counter-ion dissociation. The LMO law predicts that, with increasing ionic strength, the association of counter-ions with the surface charges becomes small. In the present case, we assume $p \rightarrow \infty$, and hence there is no ion-specific counter-ion association. The double layer can be regarded as completely dissociated (the so-called Gouy-Chapman double layer [62,63,66]).

An important characteristic of the DEDL theory is the dependence of the co-ion exclusion boundary on ionic strength, the surface charge density, separation from other charged surfaces, and on the shape of the surfaces. In the simplest case of the non-interacting, co-ion exclusion model (CX) in Fig. 5, the position of the co-ion exclusion boundary is determined by co-ion charges, temperature, dielectric constant, and in particular by ionic strength and surface charge density. The theory predicts that, as the surface charge density decreases, or as the ionic strength increases, the exclusion boundary moves towards the charge bearing surface until these surfaces coincide. Under this 'critical' or 'singular' condition, the co-ion exclusion (CX) model changes to a low potential (LP) model, where the co-ion exclusion boundary is coincident with the plane of the surface charge (Fig. 5). This transition in thermal ionic distributions, the CX/LP transition, is defined by critical values of the physicochemical parameters related by

$$\sigma_0 \left(\frac{1}{\kappa}\right) \frac{1}{\varepsilon_0 \varepsilon} = \frac{kT}{z_e} = \Psi_0 \tag{2}$$

where σ_0 is surface charge density, $1/\kappa$ is Debye's length, ε and ε_0 are permitivitties of the ionic solution and vacuum, respectively, kT is thermal energy and z_e is the charge on the co-ions in the solution. Other transitions occur when two surfaces interact with each other, as discussed elsewhere [76,77]. The theory suggests that such transitions may be involved in the activation of the transporters, particularly when they are brought about by changing surface charge density or ionic strength. This concept is developed below.

An example of the ionic strength transition (and the related ranges of electrostatic forces) is given in Table 2, where the Debye's length $1/\kappa$ can be compared to the co-ion exclusion distance, b. The range of electrostatic forces is the sum of the Debye's length $1/\kappa$ and the co-ion exclusion distance, b. We notice that in the range of ionic strengths below the critical ionic strength of 0.261 M, the range of electrostatic forces is increased by the co-ion exclusion

Table 2
The range of electrostatic forces given by the classical theory (Debye's length) and by the Maxwellian theory with monovalent ionic salt

Molarity [mol/l]	Debye length [1/κ, Å]	Co-ion exclusion [b, Å]	Surface potential [Ψ_n, mV]
0.100	9.62	5.12	43.3
0.200	6.80	0.94	29.5
0.261	5.96	0.00	25.7
0.300	5.55	0.00	24.0
0.400	4.81	0.00	20.7

For the Maxwellian model, a charge density of 534 Å^2/charge (3.00 μC/cm^2) was assumed.

distance (Fig. 5), which is not predicted by the classical double layer theory. The values of the surface potentials in the range above 25.7 mV suggest that electrostatic forces cannot be neglected at these relatively high ionic strengths. Thus, on going from 0.100 to 0.300 M, we traverse a transition from the co-ion exclusion (CX) double layer at 'critical' 0.261 molar ionic strength to the low potential (LP) double layer at higher ionic strengths. In the LP double layers there is no co-ion exclusion. The CX/LP transition is defined by the surface potential equal to 25.7 mV for monovalent anions; c.f. Eq. (2). For a mixture of monovalent and divalent anions there are two transitions predicted, the divalent anions starting to be excluded at the surface potential of 12.8 mV.

In Fig. 6 (open symbols), the critical ionic-strength is seen to decrease with increasing 'area per charge', i.e., decreasing surface charge density. This predicted qualitative trend is observed experimentally for the OpuA (closed symbols) and BetP (not shown) transporters, which require higher ionic strengths for activation, as the fraction of anionic lipids in the membrane increases (area/charge decreases). This observation lends further support to the idea that the ionic strength activation of osmoregulatory proteins is related to the predicted Maxwellian transitions in thermal ionic distributions, as characterized by the critical ionic strength; c.f. Eq. (2). These transitions occur when the surface potential reaches the value of 25.7 mV for monovalent co-ions, c.f. the value of 0.261 molar in Table 2, when the co-ion exclusion distance coincides with the charge bearing surface.

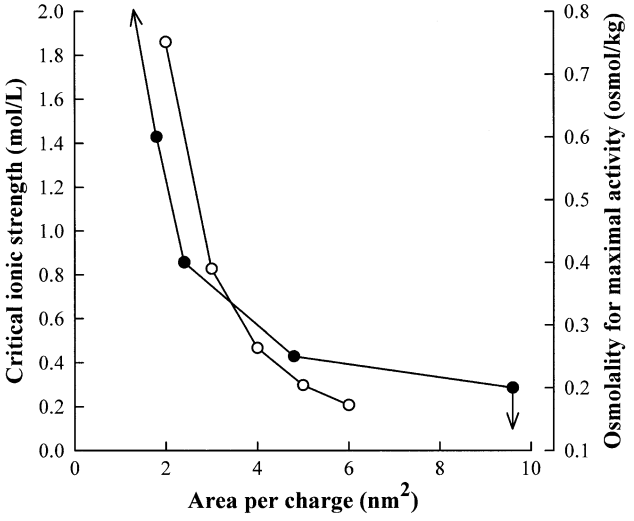


Fig. 6. Open symbols: The dependence of the critical ionic strength on area per charge according to the Maxwellian theory. Closed symbols: The dependence of the osmolality required for maximal OpuA activity on area per charge. Osmolality is roughly proportional to ionic strength in the experiments with OpuA. The area per charge was calculated for proteoliposomes with a varying fraction of anionic lipid (DOPG, see legend to Fig. 2B) and assuming a lipid surface area of 0.6 nm^2. The osmolality for maximal activity was estimated from experiments as presented in Fig. 2A (data from Ref. [55] and unpublished results). The upward arrow points to the osmolality for maximal activity at an area per charge of 1.2 nm^2, but the precise value could not be determined because the maximal activity was not yet reached. The downward arrow indicates that the osmolality value is most probably overestimated, owing to technical limitations in the assay.

The above considerations of the transition in ionic distributions and increased ranges of electrostatic forces suggest the importance of electrostatic forces. For a better understanding of such forces, detailed models of the interacting moieties (charged surfaces of polypeptides and phospholipids) need to be constructed, and the electrostatic solutions of such models would then yield the magnitudes of electrostatic forces (and energies). Currently, however, the geometries and charges of the osmosensing proteins are not well known, and detailed calculations for generalized surfaces, though possible in principle, are technically demanding. Nevertheless, some possibilities can be identified from the experimental data obtained so far, and by simplifications of a generalized model shown in Fig. 7A. Such an electrostatic model may be applicable to each of the osmoregulated transporters (OpuA, ProP, BetP). However, each transporter will have a different shape and charge density of the protein and/or lipid osmosensing surfaces, and may involve ionic strength dependence of the associating polypeptide(s) that defines the overall osmosensing surfaces or domains (e.g., ProP with ProQ). For example, charged osmosensing domains may interact with the cytoplasmic, negatively charged lipid bilayer (lipid–protein interactions) or with other surfaces of the transporter itself (180° away from the 'phospholipid' direction). Both the protein–lipid and protein–protein interactions are likely to be controlled by electrostatic forces, as suggested by the activation with ionic salts. The osmosensing domains are probably quite complex, candidate structures being described in the preceding sections.

The basic postulated mechanism of osmosensing for the lipid–protein interactions could be described as follows. As a water-soluble, osmosensing part of the transporter moves toward or away from the phospholipid charge in response to the increasing ionic strength, the conformation of the transmembrane transporter changes. It may change from a "tight" conformation to an activated, "loosened" conformation, which enables the transporter to utilize the electrochemical ion gradient, or the energy released by ATP hydrolysis, to translocate the solute molecule across the membrane. In addition, the overall membrane potential exerts a force on the proteins, potentially offsetting the ionic strength range over which the osmosensor becomes activated. Thus, in a detailed electrostatic model the total electrostatic potential profile across the membrane needs to be considered. Such a profile consists of the transmembrane (chemiosmotic) potential, a possible Donnan potential, and of the surface (double layer) potential. These potentials depend on the ionic and macromolecular composition of the 'inside' and 'outside' solutions that bathe the membrane, and on the lipid composition of the membrane.

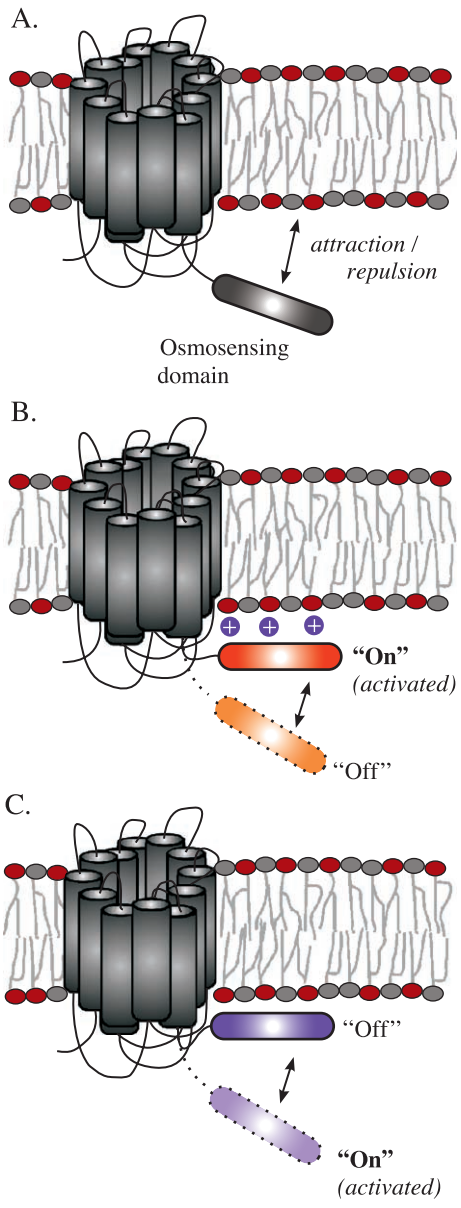


Fig. 7. (A) Generalized electrostatic mechanism of osmosensing. Anionic headgroups of lipids are indicated in red. (B) Negative osmosensor (domain in red) in activated electrostatically locked position when in contact with the membrane. (C) Positive osmosensor (domain in blue) in electrostatically locked non-activated position when in contact with the membrane. Details are described in the text.

In the following discussion, we shall simplify this general model by considering only the double layer 'switching forces' between the osmosensing domain and the phospholipid plane. These forces arise from the fixed charge distributions on the protein and from the anionic phospholipids. Three possible charge distributions of the osmosensing domain then suggest the existence of a negative osmosensor, a positive osmosensor, and an electroneutral osmosensor.

The *negative osmosensing* '*switch*' is shown in Fig. 7B. In the normal 'OFF' position ("relaxed" conformation), there are large electrostatic repulsions between the negative osmosensing peptide and the phospholipid plane. The calculations in Table 2 show the formation of a co-ion exclusion boundary (the CX model, Fig. 5) at lower ionic strengths than the critical ionic strength, when the surface potential is higher than 25.7 mV. When the critical ionic strength is reached, the screening electrolyte floods the space between the osmosensing domain and phospholipid plane, greatly reducing the negative repulsions. A 'coagulation' of the osmosensing domain onto the phospholipid plane may now occur (the "ON" position), mediated by the traditional van der Waals forces of colloidal theory, or more likely by "surface crystallization" involving available, or even specific, counter-ions. The presence of a positively charged amphipath reduces the repulsions by reducing the surface charge, and thus favours the "ON" position (shifts the activation profile, Fig. 2A, to lower ionic strength), as has been observed experimentally for OpuA (Refs. [31,55], J. Patzlaff and B. Poolman, unpublished results). Similarly, the activated state depends on the phospholipid charge density, as shown in Figs. 2B and 6. The attractive forces in the activated state may be specific to counter-ions, such as proposed for BetP [40].

The second possibility is a positive osmosensing 'switch' shown in Fig. 7C. Here there are no repulsions to be overcome. The positive protein surface tends to exclude cations and the negative surface excludes anions, when the attractive electrostatic forces are screened less at low ionic strengths, which leads to the 'locked' electrostatic normal ("OFF") position. The Debye–Hückel screening at increased ionic strength diminishes the attractions between the oppositely charged surfaces, which turns on the activated ("ON") "relaxed" conformation. Thus, the 'switching logic' of the positive and negative osmosensors is reversed. The cationic C-terminus of BetP would be expected to act in this way in an environment of negatively charged phospholipid headgroups (see Section 7). The collective data on the role of membrane lipids and ionic strength in the osmosensing of OpuA are consistent with both mechanisms. Because the 'critical' ionic strength increases with the lipid charge (fraction of anionic lipids), making the locked position stronger, we favour the possibility of OpuA being a positive osmosensor.

The third possibility is an electroneutral osmosensor that has an irregular distribution of positive and negative charges along the chain (perhaps the most realistic case). The response of the osmosensor will be a combination of the 'negative' and 'positive' responses discussed above. For instance, an electroneutral osmosensor at a 'low' ionic strength may have a 'bulging conformation' of the negatively charged loop away from the negative phospholipid plane, with a positively charged terminus 'locked' into the negative phospholipids. According to the Maxwellian theory [75–78], there may be no screening ions at all in the repulsive bulge (the negative osmosensor), as no co-ions can penetrate there if the local surface potential is more than 25.7 mV. When the ionic strength is increased, then both the repulsions

in the 'bulge' and attractions in the 'lock' decrease, causing the whole electroneutral domain to relax away into the cytoplasm by thermal motion. The Maxwellian theory now predicts an influx of co-ions (electrolyte) between the polypeptide and the phospholipid plane, if the potential drops below 25.7 mV. The C-terminal domain of *E. coli* ProP has the charge distribution expected for an electroneutral osmosensor (see Section 8 and Fig. 4). These 'electrostatic switches' (c.f. Table 2, Fig. 5), based on the transitions in the distributions of small ions, may control the 'ionic traffic' inside the cytoplasm in general [77].

The 'cartoon' models in Fig. 7 show that electrostatically controlled osmosensing function can operate only if the total osmosensing domain is charged, either negatively or positively, or if the osmosensing domain has regions of positive or negative charge distributions. Osmosensing domains that are electroneutral and have regular charge distributions are unlikely to be functional. We also stress that other physicochemical effects are likely to have a strong modulating influence. The 'electrostatic lock' conformations may redistribute or concentrate the negatively charged lipids closer to the protein domains in the membrane, leading to a general 'stiffening' of the membrane around the transporter protein, and thereby activate the osmoregulatory transport of compatible solutes. At a given membrane charge density in the 'locked position' of the negative osmosensor (Fig. 7B), it is likely that not all ions are equally effective and that there is preference for a certain type of cation, as suggested for BetP [40,58]. Even in the case of the positive osmosensor, there may be a specific ionic dependence, as the locked position is likely to be electroneutral to maximize the interaction. If the charges on the protein and phospholipid do not match, ions may be needed to equalize the charges. In addition, a macromolecular crowding effect would limit the 'relaxed' conformations, and hence a smaller energetic requirement would be needed to effect the activation. Macromolecular crowding may activate the transporter even in the absence of decreased electrostatic repulsions as observed for ProP [46]. The loss of hydration of charged moieties (lowered dielectric constant) will favor the 'locked electrostatic' conformations. However, it has to be borne in mind that the 'hydration' loss is a nonspecific modulating effect on the 'whole' cytoplasm by water efflux, and not necessarily only on the cytoplasmic membrane region, where the transporters are located.

10. Conclusions and perspectives

It is evident that bacterial cells use different mechanisms to regulate their internal osmolyte pools in response osmotic down- and upshifts, but lipid–protein interactions play a critical role in both. The osmotic downshift-activated MS channels respond to tension in the membrane, of which the lateral pressure in the acyl chain region seems most important. At sufficient tension, the transmembrane domains of the channels become tilted and a large pore is formed, which allows the osmotic pressure of the cytoplasm to be reduced. Electrostatic forces appear to be important controllers of osmotic upshift-activated transporters. These forces could act between the negative phospholipid plane and osmosensing domains or surfaces of these transmembrane proteins or they could act between protein surfaces. The osmosensing domains could be positive, negative, or electroneutral with positive and negative segments. The linear Maxwellian theory of screened electrostatic interactions predicts critical ionic-strength transitions that may define the "ON" and "OFF" protein conformations near the phospholipid membrane. The conformation may be either "thermally relaxed" or "electrostatically locked". The critical ionic strength transitions increase with surface charge densities, in accordance with the increasing ionic strengths needed to activate OpuA and BetP when they are embedded in membranes with increasing proportions of anionic lipids. The "relaxed" conformations will be restricted by macromolecular crowding effects, which would reduce the energy requirement for activation, as observed for the ProP transporter. Further development and testing of these concepts will depend upon the identification of protein residues and domains critical for osmosensing as well as definition of their interactions with one another and with membrane lipid.

Since the osmoregulation of MS channels and transporters is affected by changes in the bulk (and annular) lipids, long-term regulation of these systems could be effected via changes in the lipid composition of the membrane. Rapid activation occurring within a membrane of constant composition seems most relevant for growth in environments with rapidly changing osmolalities. However, cells 'permanently' growing in a high osmolality environment need to accumulate more compatible solutes than those residing in a low osmolality medium, and the activity of transporters could be tuned to the needs of the cells by adjusting the fraction of ionic lipids and/or the cytoplasmic ionic strength. The alternative mechanism would be to synthesize more transport protein when growing at high medium osmolality, but, this is not a necessity, and elevation of the activity of constitutively expressed transporter protein via alterations in membrane (anionic) lipid composition and/or cytoplasmic ionic strength could well explain adaptation to a high osmolality environment.

A major question to be addressed concerns the distribution of lipids within the bacterial membrane, both laterally and transversely. Given the large fraction of proteins in bacterial membranes (the weight fraction of protein and lipid is about equal), each protein will be surrounded by at most a few layers of lipids and the fraction of bulk lipids will be relatively low and much lower than in studies with proteoliposomes. As proteins could well have a preference for certain annular lipids, the

recruitment of such lipids, albeit in rapid exchange with the bulk lipids, would have a much larger impact in vivo than in the in vivo studies. In this regard, it is perhaps surprising that the osmotic regulation of MS channels and transporters in proteoliposomes mimics that in native membranes. Furthermore, there is the possibility that in vivo certain types of lipids are segregated from each other, i.e., as proposed for phosphatidylethanolamine and phosphatidylglycerol in *E. coli* and *Bacillus subtilis* [11]. On the basis of the work done with OpuA, such lipid segregation would have major consequences for the osmosensing mechanism.

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