

## Perspectives on: The response to osmotic challenges

# Bacterial responses to osmotic challenges

Janet M. Wood

Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Microbes respond to osmotic challenges in diverse, variable, and extreme natural environments

Exposure to diverse environments is a hallmark of microbial life. Microbes are everywhere; collectively, microbes experience everything. They live inside and outside eukaryotic hosts, in soil, water, and air at diverse planetary sites. They may exist as individuals (planktonic growth) or aggregates, and form biofilms on biotic and abiotic surfaces. Some survive gradual or abrupt, temporal or spatial transitions between different environments.

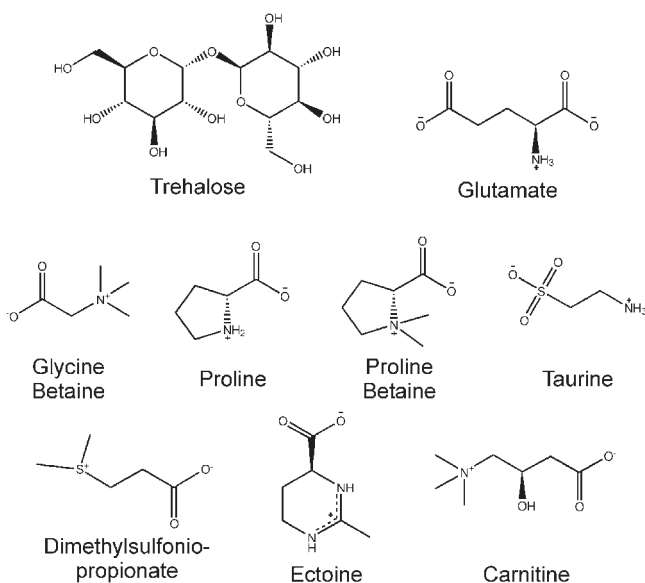
Our understanding of microbial responses to osmotic challenges is based on intensive studies of representative bacteria, archaea, and eukaryotic microbes. This Perspective focuses on bacterial responses to osmotic challenges. Among the representative bacteria for which the osmotic stress response is well characterized, *Escherichia coli* lives in terrestrial and aquatic environments as well as in the meninges and the intestinal and urinary tracts of mammals. *Bacillus subtilis* and *Corynebacterium glutamicum* are soil bacteria (*C. glutamicum* is also used to manufacture fine chemicals), and *Halomonas elongata* was isolated from a solar saltern (Wood, 2011a). Some bacteria can survive in pure water and grow at a water activity ( $a_w$ ) near 1, many thrive within human tissues (e.g., human blood,  $a_w$  of 0.995) or in seawater ( $a_w$  of 0.98), whereas others can only inhabit hypersaline environments with water activities as low as 0.75. Further examples, discussed below, illustrate the range of environments and environmental variations to which bacteria respond.

Bacteria are bounded by semipermeable cytoplasmic membranes, often including aquaporins. Most are also surrounded by a rigid, elastic, and porous cell wall (the murein or peptidoglycan layer) that determines cell shape. The cell wall of Gram-negative bacteria (such as *E. coli*) is bounded by an outer lipid membrane that includes porins like those of mitochondria. The area between the outer and cytoplasmic membranes is denoted the “periplasm.” The integrity and hydration of the cell and its compartments are dictated by their solute contents and the osmotic pressures of their environments (discussed in Altendorf et al., 2009). A decrease in

external osmotic pressure causes water influx and swelling or even lysis, whereas an increase in external osmotic pressure causes water efflux and dehydration. Water fluxes simultaneously, and almost instantaneously, perturb many cellular properties. These include cell volume (or the relative volumes of the cytoplasm and periplasm); turgor pressure; cell wall strain; and cytoplasmic membrane tension; as well as individual uncharged solute, salt ion, and biopolymer concentrations. Cells exposed consistently to a very high osmotic pressure must maintain correspondingly high cytoplasmic solute concentrations. Evidence suggests that the regulation of cytoplasmic composition and hydration is a key objective of cellular homeostasis (Wood, 2011b).

Common themes emerged as researchers characterized the osmoadaptive mechanisms of bacteria representing diverse phylogenetic groups (Wood, 2011a, and references cited therein). Cells respond to variations in external osmotic pressure by accumulating or releasing solutes, thereby attenuating water fluxes. Those solutes include inorganic ions (often  $K^+$ ), and organic molecules denoted “osmolytes” (Fig. 1). The latter are selected to minimally perturb cellular functions, even after accumulating to high (up to molar) concentrations. In turn, organisms have adapted to tolerate osmoregulatory solute accumulation. In the extreme, some halophiles accumulate KCl to molar concentrations, and their proteins function only in high salt environments. Osmoregulatory solutes accumulate via active transport or synthesis if the osmotic pressure rises and are released via mechanosensitive channels if the osmotic pressure falls. Multiple enzymes, transporters, and channels with redundant functions and specificities mediate solute accumulation and release from each organism (e.g., Fig. 2). The abundance of most osmoregulatory systems is controlled transcriptionally (Altendorf et al., 2009; Krämer, 2010). Translational regulation, mediated by small regulatory RNAs, is emerging as an important determinant of bacterial cell wall structure that may also influence the levels of osmoregulatory systems.

Correspondence to Janet M. Wood: [jwood@uoguelph.ca](mailto:jwood@uoguelph.ca)  
Abbreviation used in this paper: ABC, ATP-binding cassette.



**Figure 1.** Osmolytes. These compounds accumulate in *E. coli* in high osmotic pressure media (Altendorf et al., 2009).

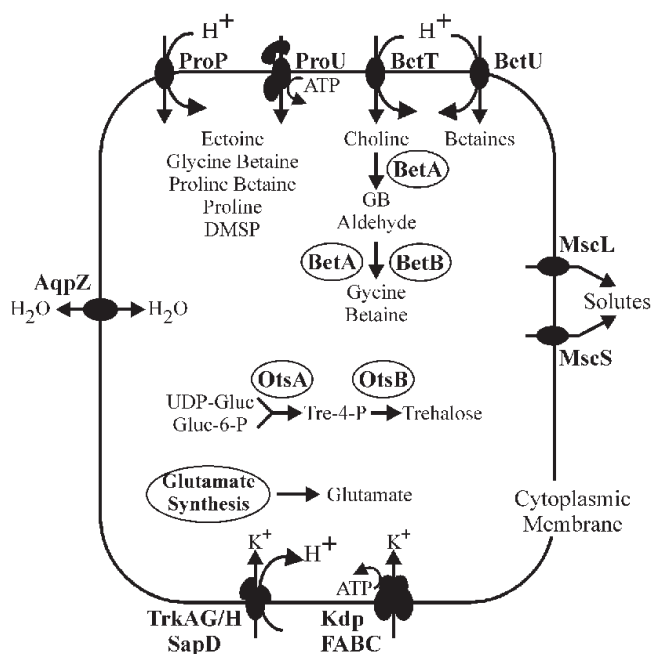
Care must be taken to differentiate osmotic stress from parallel, solute-specific effects that dominate particular environments. For example, bacteria inhabiting seawater face a higher osmotic pressure than those inhabiting most freshwater environments. Salts predominate in seawater, and marine organisms simultaneously face both a high osmotic pressure and a high  $\text{Na}^+$  concentration.  $\text{Na}^+$  fluxes are also implicated in pH homeostasis. Distinctions are also drawn between bacteria adapted to environments with extreme and stable osmotic pressures (e.g., sea water, salt lakes) and those experiencing osmotic pressure variations (e.g., those inhabiting estuarine waters or colonizing mammalian intestinal tracts).

What cellular systems limit bacterial cell and population growth rates under osmotic stress? How are osmotic stress responses orchestrated over time and space? Solute accumulation powerfully stimulates bacterial growth at high osmotic pressure, and solute release allows cells to survive osmotic downshocks. Thus, studies of bacterial osmoregulation have focused on the enzymes, transporters, and channels mediating solute accumulation and release (Krämer, 2010; Kung et al., 2010; Wood, 2011b) (Fig. 2). However, we do not fully understand how increasing osmotic pressure would limit bacterial cell or population growth in the absence of solute accumulation.

The evolution of bacterial cell and population size, protonmotive force, DNA replication, protein synthesis, and solute content were documented both after osmotic shifts and during steady-state culture of *E. coli* at various osmotic pressures, in the absence or presence of

osmoprotective solutes (Wood, 1999; Cayley and Record, 2004; Altendorf et al., 2009). Such studies revealed that the population growth rate is directly proportional to cytoplasmic hydration, and that accumulating solutes differentially affect cellular rehydration and population growth.  $\text{K}^+$  glutamate accumulation partially rehydrates cells and perturbs protein–nucleic acid interactions. It thereby offsets the impact of increased macromolecular crowding on cellular processes but does not restore growth to its pre-stress rate. In contrast, organic osmolytes rehydrate the cytoplasm and restore growth to an extent that correlates with their preferential exclusion from biopolymer surfaces (Cayley and Record, 2004) (discussed further below).

In contrast to our understanding of other stresses (e.g., oxidative stress; Imlay, 2013), we don't know what cellular properties or processes limit population growth rate when cells dehydrate. It was widely assumed that osmoregulation is necessary because turgor pressure



**Figure 2.** Osmoregulatory systems of *E. coli*. In high osmotic pressure environments, solutes accumulate in *E. coli* via synthesis (glutamate, trehalose, glycine betaine) or transport from the external medium (e.g., others shown in Fig. 1; Altendorf et al., 2009).  $\text{K}^+$ - $\text{H}^+$  symporter Trk and P-type ATPase Kdp mediate  $\text{K}^+$  uptake. Major facilitator superfamily member ProP, ABC transporter ProU, and betaine-carnitine-choline family members BetT and BetU mediate organic osmolyte uptake. ProP and ProU are similarly broad in substrate specificity, whereas BetT is choline specific (Murdock et al., 2014) and BetU is betaine specific. Mechanosensitive channels, including MscS and MscL, release solutes from the cytoplasm of osmotically downshocked bacteria. Aquaporin AqpZ exacerbates osmotic stress by accelerating transmembrane water flux. BetT and BetU are homologues of BetP from *C. glutamicum*, whereas ProU is a homologue of OpuA from *L. lactis* (see Fig. 3).

is essential for cell wall expansion and cell growth. However, evidence contradicts that assumption (e.g., *E. coli*; Cayley and Record, 2003; Rojas et al., 2014), and other cellular properties may be critical. Single-cell imaging techniques are now elucidating how osmotic stress affects cell growth and development (e.g., Pilizota and Shaevitz, 2013; Rojas et al., 2014), the composition and biophysical properties of the cytoplasm and cell membranes (Mika and Poolman, 2011; Sochacki et al., 2011; Wood, 2011b; Sévin and Sauer, 2014), and the subcellular locations of osmoregulatory systems (Romantsov et al., 2010).

Respiration, the synthesis of precursor metabolites, replication, transcription, and translation are obvious candidates for growth rate limitation (Wood, 1999). Individual strains within a species vary widely in osmotic stress tolerance (e.g., Kunin et al., 1992; Murdock et al., 2014). Analysis of new strains obtained via directed evolution and of naturally occurring variants may reveal what modifications, to what systems, extend the range of cytoplasmic hydration tolerated by an organism.

The application of high throughput “omic” technologies and cell sorting are also opening new avenues of investigation. Such tools can elucidate the orchestration of osmoadaptive mechanisms after an osmotic shift or during steady-state growth at various osmotic pressures (Withman et al., 2013, and other studies cited therein). They can also show how osmotic stress affects phenotypic variation within a microbial population. Analyses of bacterial community composition suggest that the bacterial lineages inhabiting marine and freshwater ecosystems are phylogenetically distinct, and that the capacity for osmoadaptation may be a primary determinant of that divergence (Walsh et al., 2013). Organisms adapted to a stable, high salinity marine environment may face particular barriers when transitioning to a more variable estuarine or fresh water environment. Such studies have relied heavily on genomic comparisons and annotations. Key tests of these ideas may be devised by combining physiological experiments with phylogenetic approaches.

#### How do proteins detect and respond to osmotic pressure variations?

Membrane proteins implicated in bacterial osmoregulation became the paradigms for the study of osmosensing because they retain osmotic pressure-dependent activities after purification and reconstitution in proteoliposomes (Poolman et al., 2004). Proteoliposome-based studies provided critical evidence that mechanosensitive channels and osmosensing transporters detect and respond to osmotic pressure changes in their phospholipid environments, without input from other cellular components. Studies of bacterial systems provided seminal evidence that mechanosensitive channels open in response to forces exerted by the lipid bilayer (Teng

et al., 2015). Analyses of MscL and MscS continue to elucidate mechanosensory mechanisms (Iscla and Blount, 2012; Naismith and Booth, 2012). The signal(s) to which osmosensing transporters respond remains less clear, however.

**Osmosensing transporters.** ProP of *E. coli*, BetP of *C. glutamicum*, and OpuA of *Lactococcus lactis* serve as paradigms for the study of osmosensing (Wood, 2011b) (Fig. 3). They represent different phylogenetic groups and energy-coupling mechanisms. ProP is a proton symporter and a member of the major facilitator superfamily, BetP is a Na<sup>+</sup> symporter and a member of the betaine-choline-carnitine transporter family, and OpuA is an ATP-hydrolyzing ATP-binding cassette (ABC) transporter. Available data suggest that each is similar in structure and transport mechanism to its paralogues that are not osmosensors.

The rate of osmolyte uptake via each transporter (A) is a sigmoid function of the osmotic pressure ( $\Pi$ ) or osmolality ( $\Pi/RT$ , where R is the gas constant and T is the temperature). Such data have been fit to an arbitrary relationship that implies no particular activation mechanism:

$$A = A_{\max} \left[ 1 + \exp\left(-(\Pi - \Pi_{1/2})/RTB\right) \right]^{-1}, \quad (1)$$

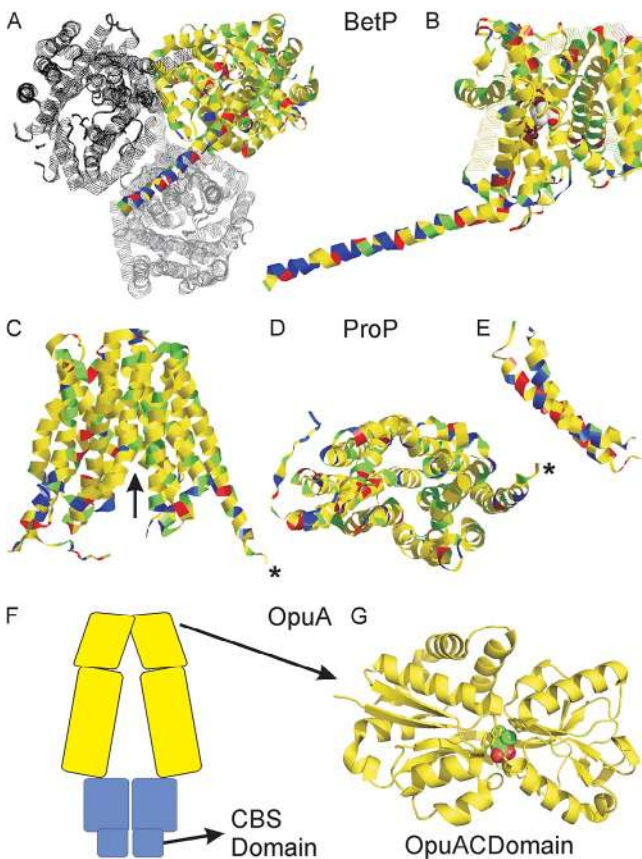
where  $A_{\max}$  is the asymptotic uptake rate, B is a constant inversely proportional to the slope of the response curve, and  $\Pi_{1/2}/RT$  is the osmolality at which activity is half-maximal. In this relationship,  $\Pi_{1/2}/RT$  can be replaced with any property that varies in parallel with the osmolality (e.g., the calculated concentration of a luminal solute in proteoliposomes). Proteoliposome data have also been fit to the Hill equation:

$$A = A_{\max} \left[ 1 + \left( K_{\text{ion}}^n / [\text{Ion}]^n \right) \right]^{-1}, \quad (2)$$

where  $K_{\text{ion}}$  is the ion concentration required to attain half-maximal activity, and  $n$  is a constant related to the slope of the curve (Mahmood et al., 2006).

To understand osmosensing, we must learn what cellular property is detected by an osmosensor and understand how variations to that property modulate osmosensor structure and function (Wood, 1999). In principle, an osmosensor would trigger a homeostatic response upon detecting deviations from a “set point” of such a critical property. Experiments performed with cells and proteoliposomes ruled out turgor pressure and membrane strain as determinants of osmosensing transporter activity (Poolman et al., 2004). Proteoliposome systems were then exploited to further assess the impacts of the external and luminal solvents on the activity of each osmosensing transporter.





**Figure 3.** Structures of osmosensory transporters. The structures of BetP from *C. glutamicum*, ProP from *E. coli*, and OpuA from *L. lactis* are illustrated. The protein backbones are colored according to amino acid side-chain polarity unless otherwise indicated: red for acidic residues Asp and Glu; blue for basic residues Arg, Lys, and His; green for polar residues Ser, Thr, Cys, Asn, and Gln; and yellow for nonpolar residues. BetP: a crystal structure of trimeric BetP (Protein Data Bank [PDB] accession no. 2WIT) as viewed from the cytoplasm (A) and of a single BetP subunit as viewed from the membrane (B). In A, the three BetP subunits are colored black, gray, and by amino acid. B shows a single subunit with residues from the N terminus through the end of transmembrane helix II as strands and residues 313–324 as a trace to reveal glycine betaine (space-filling, CPK coloring) within the substrate-binding site. ProP: a homology model of a ProP monomer (PDB accession no. 1Y8S) (C and D, residues 4–236 and 246–452 of the 500-residue ProP protein) and a nuclear magnetic resonance (NMR) structure of the C-terminal domain of ProP (PDB accession no. 1R48) (E, residues 468–497 of the 500-residue ProP protein). ProP is viewed from the membrane with the cytoplasmic surface down (C) and from the cytoplasm (D). The arrow in C marks the position of a substrate analogue in the crystal structure of homologue LacY (PDB accession no. 1PV7). The stars in C and D mark the C-terminal amino acid of the model. (E) The structure of a homodimeric peptide corresponding to residues 468–497 of ProP, determined by NMR spectroscopy (PDB accession no. 1R48). This antiparallel  $\alpha$ -helical coiled-coil and transmembrane helix XII contribute to the ProP dimer interface in vivo (Wood, 2011b). OpuA: a schematic representation of transporter OpuA (F) and the structure of periplasmic-binding protein domain OpuAC (G). In F, two cytoplasmic ATP-binding OpuAA subunits, including C-terminal cystathionine- $\beta$ -synthase (CBS) domains, are blue. Two transmembrane OpuAB domains and the contiguous substrate-binding OpuAC domains are yellow.

*Merits and liabilities of proteoliposome systems.* The interpretation of proteoliposome data are supported by evidence that secondary transporters ProP and BetP reconstitute predominantly with their cytoplasmic surfaces facing the lumen, and the direction of transport is determined by an imposed ion motive force. Studies of ABC transporter OpuA exploit the fact that the direction of transport can be controlled by supplying ATP in either the external or the luminal medium (Wood, 2011b). To date, functional tests have been the primary indicators of osmosensing (i.e., solute uptake assays as opposed to spectroscopic indicators of transporter conformation). The requirements to maintain the membrane permeability barrier and to meet energy requirements for transport restrict the range of luminal and external solvent compositions accessible for these studies. The  $A_{\max}$  values obtained with proteoliposomes are variable because transporter purification, reconstitution, and solute loading are intrinsically variable procedures. A recent comparison of the molecular activities of BetP in cells and proteoliposomes indicated that only 2.4% of BetP molecules in proteoliposomes were active (Maximov et al., 2014), reinforcing the need for careful interpretation of proteoliposome data. In contrast,  $\Pi_{1/2}/RT$ , B, and  $K_{\text{ion}}$  values are independent of transporter quantity, more reproducible, and hence presumed to be more reliable indicators.

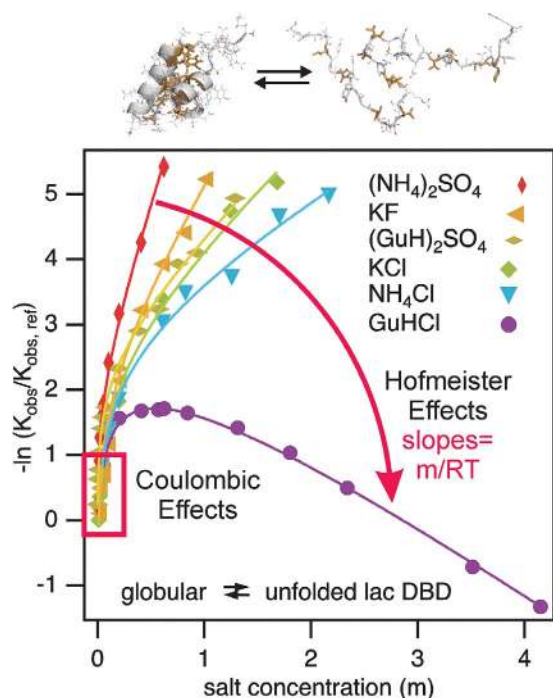
*Solvent effects on biopolymer structures.* Current knowledge of solvent effects on biopolymer structures provides a useful context for the analysis of osmosensory mechanisms. Soluble proteins and DNA have been the primary foci of such studies, which explore the thermodynamic nonideality inherent to physiological systems and their models (Record et al., 1998b, 2013). Solvent additives can affect biopolymer processes by binding as ligands at specific sites, via preferential interactions with buried or exposed biopolymer surfaces (Hofmeister effects, involving both uncharged and charged solutes) and via conformation-specific, Coulombic interactions with fixed biopolymer charges (charged solutes only). Thus, solutes may act individually (high affinity ligand binding at one or a few specific sites) and/or collectively (weak interactions at many sites).

Collective solute effects modulate the equilibrium constant (K) for any process that changes the amount of biopolymer surface interacting with a solute. The magnitudes and functional forms of these collective effects are determined by the nature of the solute excluded

G shows a crystal structure of domain OpuAC (yellow; PDB accession no. 3L6H) in complex with glycine betaine (spheres). The binding pocket includes three Trp residues (W330, W377, and W484, shown as sticks) that coordinate the trimethylammonium group of glycine betaine.

from or concentrated at the biopolymer surface (particularly whether it is charged or uncharged) and of the exposed or buried biopolymer surface (e.g., that of a high charge density polyelectrolyte like DNA or a low to no charge density biopolymer like a typical protein). When salt concentrations are low, the collective effects are primarily Coulombic (salts weaken charge–charge interactions). When salt or uncharged solute concentrations are high, their contributions to Hofmeister effects become dominant (e.g., Fig. 4; Record et al., 2013). The salt concentration ranges over which Coulombic and Hofmeister effects dominate for DNA and protein processes differ because DNA is a polyelectrolyte.

If a process changes the amount of uncharged or weakly charged biopolymer surface exposed to a preferentially



**Figure 4.** Effects of Hofmeister salts on protein unfolding. The effects of low and high concentrations of salts spanning the Hofmeister series on unfolding of the lac repressor DNA binding domain at 40°C.  $K_{\text{obs}}$  is the unfolding equilibrium constant in the presence of salt at concentration  $X$ , and  $K_{\text{obs,ref}}$  is the reference equilibrium constant in low salt buffer. At low salt concentration, all salts exert similar stabilizing effects. These are Coulombic in origin and vary nonlinearly with salt concentration. At high salt concentration, different salts exhibit a wide range of stabilizing (e.g.,  $(\text{NH}_4)_2\text{SO}_4$ , KF) to destabilizing (guanidinium HCl (GuHCl)) effects, which are linear in salt concentration and follow the traditional Hofmeister series. The slopes of these high salt linear regions ( $m$ -value/ $RT$ ) correlate with the magnitude and chemical composition of the protein surface that is exposed to the solution in unfolding and the chemical properties of the salt (the places of the cation and anion in the Hofmeister series). Fitted curves allow a separation of Coulombic and Hofmeister effects of these salts. Adapted from Fig. 6 of Record et al. (2013) with permission of The Royal Society of Chemistry (<http://dx.doi.org/10.1039/C2FD20128C>).

interacting solute, then the free energy (or the logarithm of the equilibrium constant,  $K$ ) for that process is a linear function of the solute concentration with a proportionality constant (the thermodynamic  $m$ -value) that reflects the properties of the solute and the magnitude of the exposed or buried biopolymer surface. Such effects are very weak at low solute concentration. In contrast, if a process changes the amount of a (polyanionic) DNA surface exposed to ionic solutes, then the logarithm of the equilibrium constant ( $K$ ) varies with a power of the logarithm of the ion concentration. Such Coulombic effects are large even at low salt concentrations. The latter analysis supersedes the Debye–Hückel approximation, based on ionic strengths calculated as a function of ion concentrations and valencies, which has much more limited application. Principles governing protein–membrane interactions have not been analyzed in this way, but interactions of proteins with polyanionic membrane surfaces can be expected to share characteristics with protein–DNA interactions.

The principles outlined above were established primarily with *in vitro* systems. There is also evidence that cytoplasmic solutes collectively influence cellular processes, particularly as osmotic pressure changes alter cytoplasmic hydration (Record et al., 1998a,b). Small cytoplasmic solutes (e.g.,  $\text{K}^+$ , glutamate, and other metabolites) are preferentially excluded from nonpolar biopolymer surfaces that become exposed in unfolding. Increasing concentrations of these solutes will favor conformational changes that bury nonpolar surfaces (Record et al., 2013). At the same time, condensation of  $\text{K}^+$  as a DNA counterion impedes processes involving protein–DNA interactions. In addition, increased concentrations of cytoplasmic biopolymers favor folding, especially if folding is coupled to oligomerization, by an excluded volume effect (sometimes denoted as “macromolecular crowding”; Cayley and Record, 2004).

*Conceptual framework for the analysis of osmosensing.* In proteoliposomes, osmosensory transporters become active as luminal solute concentrations approach 0.5 M. This suggests that both Coulombic and Hofmeister effects may participate in transporter activation and that resolution of Coulombic and Hofmeister effects will be challenging (c.f. Fig. 4). The sigmoid relationship between  $A$  and  $\Pi_{1/2}/RT$  implies that transporter molecules are systematically converted from an inactive to an active conformation as the osmolality increases:



If so, the fraction of transporter active at a particular osmolality may be represented by:

$$f = A/A_{\text{max}} \quad (4)$$

where  $A$  is the initial rate of substrate uptake at a given osmolality, and  $A_{\max}$  is the asymptotic initial rate approached at high osmolality. Then the equilibrium constant  $K$  for this transition at a particular osmolality is:

$$K = f/(1-f). \quad (5)$$

If the activating conformational change were triggered only by solute exclusion from nonpolar transporter surfaces that were exposed in the inactive and buried in the active transporter (a Hofmeister effect), the logarithm of the equilibrium constant  $K$  would be expected to vary linearly with the solute concentration  $X$  (Record et al., 2013):

$$\ln K = \ln K_0 - (m/RT) X \text{ or } K = K_0 \exp \left( -(m/RT)X \right). \quad (6)$$

In this equation,  $K_0$  would be the equilibrium constant at  $X = 0$ , where the transporter activity is undetectably small, and  $m/RT$  would be a thermodynamic parameter (the thermodynamic  $m$ -value) characteristic of the solute and the conformational change. To obtain  $m$  and  $K_0$  for transporter activation, values of  $A$  at each  $X$  would be fit to the following combined relationship:

$$A = A_{\max} \exp \left( -(\ln K_0 + (m/RT)X) / [1 + \exp \left( -(\ln K_0 + (m/RT)X) \right)] \right). \quad (7)$$

The  $m$ -values for an array of solutes would follow the Hofmeister series (a ranking of solutes according to their effects on diverse biopolymer processes; Record et al., 2013). Eq. 7 has the same form as Eq. 1, but it provides a thermodynamic interpretation of the resulting parameters. If the activating conformational change were triggered only by interactions of ions with charged surfaces (a Coulombic effect), the logarithm of the equilibrium constant  $K$  would be expected to vary with a power ( $n$ ) of the logarithm of the solute concentration  $X$  (Record et al., 2013):

$$\ln K = \ln K_0 - (m/RT)[\ln X]^n. \quad (8)$$

A relationship analogous to Eq. 7 would then reflect the dependence of  $\ln K$  on  $[\ln X]^n$ , and  $\ln K_0$  would be the value of  $\ln K$  at an ion concentration ( $X$ ) of 1 M. It is critical to note that reliable estimates of  $m/RT$ , the most informative parameter, can only be obtained from data that define the full range of  $f$  values.

*Proteoliposome-based analysis of osmosensing by ProP, BetP, and OpuA (Wood, 2011b).* All tested membrane-impermeant solutes had similar effects on transporter activity when applied to attain the same osmolality at the external transporter surface. This response was phospholipid sensitive: the osmolality at which each transporter activates was a direct function of the anionic lipid content of the host membrane (both in vitro and in vivo).

All three transporters became active as inorganic ions were concentrated at their cytoplasmic surfaces from  $\sim 0.1$  to  $\sim 0.5$  M. Differences emerged when diverse ions were used, however.

ProP activity correlated with luminal cation concentration but not luminal  $K^+$  concentration. For proteoliposomes loaded with  $K$  phosphate plus the  $K$  salts of various anions, the osmolality yielding half-maximal ProP activity ( $\Pi_{1/2}/RT$ ) followed the Hofmeister series. ProP activity was enhanced when proteoliposomes were loaded with high molecular weight polymers (polyethyleneglycols or bovine serum albumin) at concentrations that simulated the volume exclusion occurring in the bacterial cytoplasm (Culham et al., 2012). Culham et al. (2012) concluded that ProP activity is determined by the concentrations of Hofmeister anions and macromolecular crowding.

Internal  $K^+$  phosphate, glutamate or chloride,  $Rb^+$ , or  $Cs^+$  chloride activated BetP to varying degrees, whereas  $Na^+$  (the coupling ion),  $NH_4^+$ , or choline chloride did not.  $K^+$  salts yielded the strongest stimulations (Krämer, 2010). However,  $K^+$  dependence did not fully account for the osmotic activation of BetP in vivo, leading Maximov et al. (2014) to conclude that BetP senses  $K^+$  concentration and a signal from the membrane. The effects of crowding agents on BetP activity have not been reported.

The rate of glycine betaine uptake via OpuA was enhanced similarly by  $K^+$ ,  $Na^+$ ,  $Li^+$ , or  $NH_4^+$  chloride. OpuA was further activated by  $MgCl_2$  and  $BaCl_2$  and inhibited by  $RbCl$  and  $CsCl$ . Ions and a large polyethylene glycol (PEG600) acted synergistically to stimulate substrate-dependent ATP hydrolysis by OpuA in nanodiscs (Karasawa et al., 2013). Karasawa et al. (2013) concluded that OpuA responds synergistically to the ionic strength and macromolecular crowding.

These reports evoke critical roles for electrolytes, for a membrane with a polyanionic surface, and possibly for cytoplasmic volume exclusion in transporter activation. All are likely to result from some combination of collective Coulombic and Hofmeister effects of luminal solutes on changes to cytoplasm-exposed membrane and transporter surfaces. Unfortunately, the reported data are insufficient to clearly delineate the relative contributions of Coulombic and Hofmeister effects.

It is challenging to deduce the structural mechanism of osmosensing because membrane proteins are refractory to structural analysis. An impressive series of crystal structures has made enormous contributions to our understanding of the transport mechanism for BetP and related systems (Perez et al., 2014). However, conformational differences between inactive and osmotically activated BetP conformers remain to be defined. Data outlined above suggest that BetP is a chemosensor, possessing one or more cytoplasm-exposed,  $K^+$ -specific regulatory sites, but those sites have not been identified. By



comparison, our structural knowledge of ProP and OpuA is limited (Fig. 3).

We do know that each transporter is an oligomer (ProP and OpuA are dimers; BetP is a trimer; Wood, 2011b). The role of oligomerization in osmosensing by BetP has been explored experimentally but remains uncertain (Becker et al., 2014). The roles of oligomerization for ProP and OpuA remain unknown. Each transporter possesses an extended cytoplasmic C terminus (Fig. 3). The C termini of some ProP orthologues form antiparallel, intermolecular coiled-coils, whereas the extended C termini of other orthologues do not include coiled-coil motifs. The C terminus of BetP forms a long  $\alpha$  helix that mediates inter-monomer interactions within BetP trimers, and the C termini of the two ATP-binding subunits of OpuAA include dual cystathionine- $\beta$ -synthase domains with anionic tails. Structural changes to the C-terminal domains modulate the osmoregulatory response (they shift the osmolalities at which the transporters become active). It has been proposed that the cytoplasmic C termini mediate osmosensing via salt-sensitive interactions with other transporter elements (protein-protein interactions) and/or the polyanionic membrane surface. Osmotically induced variations in membrane surface charge density would also modulate protein-membrane interactions. Each of these interactions would have a characteristic thermodynamic signature, and clear dominance of Coulombic or Hofmeister effects would support distinct structural models. Thus, osmosensing may provide a paradigm for the regulation of membrane protein structure and function through protein-solvent interactions, involving solute exclusion from or accumulation at extensive protein and/or membrane surfaces.

This Perspective series includes articles by [Andersen](#), [Sachs and Sivaselvan](#), and [Haswell and Verslues](#).

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