Osmotic regulation of transport processes in Lactobacillus plantarum
Glaasker, Erwin

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Regulation of Compatible Solute Accumulation in Bacteria

Bert Poolman and Erwin Glaasker

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ABSTRACT

In their natural habitats microorganisms are often exposed to osmolality changes in the environment. The osmotic stress must be sensed and converted into an activity change of specific enzymes and transport proteins and/or it must trigger their synthesis such that the osmotic imbalance can be rapidly restored. On the basis of the available literature we conclude that representative Gram-negative and Gram-positive bacteria use different strategies to respond to osmotic stress. The main focus of this paper is on the initial response of bacteria to hyper and hypo-osmotic conditions, and in particular the osmosensing devices that allow the cell to rapidly activate and/or to synthesize the transport systems necessary for uptake and excretion of compatible solutes. The experimental data allow us to discriminate the transport systems by the physicochemical parameter that is sensed, which can be a change in external osmotic pressure, turgor pressure, membrane strain, internal osmolality and/or concentration of specific signal molecule. We also evaluate the molecular basis for osmosensing by reviewing the unique structural features of known osmoregulated transport systems.

1. Introduction
The cytoplasmic membrane of bacteria is permeable to water but forms an effective barrier for most solutes in the medium and metabolites in the cytoplasm. A lowering of the external water activity (hyper-osmotic conditions) causes a rapid efflux of water and loss of turgor; ultimately the cells may plasmolyse, i.e. the cytoplasmic membrane retracts from the cell wall. Similarly, upon hypo-osmotic shock water flows into the cell and increases the cytoplasmic volume and/or turgor pressure. To survive osmotic stresses, the cells need to adapt by accumulating specific solutes under hyper-osmotic conditions and releasing them under hypo-osmotic conditions. Such solutes include K\(^+\), amino acids (e.g. glutamate, proline), amino acid derivatives (peptides, N-acetylated amino acids), quaternary amines (e.g. glycine betaine, carnitine), sugars (e.g. sucrose, trehalose), and tetrahydropyrimidines (ectoines) (Csonka, 1989; Galinski and Trüper, 1994). These solutes are often referred to as compatible solutes because they can be accumulated to high levels by de novo synthesis or transport without interference with vital cellular processes. In fact, many compatible solutes proved to be effective stabilizers of enzymes, providing protection not only against high
salt, but also against high temperature, freeze-thawing and drying (Yancey et al., 1982). Solutes that are non-charged or zwitterionic are generally more favorable to protein stability than ionic solutes, and, consistently, upon accumulation of neutral compatible solutes one often observes an iso-osmotic displacement of ionic solutes such as K⁺ and glutamate from the cell. In most eubacterial species glycine betaine is the preferred compatible solute and generally provides the highest level of osmotolerance, which may reflect -among others- its favorable interaction with macromolecules.

Osmoprotectants are those solutes that alleviate the inhibitory effect of hyper-osmotic stress on the microorganisms when they are added to the medium, but often this term is also used for any solute that can overcome osmotic inhibition. Since the term osmoprotectant is vague and not well-defined, it is preferable to only use compatible solute for any compound that offers protection to high osmolality by accumulating to high cytoplasmic concentrations either by uptake from the medium or de novo synthesis. Finally, osmolality describes the osmotic pressure of a solution in osmoles of osmolytes per kg of solvent. Notice that the osmole is not a defined number because the osmotic pressure is not solely determined by the particle number but also by their size, shape and charge (see Sweeney et al., 1993 for measurement of these parameters).

The cells may synthesize (some of the) compatible solutes following an osmotic upshock and degrade them following an osmotic downshift, but the initial response is much more rapid if compatible solutes can be taken up from the medium and/or released into the medium via semi-constitutive transport systems. The transport systems involved in osmoregulation can be subdivided in specific uptake systems, stretch-activated channels, specific efflux systems and aquaporins. The regulation of the activity of these systems forms the topic of this chapter. In many cases the synthesis of the transport systems is also under osmotic control, but often the effects of hyper and hypo-osmotic conditions on expression and activity are not adequately discriminated or poorly described. We prefer to use the term (in)activation to indicate a change in activity of a transport system or open probability of a channel protein, and use induction/repression to describe the phenomena that lead to an altered expression. In this paper, we evaluate the main findings pertinent to the immediate or short-term response of transport systems for compatible solutes following a shift in medium osmolarity. When appropriate we compare the mechanism of activation of the system with the mechanism of induction of the corresponding gene(s). A more general
overview of the genetics and physiology of prokaryotic osmoregulation is given in Csonka (1989), Csonka and Hanson (1991), and Lucht and Bremer (1994).

2. What are the regulatory signals for (in)activation?
Upon a change in the medium osmolality, a membrane transport system may be (in)activated by one or more of the following physicochemical parameters (Fig. 1): (i) external osmolality or water activity; (ii) turgor pressure, i.e. the difference between the extra- and intracellular potential of all osmotically active solutes, which affects the compression of the membrane against the cell wall; (iii) membrane-strain, which occurs in response to a change in turgor pressure and affects the compression/expansion of the bilayer in the plane of the membrane; (iv) internal hydrostatic pressure; (v) internal osmolality or water activity; (vi) cytoplasmic volume; and (vii) concentration of specific cytoplasmic signal molecule(s). Not all these parameters will act on the same time scale, which may be diagnostic in distinguishing some of the sensing mechanisms. The first three of the physicochemical parameters or signals are thought to act more rapidly than the latter four as it will take some time to elicit significant changes in cytoplasmic concentration and volume (Csonka and Hanson, 1991; Stock et al., 1977). For instance, upon osmotic upshock, the increase in external osmolality will be instantaneous, the decrease in turgor pressure will be rapid but the intracellular volume may only decrease after turgor has fallen to zero. However, as pointed out by Csonka and Hanson, 1991, what actually happens with the cell volume is highly dependent on the rigidity of the cell wall (peptidoglycan layer or sacculus). Since the cell wall of Gram-negative bacteria is much thinner and the degree of cross-linking of the disaccharide units by peptides in the peptidoglycan is much lower than in Gram-positive bacteria, one would anticipate that its mechanical properties are quite different (Dijkstra and Keck, 1996). On the basis of studies with peptidoglycan purified from Gram-negative and Gram-positive bacteria, it is now well established that the peptidoglycan sacculus is an elastic, flexible, polyanionic restraining network (that behaves as a viscoelastic polymer) and not an inherently rigid structure as is often described in textbooks (Doyle and Marquis, 1994; Koch and Woeste, 1992; Thwaites and Mendelson, 1989). The cell wall is thus able to stretch as water flows into the cell, and shrink when water flows out of the cell, which will result in changes in the cytoplasmic volume (and related parameters) along with the osmolality changes in the medium. Thus, opposite to what is often thought, volume changes may already occur when there is still turgor pressure. After the turgor pressure has dropped to zero, the bacteria may plasmolyse, i.e. the cytoplasmic membrane separates from the cell wall and the volume decreases further; at this point the cells behave as
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osmometers. In contrast to Gram-negative bacteria, Gram-positive do not plasmolyse (Mitchell and Moyle, 1956; Whatmore and Reed, 1990), which implies that the ultimate cytoplasmic volume under hyper-osmotic conditions is set by the rigidity of the cell wall. The reason for the failure of Gram-positive bacteria to plasmolyse might be the strong adhesion between the cytoplasmic membrane and peptidoglycan. Alternatively, it has been proposed that these bacteria do not plasmolyse because of their very high internal osmotic pressure (turgor pressures of 15 to 25 atm), which means that much higher external osmolalities are needed before the turgor drops to zero than in Gram-negative bacteria (turgor pressures of 1 to 5 atm). Besides these anticipated differences between Gram-negative and Gram-positive bacteria in experiencing osmolality changes, it is obvious that most of the physicochemical parameters associated with an osmolality change of the medium are not mutually exclusive but rather interrelated, and these will change in parallel upon an osmotic shift.

Changes in each of heretofore mentioned physicochemical signals may trigger the activation of osmoregulated transport proteins or alter the open probability of channel proteins, but they will not solely determine the fluxes of compatible solutes across the membrane. The ultimate activity of a system following the activation by a shift in medium osmolality will depend on the state of the cells with respect to (i) the internal osmotic pressure or related parameter at the time of the shift (as described above); (ii) the internal concentration of the compatible solute (and structural analogs), which may inhibit through ‘feedback’ or ‘trans’ inhibition; and/or (iii) physiological parameters such as the energy status and the internal pH of the cell.

As a result of a change in the external osmolality or water activity the structure of a membrane protein or a portion thereof (external sensing domain) could be deformed, which in turn may affect the activity of the protein. Similarly, as a result of a change in turgor pressure an integral membrane protein could be deformed through changes in protein-lipid interactions as a result of membrane-stretch/tension or compression of the protein against the peptidoglycan layer. The other mechanisms may require a specific internally located domain that senses changes in hydrostatic pressure, intracellular osmolality or concentration of a specific solute. Below we describe the likely mechanism(s) of osmotic regulation of well-studied uptake and efflux systems that, in our opinion, exemplify many other osmoregulated systems as well (Table I).
Chapter 2

3. The initial response to osmotic upshift: Gram⁻ versus Gram⁺ bacteria
The initial response of enteric bacteria to an osmotic upshock involves the uptake of potassium via Kdp and Trk, the main transport systems for K⁺. With Trk we refer to the system that uses either TrkG or TrkH as the pathway for K⁺ uptake in *Escherichia coli* (Bakker, 1992; Schlösser *et al*., 1995). To maintain electroneutrality, the accumulation of K⁺ is accompanied by increases in the glutamate pool by *de novo* synthesis during growth at high osmolarity (Caylay *et al*., 1991; McLaggan *et al*., 1994), but other processes such as accumulation of other anions, putrescine excretion and proton efflux may contribute as well (Csonka, 1989). Although potassium transport has been studied most thoroughly in *E. coli* and *Salmonella typhimurium*, similar turgor-sensitive transport systems may be present in other organisms (Whatmore *et al*., 1990), and the initial response to high osmolarity is generally associated with potassium uptake. Displacement of K⁺ (and counterion(s)) for neutral compatible solutes may occur shortly after the accumulation of these ions, e.g. through the uptake of glycine betaine or proline via semi-constitutive transport systems such as ProP. Although K⁺ and glycine betaine or proline uptake may proceed simultaneously, the accumulation of K⁺ via Trk is more rapid and quantitatively most important in the initial phase of hyper-osmotic stress. Other systems, e.g. those for trehalose synthesis and high affinity uptake of glycine
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betaine (proU), require induction and contribute to the restoration of turgor at later times of hyper-osmotic stress (Booth, 1992; Lucht and Bremer, 1994).

Recent studies in Lactobacillus plantarum indicate that in this organism most of the potassium is bound (Glaasker et al., 1996a; 1998), which reduces its osmotic significance. Moreover, increases in the cellular concentrations of \( K^+ \) are only observed in KCl-stressed cells and not with equi-osmolar concentrations of NaCl or sugar. It is, therefore, thought that this Gram-positive bacterium binds a large fraction of the \( K^+ \) extracelluarly. Unlike the enteric bacteria and Bacillus subtilis, L. plantarum and other lactic acid bacteria have limited or no possibilities to synthesize compatible solutes. Most of these organisms are multiple amino acid auxotrophs and reside in environments that contain these amino acids in some form as well as glycine betaine (plant origin) or carnitine (animal origin). The only way out to recover from hyper-osmotic stress is via the uptake of compatible solutes such as glycine betaine, carnitine and/or proline, and the initial osmotic response in L. plantarum is formed by activation of the high capacity-high affinity semi-constitutive glycine betaine/carnitine/proline transport system QacT.

The findings made for L. plantarum may represent the basis for osmoregulation in Gram-positive bacteria in general or at least in simple anaerobes such as the lactic acid bacteria. In fact, already in 1975 it was observed that in response to osmotic stress Gram-negative bacteria accumulate large amounts of glutamate (together with \( K^+ \)), whereas the Gram-positive bacteria investigated accumulate proline (Measures, 1975). Under non-stressed conditions, the Gram-positives already have a high amino acid pool, of which a large proportion is glutamate. Similarly, the cellular concentrations of \( K^+ \) in non-stressed Gram-positive bacteria are usually much higher (often around 1 M) than in Gram-negative bacteria (Glaasker et al., 1996a and 1998; Measures, 1975; Kakinuma and Igarashi, 1988; Poolman et al., 1987b; Caylay et al., 1991; McLaggen et al, 1994), which is also reflected in the higher turgor pressure of the organisms (Csonka, 1989). It should be stressed, however, that the fractions of ‘free’ and ‘bound’ potassium have only been determined in a few cases (Caylay et al., 1991; McLaggen et al, 1994; Glaasker et al., 1998). Given the differences in osmolality and the high concentrations of electrolytes, it seems that under hyper-osmotic conditions, in particular Gram-positive bacteria are better off by accumulating proline or glycine betaine (or quaternary amines in general) than the electrolyte pair K-glutamate. Whereas some (Gram-positive) bacteria can synthesize proline and glycine betaine (from choline), others rely completely on
uptake of these or other compatible solutes from the medium. We emphasize that the term accumulation as used in this section reflects increases in pool sizes through uptake and/or synthesis. In several cases (e.g., Measures, 1975), the cells were grown in rich media containing amino acids and peptides and accumulation through uptake or synthesis cannot be discriminated.

4. Osmotic regulation of uptake of potassium ions
The osmotic regulation of the Kdp system occurs at the level of catalytic activity as well as protein expression. There is evidence that Kdp mediates potassium exchange without net movement under osmostatic conditions, whereas the system switches to net uptake when turgor is low (Epstein, 1992). However, compared to the regulation of Kdp activity much more is known about the transcriptional regulation of the kdp genes, which represents one of the best-understood systems in terms of osmosensing (Booth, 1992; Nakashima et al., 1993; Sugiura et al., 1994; Jung et al., 1997). The induction of the kdp operon is mediated by a sensor kinase (KdpD)/response regulator (KdpE) system, but the sensing mechanism may be paradigmatic of that of some transport and channel proteins that are described below. A decrease in turgor pressure or related parameter results in autophosphorylation of KdpD. Subsequent transfer of the phosphoryl group to the transcription factor KdpE results in increased transcription of the kdp genes. Since amphipaths that intercalate into the lipid bilayer elicit a similar effect as an osmotic upshock, it has been proposed that the osmotic signal that is sensed by KdpD corresponds to membrane stretch. In contrast to Kdp, the osmotic regulation of Trk is mainly at the level of transport activity but the signal for activation may be very similar to that of KdpD. The activity of Trk increases upon an osmotic upshift but the initial rate of influx does not depend on the size of the external osmolarity increase (Meury et al., 1985). Instead, it seems that the activity of Trk depends on the intracellular osmolality at the time the external osmolality is increased. Since in the experimental setup intracellular osmolality and potassium concentration are not well-separated it is also possible that the actual rate is determined by the intracellular potassium concentration through feedback regulation. Nevertheless, the instantaneous activation of K\(^+\) uptake upon an osmotic upshift and the dependence of the accumulation level on the size of the osmotic shift strongly suggests that the system is turgor-controlled; the internal osmolality and/or K\(^+\) concentration modulates the activity (Meury et al., 1985).

5. Osmotic regulation of uptake of organic compatible solutes
Glycine betaine and proline uptake in enteric bacteria follow the uptake of potassium(-glutamate), provided these compatible solutes are present in the medium, and thereby replace the electrolytes in time. The uptake of these compatible solutes is effected by the proton motive force-driven ProP and ATP-driven binding protein-dependent ProU system. Upon osmotic upshock the ProP protein is activated within seconds, the time resolution of the experiment, but the stimulation of activity requires the presence of K\(^+\) in the medium (Koo et al., 1991). Since ‘activated’ uptake occurs irrespective of whether the turgor has been restored via the uptake of K\(^+\), it seems unlikely that ProP senses turgor \textit{per se}. The requirement for K\(^+\) is indicative for an important role of the internal pH in regulating ProP (Poolman et al., 1987ab), but, as pointed out before, the increase in intracellular pH upon K\(^+\) uptake is usually transient whereas the ‘activated’ uptake lasts as long as the medium osmolarity is high (Koo et al., 1991). On the other hand, it should be stressed that an increased internal pH can be maintained when counterions that balance the charge during the uptake of K\(^+\) are absent (McLaggan et al., 1994). In this regard, the putative activation of ProP by potassium ions requires further exploration. The osmotic regulation of ProP has also been studied in membrane vesicles and although the authors conclude that the system is activated by low turgor pressure (Milner et al., 1988), the fact that membrane vesicles cannot withstand any (or at most very small) pressures argues against regulation by turgor pressure. The imposed osmotic shifts transiently disrupt the barrier function of the membrane and during this period external and internal solutes will equilibrate, thereby minimizing the turgor pressure (Glaasker and Poolman, unpublished). In our opinion the data obtained with the membrane vesicles are most consistent with regulation by external osmotic pressure or related parameter, which also fits best the observations made by Koo et al., 1991.

The hyper-osmotic activation of ProU takes several minutes (Faatz et al., 1988), which makes it unlikely that increased external osmotic pressure or reduced turgor pressure triggers the activation. A closer look at the data suggests that activation of ProU is also dependent on the presence of substrate (glycine betaine) as the transport activity increases up to 3 min after an osmotic upshock in the absence but not in the presence of glycine betaine (Faatz et al., 1988). Despite the peculiarities in the time dependence of activation of the ProU system, the regulation of transport activity is clearly different from that of Trk and ProP and resembles that of transcription of \textit{proU}, i.e., both are delayed upon an upshift. Initially it was thought that K\(^+\) and glutamate served a role as ‘second messengers’ in enhancing the \textit{proU} transcription (Ramirez et al., 1989; Booth,
1992), but other studies have indicated that a large glutamate pool is not necessary for induction (Jovanovich et al., 1989; Csonka et al., 1994). It has been proposed (Csonka et al., 1994) that the stimulation of transcription of proU in vitro by K⁺-glutamate is a manifestation of its favorable effect on macromolecular function (e.g., RNA polymerase-promoter interaction) and not unique to osmotic regulation of the proU promoter. In this view, the steady state level of expression of proU increases with increasing intracellular osmolality, which takes time when the medium osmolarity is suddenly raised. The time dependence of activation of ProU is also consistent with an increased intracellular osmolality as signal for increased ProU activity, which in our opinion represents a more sensible (general) type of regulation of transport than one that requires some specific intracellular signal. Despite the attractiveness of a mechanism involving the sensing of intracellular osmolality by ProU, it needs to be said that the experimental evidence for this is limited and that even for the transcriptional regulation of proU some questions about the specificity of the osmosensing device still persist (see also Rajkumari et al., 1996).

In *L. plantarum* glycine betaine, carnitine, other quaternary ammonium compounds and proline are taken up via one and the same system (QacT) that is activated upon osmotic upshock (Glaasker et al., 1996ab; unpublished results). Since the activated state lasts until the turgor is restored (Glaasker et al., 1998), e.g., via the uptake of sugars, this seems to be a clear case of turgor-regulated activity. Consistent with a turgor-controlled mechanism, the ‘activated’ state of the system lasts longer as the increase in medium osmolarity is higher. The increase in uptake rate from ‘basal’ to ‘activated’ mainly reflects an increase in maximal activity (V_max increases 5 to 10-fold), but it involves more than a single effect. It appears that the increase in V_max upon osmotic upshock is due to a diminished trans-inhibition (by proline and/or glycine betaine) as well as an effect that is independent of intracellular substrate (Glaasker et al., in preparation). The inhibition by intracellular substrate forms an additional level of control against excessive accumulation of glycine betaine, carnitine and proline. The linkage of the trans-inhibitory effect to the osmotic strength of the environment is also observed in other bacteria (next paragraph), and it may form a general strategy to tune the intracellular osmolality and maintain the cell turgor within certain limits.

In *Listeria monocytogenes* Scott A the structural analogs glycine betaine and carnitine are taken up via separate systems that respond to an osmotic upshock only when the cells have been pregrown in the presence of glycine betaine or
carnitine (Verheul et al., 1997). These systems, although highly specific for their substrates, are inhibited by both glycine betaine and carnitine at the cytoplasmic face (trans site) of the membrane. Without intracellular glycine betaine and/or carnitine the activity is maximal and not affected by medium osmolality. The inhibition by intracellular glycine betaine and carnitine is relieved upon osmotic upshock, which allows the cells to accumulate these compatible solutes further and restore turgor more rapidly. In kinetic terms, the activation of the glycine betaine and carnitine uptake systems of L. monocytogenes upon osmotic upshock is thought to reflect an increase in $K_i^{\text{App}}$ for the compatible solutes at the inner surface of the membrane. Apparently, a decrease in turgor alters the internal binding site for glycine betaine and carnitine, a phenomenon that is observed for both the ATP-driven carnitine and the ion motive force-driven betaine uptake system (Verheul et al., 1997).

6. Efflux of compatible solutes
Specific efflux systems. It has been shown for a number of microorganisms that compatible solutes are rapidly released from the cells upon a hypo-osmotic shock. For instance, in E. coli a rapid release of $K^+$, glutamate and trehalose is observed upon a osmotic downshock, whereas solutes such as alanine, lysine, arginine and sucrose are fully retained by the cells (Schleyer et al., 1993). When L. plantarum is subjected to an osmotic downshock, a rapid efflux of glycine betaine, proline and some glutamate occurs whereas the pools of other amino acids remain unaffected (Glaasker et al., 1996a). Osmoregulated efflux activity with specificity for compatible solutes has also been described in Corynebacterium glutamicum (Lambert et al., 1995; Ruffert et al., 1997). Although the molecular nature of these efflux activities is unknown, the systems exhibit properties that mimic mechanosensitive channels (Sukharev et al., 1997; see below). Some features that discriminate the systems from ‘ordinary’ secondary carrier proteins (Poolman and Konings, 1993) are the following: (i) efflux is extremely fast and effected by an osmotic downshock as well as amphipaths that insert into the membrane; (ii) efflux is independent of metabolic energy; (iii) efflux is unaffected by substrate at the trans site of the membrane; (iv) in many cases efflux is inhibited by gadolinium ions (Gd$^{3+}$), an unspecific channel blocker (Berrier et al., 1992; Glaasker et al., 1996b; Lambert et al., 1995; Ruffert et al., 1997). In the case of the glutamate excretion system from C. glutamicum, there is direct evidence that the effects of the osmotic gradients and the amphipath tetracaine are mutually compensative, i.e., the higher the medium osmolality the more tetracaine is needed to elicit efflux (Lambert et al., 1995). These and other experiments suggest that osmotic changes and
amphipaths exert a similar type of mechanical stress on the membrane (Martinac et al., 1990; Sheetz et al., 1976; Sheetz and Singer, 1974).
### TABLE I. Properties of osmoregulated transport systems in bacteria.

<table>
<thead>
<tr>
<th>System</th>
<th>Organism</th>
<th>Activation Signal</th>
<th>Parameters That Affect the Activity</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>External</td>
<td>Internal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Osmolality</td>
<td>Osmolality</td>
</tr>
<tr>
<td><strong>Osmosensor</strong></td>
<td>E.coli</td>
<td>Low Turgor (membrane strain)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KdpD</td>
<td>E.coli</td>
<td>Low Turgor</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased Ext.Osm.Press.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ProP</td>
<td>E.coli/S.typhimurium</td>
<td>Low Turgor</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ProU</td>
<td>E.coli/S.typhimurium</td>
<td>Internal Factor</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BetP</td>
<td>C.glutamicum</td>
<td>Low Turgor? (membrane strain)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td>QacT</td>
<td>L.plantarum</td>
<td>Low Turgor</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>Betaine</td>
<td>L.monocytogenes</td>
<td>Low Turgor?</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carnitine</td>
<td>L.monocytogenes</td>
<td>Low Turgor?</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Efflux</strong></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Comp.Solutes</td>
<td>E.coli/S.typhimurium</td>
<td>High Turgor (membrane strain)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Lb.plantarum</td>
<td></td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>C.glutamicum</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MscL</td>
<td>E.coli</td>
<td>High Turgor (membrane strain)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

[-], decreases the activity; [+], increases the activity; ?, uncertain; -, unknown; pH_in, internal pH; ¹not studied in all cases
The efflux of glycine betaine and proline by *L. plantarum* upon osmotic downshock is characterised by two kinetic components, i.e., the one described above with a half time \( t_{1/2} < 1 \) s and a slow one with a \( t_{1/2} \) of 4-5 min. Similar observations have been made for the efflux of glycine betaine and carnitine in *L. monocytogenes*. The component with the slow kinetics is affected by the metabolic state of the cell and may represent a specific efflux system or, alternatively, exit of compatible solutes via the same system that mediates the uptake. The glycine betaine uptake system of *L. monocytogenes* is a secondary transport protein that under the appropriate conditions (e.g., hypo-osmotic shock) may have a role in the release of compatible solutes. Secondary transport systems usually catalyze a reversible transport, of which the direction of net flux depends on the magnitudes of the ion-motive force and the solute concentration gradients (Poolman and Konings, 1993). Thus, a lowering of the ion motive force as a result of an osmotic downshock would lead to net efflux. The QacT system of *L. plantarum* and the carnitine uptake system of *L. monocytogenes* are ATP-driven, and these systems are generally thought to operate unidirectionally and not to mediate efflux. However, it is worth mentioning that in *Rhizobium leguminosarum* an ABC-type binding protein-dependent amino acid uptake system (Aap) has been described that also affects efflux (Walshaw and Poole, 1996). At present it cannot be excluded that Aap-mediated efflux is indirect, i.e., through regulation of another channel or transport system, but the experimental data favor a direct role as the system mediated uptake and efflux in the heterologous host *E. coli*.

**Mechanosensitive channels.** The one mechanosensitive channel in the cytoplasmic membrane of a bacterium that has been studied in great detail is MscL of *E. coli* (Sukharev et al., 1997). Tension in the lipid bilayer is conveyed to MscL which increases the open probability of the channel by several orders of magnitude (Sukharev et al., 1994). The pressure sensitivity of MscL is voltage dependent, i.e., the channel is more sensitive to membrane tension at more negative membrane potentials (Blount et al., 1997); the channel is blocked by Gd\(^{3+}\). Support for the idea that the mechanical gating force comes from the lipids surrounding the protein is three-fold (Martinac et al., 1990; Sukharev et al., 1997).
1994; Blount et al., 1997): (i) the purified MscL is fully functional after reconstitution into artificial lipid membranes, i.e., a bilayer is necessary and sufficient for mechanosensation; (ii) mutations within TMS1 of MscL alter the gating of the channel protein; (iii) amphipaths that partition differently into the outer and inner leaflet of the membrane also activate the channel. It has been proposed that the lipid leaflet that has the highest tension gates the channel (Markin and Martinac, 1991). In addition to MscL, E. coli has other channel activities with conductances smaller than those of MscL (MscL has a conductance of ~2.5 nS). It is possible that one or more of these channel activities correspond(s) to the observed efflux of compatible solutes upon hypo-osmotic shock as described above under ‘Specific efflux systems’. In fact, mutations within the channel protein (K31D and K31E) evoke a strongly reduced growth rate that can partially be rescued by increasing the osmolality of the medium (Blount et al., 1997). The growth phenotype of the mutants parallels an increased loss of K\(^+\), which can largely be reversed by an increase in osmolality.

7. Aquaporins
One aspect of osmoregulation in bacteria that has been poorly documented concerns the molecular pathway of water movement across the cytoplasmic membrane. Although water may move passively across the membrane, it is evident from work in mammalian cells that aquaporins are crucial for the osmotic flow of water (Chrispeels and Agre, 1994). Bacterial aquaporins are only known since the cloning and molecular characterization of aqpZ, the gene encoding the E. coli aquaporin. AqpZ is homologous to eukaryotic aquaporins and belongs to the major intrinsic protein (MIP) family which also includes the glycerol facilitator GlpF (Calamita et al., 1995), but its physiological role in osmoregulation is unknown. Important insight into the structure and functioning of aquaporins has recently been obtained with the cryo-electron microscopy structure at 0.6 nm resolution of aquaporin-1 (Walz et al., 1997).

8. Regulation of pool sizes
In contrast to the K\(^+\) uptake systems there appears to be no feedback regulation (‘trans inhibition’) of the ProP and ProU systems in enteric bacteria, and the magnitude of the glycine betaine and proline pools is controlled by separate uptake and efflux systems. In the Gram-positive bacteria L. plantarum and L. monocytogenes the major osmoregulated transport systems for compatible solutes are feedback regulated which prevents excessive uptake, but at the same time these organisms possess specific efflux/channel activity. It seems that irrespective of whether an organism can regulate the accumulation of compatible
solutes through feedback inhibition of the corresponding uptake system, there is always a need for separate efflux pathways under conditions that the turgor pressure becomes so great that the cells may break. To prevent futile cycling, the efflux systems must be highly regulated (‘shut off’) under normal osmotic and hyper-osmotic conditions, whereas they must rapidly open to release turgor upon a sudden osmotic downshock. In fact, it has recently been shown that at osmostasis \textit{L. plantarum} maintains basal flux of glycine betaine (but no net uptake or efflux) that amounts to about 10\% of the maximal rate of uptake following an upshock (Fig.2). Upon osmotic upshock the glycine betaine uptake system is rapidly activated to 100\% and the basal efflux is completely inhibited (Glaasker 	extit{et al.}, 1996b).

\textbf{9. Molecular basis for osmosensing}

So far, we have described what happens phenomenologically when a transport system experiences a change in medium osmolarity. How the environmental osmolality is sensed at the molecular level and converted into an altered activity is largely unexplored. The best-studied osmosensors are the KdpD and MscL proteins of \textit{E. coli} (Sugiura \textit{et al.}, 1994; Blount \textit{et al.}, 1996). By mutational analysis it has been shown that the transmembrane segments (TMSs) 3 and 4 (together with short flanking regions) of KdpD are critical for the sensing of potassium (Sugiura \textit{et al.}, 1994). Moreover, the sensing of K$^+$ could be separated mechanistically from medium osmolarity signals as mutants that failed to perceive the K$^+$ signal responded normally to hyper-osmotic stress. The autophosphorylation of wild-type KdpD is negatively regulated by K$^+$, whereas medium osmolarity has a positive effect. Since amphipathic compounds such as chlorpromazine and procaine effect the phosphorylation of KdpD in a similar manner as high medium osmolarity, it is thought that the transmembrane segments of the protein sense osmolarity changes through stretch forces in the cytoplasmic membrane.

The mechanosensitivity of MscL is confined to the hydrophobic core (2 TMSs) and periplasmic loop in between the TMSs (Blount \textit{et al.}, 1996). The observation that amphipathic compounds activate MscL with an effectiveness that corresponds to their lipid solubility is taken as additional evidence that the mechanical gating force comes from the surrounding lipids (membrane stretch/tension) and that the signal is transferred to the TMSs (Martinac \textit{et al.}, 1990). The isolation of the K31D and K31E mutants with increased sensitivity to mechanical stress supports this contention as Lys-31 is located near the middle of
TMS1 (Blount et al., 1997). Patch clamp studies have shown that the mutant channel proteins open more easily upon membrane tension as effected by osmotic downshifts. Finally, screening of MscL mutants with a ‘slow’ or ‘no growth’ phenotype has indicated that 14 of a total of 19 map in a region that constitutes 2/3 of TMS1. From a structural point of view the ‘osmolality-sensing’ TMSs of KdpD and those of MscL cannot be discriminated from those of any non-osmotically regulated membrane transport on the basis of primary sequence (motifs) or predicted secondary structure.

What are the putative osmosensing parts of other transport proteins that respond to changes in medium osmolarity? The known transport systems for the compatible solutes glycine betaine and proline can be subdivided in three families: 1) the binding protein-dependent ATP-driven systems that include ProU of *E. coli* and *S. typhimurium*, and OpuA and OpuC of *B. subtilis*; 2) the ion motive force-driven transporters exemplified by ProP of *E. coli* and OusA of *Erwinia chrysanthemi*; 3) the ion motive force-driven transporters exemplified by OpuD of *B. subtilis*, BetT and CaiT of *E. coli*, BetP of *C. glutamicum*, and the BetT-like protein of *Haemophilus influenzae*. The family 1 transporter ProU is activated by an osmotic upshock (Faatz et al., 1988), but the protein(s) / site(s) of osmosensing are unknown. The ProW protein, that forms the putative translocation pathway of ProU, has an unusually long periplasmic tail that is predicted to form an amphiphilic α-helix (Haardt and Bremer, 1996). This protein domain has been implicated in osmosensing by monitoring alterations in membrane tension, but as we discussed above the physiological data are most compatible with the regulation of activity through changes in intracellular osmolality which may not be sensed by the periplasmic tail. Most of the extended hydrophilic amino-terminal region is lacking in OpuAB (homolog of ProW and part of the OpuA system) (Kempf and Bremer, 1995), but, unfortunately, the osmotic activation of OpuA has not been studied. The hyper-osmotic stress-activated QacT transporter of *L. plantarum* and the carnitine transporter of *L. monocytogenes* are dependent on ATP and have all the properties of transport systems that belong to the ABC superfamily, but structural information of the components is not available. It is clear that more decisive experiments are needed to resolve the structure-osmoregulation relationships in these proteins.

The family 2 transporters ProP and OusA form part of a larger family that includes citrate and α-ketoglutarate transport systems but differ from these proteins by the presence of an extended central hydrophilic loop and a carboxyl-terminal extension that is predicted to from an α-helical coiled coil; both
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structural elements are located internally (Culham et al., 1993; Gouesbet et al., 1996). It is tempting to speculate that the α-helical coiled coil is relevant for the osmoregulation of activity (Culham et al., 1993), but, unlike ProP, there is (preliminary) evidence that OusA is not affected by the osmolarity of the medium (Gouesbet et al., 1996). Since possible activation of OusA has only been studied by comparing assay media with and without 0.5 M NaCl, the conclusion should be taken with caution.

The family 3 transporters are generally quite specific for one or a few quaternary ammonium compounds, whereas the family 1 and 2 transporters facilitate the uptake of glycine betaine, carnitine, proline, ectoine, pipecolic acid and other related compounds. Of the family 3 transporters, OpuD, BetT and BetP are activated by hyper-osmotic conditions, whereas CaiT is not (Kappes et al., 1996; Lamark et al., 1991; Peter et al., 1996; Eichler et al., 1994). Of these transporters, BetT, BetP and BetT-like of H. influenzae have a carboxyl-terminal extension reminiscent of that in ProP and OusA, whereas such a ‘domain’ is lacking in OpuD and CaiT. Again a correlation between osmoregulated activity and putative osmosensor ‘domain’ is lacking. It should be stressed, however, that osmoregulated activity can be overlooked easily as we have shown for glycine betaine uptake in L. monocytogenes (Verheul et al., 1997), where the osmotic response is dependent on the internal concentration of glycine betaine and carnitine. Clear evidence that the carboxyl-terminal extension has a role in osmosensing comes from studies of the BetP protein of C. glutamicum (Peter et al., 1998). The carboxyl-terminal extension is 55 amino acids long and has a large excess of positively charged residues. Deletions in this putative domain result in complete loss of regulation. However, deletions in the hydrophilic amino-terminal tail also affect the osmoregulation of BetP by shifting the optimum of activation from 1.3 to 2.6 osmol/kg; the amino-terminal domain is 62 amino acids long, and has a large excess of negatively charged residues. These data could be interpreted as regulation through a single osmosensing device that is build up of the two extremities of the protein. On the other hand, the amino-terminal region is not conserved in the osmoregulated OpuD and BetT proteins of the family 3 transporters, which points to a central role of the carboxyl-terminal region as the osmosensing device.

CONCLUDING REMARKS
With the exception of Trk and ProP in enteric bacteria, BetP in \textit{C. glutamicum}, and the transport systems for compatible solutes in \textit{L. plantarum} (and \textit{L. monocytogenes}), the hyper-osmotic ‘activation’ of other uptake systems is in general only poorly described, and often based on one or a few experiments in which either a salt or sugar is used to raise the medium osmolality. Nevertheless, it is clear that some osmosensing mechanism forms an inherent property of the osmoregulated transport systems and channel proteins. A more rigorous kinetic analysis of the activation mechanism in relation to structural analysis of the system components should indicate whether or not ‘added’ loops and/or tails, present in a number of osmoregulated transport proteins, or specific TMSs have a role in osmosensing. It should be stressed, however, that the osmosensing parts of the proteins may not easily be identified without the isolation of the appropriate mutants as suggested by the work on KdpD and MscL. Despite a lack of structural information, there are strong indications that the physicochemical signal that is sensed upon an osmolality change is not the same in all these systems, and that either external osmolality, turgor pressure (or a derived parameter such as membrane tension), and/or internal osmolality may be the trigger for activation. This implies that the molecular mechanism of osmosensing will vary among different transport systems, an area of research that is largely unexplored and deserves more attention than it has received thus far.

REFERENCES


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