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# Cation specificity of osmosensing by the betaine carrier BetP of *Corynebacterium glutamicum*

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**Abstract The $Na^+$/betaine carrier BetP from *Corynebacterium glutamicum* was purified and reconstituted in *Escherichia coli* phospholipid liposomes and its osmosensory properties were studied with respect to the cation specificity of osmotic activation. To dissect the influence of the co-substrate $Na^+$ on the energetics of uptake from its possible role as a putative trigger of osmolality-dependent BetP activation, the internal $Na^+$ concentration was varied without changing $\Delta pNa^+$. Studying betaine uptake at increasing luminal $Na^+$ or $K^+$ revealed that BetP activity was triggered by $Na^+$ only to a negligible extent compared to activation by $K^+$. We conclude that activation of BetP in proteoliposomes depends solely on $K^+$, both in mechanistic and in physiological terms.**



*Key words:* Ion specificity; Osmosensor; Osmoregulator; Potassium; Sodium; *Corynebacterium glutamicum*

## 1. Introduction

A widely distributed strategy of eubacteria to overcome hyperosmotic stress is the accumulation of compatible solutes, by biosynthesis and/or uptake, thereby avoiding dehydration of the cytoplasm [1–3]. Since uptake of compatible solutes is faster and energetically cheaper than de novo synthesis, normally a fast activation of uptake systems for compatible solutes occurs under hyperosmotic conditions [4].

*Corynebacterium glutamicum* possesses four carriers for the uptake of compatible solutes, which are osmoregulated at the activity level ([4–6]; unpublished results). The $Na^+$-dependent betaine uptake system BetP is the best-studied carrier involved in osmoadaptation of *C. glutamicum* [6–8]. Functional reconstitution of purified BetP demonstrated that this transporter harbors three different functions: (i) catalytic activity of betaine transport, (ii) sensing of osmotic stress, and (iii) osmoregulation, i.e. adjustment of the transport rate to the extent of stress [8].

Two other uptake systems for compatible solutes have also been shown to possess osmosensory and osmoregulatory functions, namely ProP from *Escherichia coli* [9] and OpuA from *Lactococcus lactis* [10]. The underlying mechanisms as to how osmotic stress is actually sensed are a matter of current research [3]. In all three cases changes of luminal conditions of the proteoliposomes seem to be used as a measure for hyperosmosis. OpuA and ProP appear to be activated by rather unspecific triggers, namely changes in the ionic interaction between the carrier and the charged phospholipids of the membrane induced by increasing luminal ion concentrations in the former and an increase of the internal solute concentration (i.e. ions or cytoplasmic macromolecules) in the latter case [11,12]. In contrast, activation of BetP seems to be characterized by a higher ion specificity. We showed that BetP is triggered by an increase of the internal $K^+$ (and $Rb^+$ or $Cs^+$) concentration as a measure of hypertonicity, but not by other cations, such as ammonium or choline [13]. Unfortunately, $Na^+$ could not be ruled out as a stimulating cation due to the fact that $Na^+$ is not only a potential activator of BetP, but also a co-substrate for betaine uptake.

It does not seem very likely that a change in the cytoplasmic $Na^+$ concentration is an important stimulus in response to osmotic stress in physiological terms, since the concentration of cytoplasmic $Na^+$ is in general much lower than that of $K^+$. In mechanistic terms, however, i.e. for a correct understanding of osmolality-dependent carrier activation at the molecular level, it is important to define the ion specificity of activation to obtain more information about possible binding processes involved.

Since $Na^+$ is the co-substrate and the electrochemical $Na^+$ potential is the driving force for betaine uptake, an assay was developed in this study which made it possible to dissect the influence of $Na^+$ on the energetics of BetP from its putative function as an osmotic trigger. Using this assay, we were able to show that the activation power of $Na^+$ is low in comparison to $K^+$, the major stimulus of BetP in response to osmotic stress.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids and growth conditions

*E. coli* DH5αmcr [14] was used for *strepbetP* expression. The plasmid used was pAcl1 [13] in which *strepbetP* is under the control of the *tet* promoter. This plasmid encodes a Cys-less version of BetP, which shows identical properties to the wild type protein (unpublished results). *E. coli* cells were grown at 37°C in LB medium supplemented with carbenicillin (50 μg/ml).

### 2.2. Purification, reconstitution and transport assays of BetP

Purification of Strep-BetP, reconstitution into *E. coli* phospholipids and transport measurements were carried out as described previously [8]. Briefly, for the exchange of the internal buffer (100 mM $KP_i$, pH 7.5), 15–30 μl preformed proteoliposomes were suspended in 500 μl buffer with varying $KP_i$ concentrations ranging from 19 to 183 mM. In addition, the buffer contained different NaCl concentrations (up to 200 mM). This suspension was extruded 13 times through a polycar-

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bonate filter (400 nm pore size) to exchange the internal buffer [13]. Subsequently, proteoliposomes were collected by centrifugation and suspended in the same buffer which was used for the exchange of the internal buffer (mentioned above) to a concentration of approx. 60 mg lipid/ml. A volume of 2.5 μl of proteoliposomes was diluted 200-fold into potassium-free buffer (20 mM $NaP_i$, pH 7.5 and varying NaCl concentrations) containing 15 μM [$^{14}$C]betaine and 1 μM valinomycin to create an outwardly directed $K^+$ diffusion potential. Either to establish hyperosmotic conditions or to balance the internal osmolality, sorbitol was added to the external buffer. After several time intervals samples were taken and filtered rapidly through 0.45 μm nitrocellulose filters (GS, Millipore, Eschborn, Germany). The filters were washed with 100 mM LiCl and the radioactivity was determined. Under conditions where the $Na^+$ concentrations in the transport assay were far below the $K_M$ of the co-substrate $Na^+$, $V_{max}$ values were calculated according to the Michaelis–Menten equation.

## 3. Results and discussion

In this study, we used a Cys-less version of BetP, in which cysteine at position 252 was changed to threonine, in order to be able to introduce cysteines at strategic places in future experiments. The two variants differ in the $V_{max}$ values by about 50% (Cys-less version higher), but both are identical in terms of the $K_M$ for sodium and betaine and their regulatory properties (identical activation by osmotic stress). Therefore, the mutant BetP C252T is well suited for the characterization of BetP (unpublished results).

To analyze the influence of internal $Na^+$ as a potential trigger for the activation of BetP, we first applied conditions of a constant chemical $Na^+$ potential. For that purpose internal NaCl was varied between 1 and 15 mM in proteoliposomes containing three different internal $KP_i$ concentrations (Fig. 1). At the start of the transport measurement, the proteoliposomes were diluted into a buffer with a 2.7-fold higher osmolality, which led to liposome shrinkage by a factor of 2.7 and consequently to an increase in the concentrations of the internal solutes. Since the $Na^+$ concentration of the external buffer was varied between 10 and 150 mM, an identical 3.7-fold $Na^+$ gradient and therefore an identical chemical $Na^+$ potential was present in each individual proteoliposomal preparation. The dilution ratios of $K^+$-loaded proteoliposomes into $K^+$-free buffer and thus the $K^+$ diffusion potential were kept constant for all measurements. As shown in Fig. 1A,B,

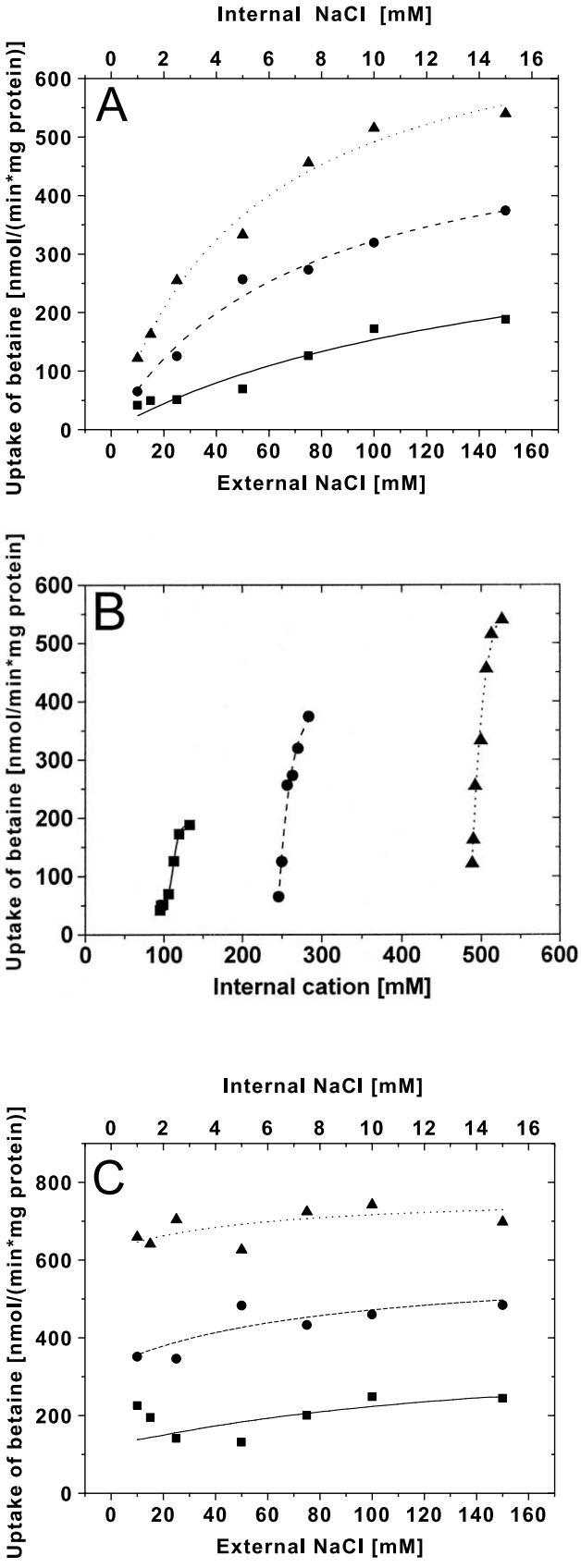


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Fig. 1. Stimulation of BetP by internal $Na^+$ and $K^+$. A: BetP activity at different sodium concentrations in proteoliposomes preloaded with different $KP_i$ buffers. The internal NaCl concentration, which varied between 1 and 15 mM (pre-shock values), was 10 times lower than the external concentration. Proteoliposomes were preloaded either with 19 (squares), 50 (circles) or 100 mM $KP_i$ buffer (triangles). At the start of the uptake measurement, proteoliposomes were exposed to an osmotic upshift leading to a 2.7-fold shrinkage and thus to a 2.7-fold increase of internal solute concentrations. In detail this means that the external osmolality was varied either between 140 and 216 mosmol/kg, 297 and 378 mosmol/kg or 599 and 675 mosmol/kg, respectively. The data points were fitted according to Michaelis–Menten (dotted, dashed and solid line, respectively). B: Same data as shown in A, but plotted against the internal cation concentrations (post-shock concentrations), which represent the sum of $K^+$ and $Na^+$ ions. C: $V_{max}$ of betaine uptake was calculated using the data and the fitted curves shown in A as well as the apparent $K_M$ for $Na^+$ of 44 ± 2 mM. The absolute values for $V_{max}$ were calculated according to the Michaelis–Menten equation for the three different conditions (pre-shock $KP_i$ concentrations of 19, 50 and 100 mM).

BetP activity increased with increasing internal concentrations of both $Na^+$ and $K^+$.

Whereas stimulation by internal $K^+$ (and $Rb^+$ or $Cs^+$) as the main trigger for osmolality-dependent activation of BetP was previously described [13], activation by $Na^+$ was unexpected. The external buffer contained $Na^+$ concentrations between 10 and 150 mM which were around or below the $K_M$ for $Na^+$ as co-substrate. Consequently, the observed stimulation is a combination of the increase in activity due to increasing saturation by external $Na^+$ as a substrate and the putative direct BetP activation by internal $Na^+$. The former contribution can be quantified if the exact $K_M$ for $Na^+$ is known. It turned out that this value depended to some extent on the phospholipid preparations used which can be rationalized by the fact that the share of negatively charged phospholipids may vary in the *E. coli* polar lipid extract used. In a series of eight experiments using different lipid preparations we determined the affinity for sodium in liposomes preloaded with 100 mM $KP_i$, pH 7.5 which were diluted into a buffer at a fixed external osmolality of 600 mosmol/kg containing 20 mM Tris/MES (pH 7.5) and varying $Na^+$ (2.5–150 mM) and sorbitol concentrations. We measured a sodium $K_M$ of 44 ± 2 mM. This value is a factor of 3 higher than the previously published $K_M$ [8]. The former value was determined in one single lot of *E. coli* phospholipids, thus the newly determined $K_M$ for sodium is more reliable since it is based on several determinations in which different phospholipid preparations were used. When calculating maximum rates of betaine transport, an additional source of potential misinterpretation has to be considered, namely the possibility that the $K_M$ for $Na^+$ may depend on the osmolality applied in different experiments. An eightfold increase in the substrate affinity for proline in dependence on the extent of the osmotic shifts has in fact been shown for the compatible solute carrier ProP of *E. coli* [15]. We found, however, that the $K_M$ of BetP for $Na^+$ varied at most by a factor of two, between 35 ± 9 mM and 84 ± 14 mM, within the range of osmolality used in these experiments. Furthermore, the $K_M$ did not change systematically in dependence on the extent of osmotic stress applied (results not shown). Therefore, the osmostress-dependent activity regulation of BetP from *C. glutamicum* and ProP from *E. coli* differs in this important mechanistic aspect.

Based on the $K_M$ of BetP for $Na^+$, the $V_{max}$ values of betaine transport for infinite external $Na^+$ concentrations for each experiment shown in Fig. 1A were calculated, revealing the limited potential of $Na^+$ to trigger BetP activity (Fig. 1C). Only a marginal activating influence of internal $Na^+$ was detectable if liposomes preloaded with 100 mM $KP_i$ (corre-

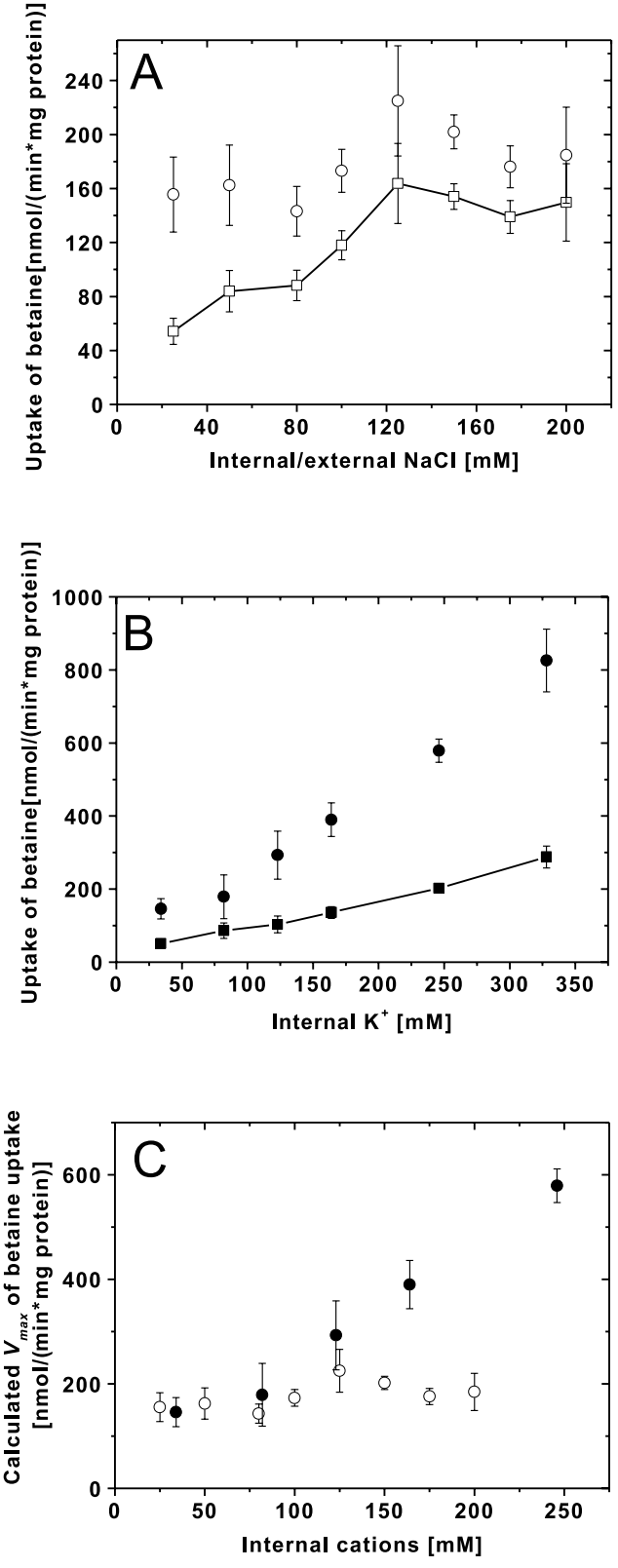


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Fig. 2. Activity regulation of reconstituted BetP C252T at increasing $Na^+$ and $K^+$ concentrations. Betaine uptake was determined under iso-osmotic conditions and in the absence of a $Na^+$ gradient. A: Effect of increasing $Na^+$ concentrations on betaine uptake. The internal buffer contained 19 mM $KP_i$, pH 7.5, and 25, 50, 80, 100, 125, 150, 175 or 200 mM NaCl. The external buffer contained 50 mM Tris/MES, pH 7.5, and 25, 50, 80, 100, 125, 150, 175 or 200 mM NaCl. The directly measured rates (open squares) were extrapolated to $V_{max}$ values for infinite $Na^+$ concentration using a $Na^+$ $K_M$ of 44 ± 2 mM (open circles). B: Effect of increasing $K^+$ concentrations on betaine uptake. The internal buffer contained 25 mM NaCl and 19, 45.6, 68.4, 91.2, 136.8, or 182.4 mM $KP_i$, pH 7.5, corresponding to internal $K^+$ concentrations of 34, 82, 123, 164, 246, and 328 mM. The external buffer basically contained 50 mM Tris/MES, pH 7.5, 25 mM NaCl (100 mosmol/kg). Iso-osmolality between the internal and external space was adjusted by addition of sorbitol to the external buffer. Direct measurement (solid squares) and extrapolated values (solid circles) are shown. C: Comparison of the effect of $Na^+$ (open circles) and $K^+$ (closed circles) on betaine uptake on the basis of $V_{max}$ values of transport extrapolated for infinite $Na^+$ concentrations.

sponding to a $K^+$ concentration of 490 mM after the osmotic upshift) were used. Obviously, BetP function at or near $V_{max}$ conditions due to the high internal $K^+$ concentrations is only restricted by the limited availability of external $Na^+$ as a co-substrate (cf. Fig. 1B,C). In proteoliposomes prepared at an internal concentration of 50 or 19 mM $KP_i$ (corresponding to a post-shock $K^+$ concentration of 240 or 90 mM $K^+$), stimulation of BetP by $Na^+$ was stronger, but never exceeded a factor of two even at the lowest $K^+$ concentrations tested which led under standard conditions only to a low preactivation of BetP [13]. In the same publication we showed furthermore that starting from an internal $K^+$ concentration of 90 mM the activity of BetP can be increased 8–12-fold by increasing internal $K^+$ concentrations (as shown in figs. 4 and 5 of [13]). Thus, in the experiment shown in Fig. 1 the preactivation of BetP at an internal $K^+$ concentration of 90 mM was low enough to detect an additional activation by $Na^+$. The $V_{max}$ correction, however, shows (Fig. 1C) that even at low internal potassium concentrations $Na^+$ has a very limited ability to increase BetP activity.

In order to exactly quantify the extent of $Na^+$-dependent activation, we applied conditions of low internal $K^+$ concentrations at simplified experimental conditions to reduce possible influences of other parameters. For this purpose, we used a proteoliposomal system with an internal buffer containing only 19 mM $KP_i$ and worked under iso-osmotic conditions in the absence of any $Na^+$ gradient. Consequently, betaine uptake into proteoliposomes was only driven by the $K^+$ diffusion potential of $\Delta\Psi = -136$ mV, and $K^+$ was far below a stimulating level. As described above, the activating effect of $Na^+$ was separated from its influence as a co-substrate by extrapolation to $V_{max}$ conditions (Fig. 2A). The $V_{max}$ values revealed a low activation of betaine uptake as a consequence of the increase in internal $Na^+$ from 25 to 200 mM. As a comparison, stimulation in dependence on internal $K^+$ is shown in an identical type of experiment (Fig. 2B). The absolute transport rates were lower than those observed in the presence of both a chemical $Na^+$ potential and a membrane potential (cf. Fig. 1), nevertheless, stimulation by $K^+$ was much stronger. The $V_{max}$ values for transport in dependence on the internal cation concentration ($Na^+$ and $K^+$) for infinite $Na^+$ concentration are combined in Fig. 2C. Whereas the 1.4-fold stimulation observed for $Na^+$ already leveled off around 120 mM, activation by internal $K^+$ was fivefold in a concentration-dependent manner, indicating that BetP is indeed able to discriminate between the two cations. Recently, it was shown in vitro that the membrane-anchored histidine kinases KdpD and EnvZ from *E. coli* can also distinguish between internal cations in the activation process [16,17]. Using right-side-out membrane vesicles it was demonstrated that luminal $K^+$ ions either inactivate in the former or activate in the latter case, whereas ions other than $K^+$ had an opposite effect.

Taken together, arguments against cytoplasmic $Na^+$ being a relevant stimulus for BetP are twofold. By functional analysis we have quantitatively discriminated the stimulation by $K^+$ and $Na^+$ of BetP-mediated betaine uptake. In a proteoliposomal system consisting of purified BetP reconstituted in *E. coli* lipids the extent of stimulation by $Na^+$ was small, and only visible in the presence of low $K^+$ concentrations. Besides these arguments in mechanistic terms, physiological considerations are equally valid. The cytoplasmic $K^+$ concentration is in general far above the level where any $Na^+$-dependent effect becomes obscured [18]; moreover, it is unlikely that *C. glutamicum* cells ever reach internal $Na^+$ concentrations high enough to cause stimulation of BetP [5].

This study reinforces the concept of $K^+$ (or $Rb^+$ and $Cs^+$) being a specific stimulus for BetP activation, and thus argues for the fact that BetP is primarily a $K^+$ sensor and not an osmosensor. Furthermore, the effective discrimination between different cations, like ammonium, choline [13] or $Na^+$, indicates the presence of a $K^+$ (or $Rb^+$ or $Cs^+$) binding site, which seem to be linked to the C-terminal domain of BetP [7]. However, in proteoliposomes half-maximal activation of BetP was found at an internal $K^+$ concentration of 220 mM [13], which appears to be rather high for a specific binding site. On the other hand, this value may reflect the fact that in *C. glutamicum*, as in many other bacteria, high internal $K^+$ concentrations are in general present [18,19] which further increase under hyperosmotic conditions due to the loss of water and the active uptake of $K^+$ [18]. It thus seems to be reasonable that potassium triggers BetP activity in a concentration range which was determined in the *E. coli* proteoliposome system. Consequently, the presence of a regulatory $K^+$ binding site in BetP must be postulated. Since the sequence of BetP does not include a motif known to be involved in $K^+$ binding/recognition, it remains to be elucidated how $K^+$ binding specificity is mediated. Possible scenarios include (i) sensing by a single BetP protein, (ii) $K^+$ sensing by oligomers of BetP, or (iii) involvement of the membrane together with BetP in creating $K^+$ specificity. ProP of *E. coli* and OpuA from *L. lactis* [9,10], which also fulfill the criteria of being an osmosensor and osmoregulator, seem to rely on a different kind of triggering mechanism. Although changes of internal conditions, solute (or macromolecule) concentration in the former case and changes in the protein/membrane interaction induced by increased ion concentrations in the latter case, have also been identified as a measure for hypertonicity, no ion specificity could be detected [11,12]. This indicates that osmosensing by ProP and OpuA is rather unspecific, and thus different from that of BetP, which seems to sense the increase of the internal $K^+$ concentration. It is thus interesting to note that all examples of bacterial osmoregulated uptake systems investigated so far in detail actually perceive changes in internal solute concentrations; the mechanisms of signal perception, however, seem to be different.

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