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Osmoregulated ABC-transport system of *Lactococcus lactis* senses water stress via changes in the physical state of the membrane

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An osmoregulated ABC transporter (OpuA) with novel structural features has been identified that responds to water stress. This glycine betaine transport system consists of an ATP-binding/hydrolyzing subunit (OpuAA) and a protein (OpuABC) that contains both the translocator and the substrate-binding domain. The components of OpuA have been overexpressed, purified, and functionally incorporated into liposomes with an ATP-regenerating system in the vesicle lumen. A transmembrane osmotic gradient (outside hyperosmotic relative to the inside) of both ionic and nonionic compounds was able to osmotically activate OpuA in the proteoliposomal system. Hypoosmotic medium conditions inhibited the basal activity of the system. The data show that OpuAA and OpuABC are sufficient for osmoregulated transport, indicating that OpuA can act both as osmosensor and osmoregulator. Strikingly, OpuA could also be activated by low concentrations of cationic and anionic amphipaths, which interact with the membrane. This result indicates that activation by a transmembrane osmotic gradient is mediated by changes in membrane properties/protein-lipid interactions.

In their natural habitats, microorganisms are often exposed to changes in the concentrations of the solutes in their environment, whereas the internal concentrations of nutrients need to be relatively constant. A sudden increase in the osmolality of the environment results in the movement of water from the cell to the environment, which causes loss of turgor pressure, changes in intracellular solute concentrations, and cell volume changes. Such hyperosmotic conditions are detrimental to any living cell. Osmotic stress can also involve hypoosmotic conditions, which may cause cell lysis. Bacteria counteract hyperosmotic stress by accumulating compatible solutes by uptake and/or synthesis. This group of solutes includes a whole range of compounds, including amino acids (and analogs), polyols, sugars, quaternary ammonium compounds, and potassium, and these compounds can be accumulated to high intracellular concentrations without affecting vital cellular processes. On hypoosmotic stress conditions, these compatible solutes are released from the cell. Among the most potent compatible solutes are the quaternary ammonium compounds glycine betaine and carnitine, which are preferentially used by most prokaryotes and eukaryotes (1). The osmoregulated transporter for quaternary compounds (OpuA) in *L. lactis* constitutes the main system to protect the organism against hyperosmotic stress (2, 3), and equivalent activities have been described for other organisms (1, 4).

A major question in the field of osmoregulation concerns the mechanism by which the cell senses osmotic stress and transduces the signal(s) to the osmoregulators. The physicochemical parameters that (could) change on a change in medium osmolality and thus participate in the sensing mechanism include: external water activity, turgor pressure, membrane strain, internal hydrostatic pressure, internal water activity, cytoplasmic volume, or concentration of specific signal molecule(s) (1, 4, 5). The present study eliminates most of these physicochemical factors as a signal that is sensed by the glycine betaine transporter of *L. lactis*. In fact, this work indicates that

changes in the transmembrane osmotic gradient are transmitted to the OpuA system via distortions in the membrane bilayer, which is consistent with a role for membrane strain (or curvature stress) in the activation mechanism. To address questions pertinent to osmosensing and osmoregulation, the genes encoding the system were cloned and overexpressed, and the proteins were purified and functionally reconstituted into liposomes in which an ATP-regenerating system was entrapped.

Experimental Procedures

Bacterial Strains, Growth Conditions, and Isolation of Membrane Vesicles. *L. lactis* strain NZ9000 (6) was cultivated semianaerobically at 30°C in M17 broth, pH 6.5, supplemented with 1.0% (wt/vol) glucose (GM17 medium) and 5 µg/ml chloramphenicol when carrying pNZopuAhis or derivatives. For isolation of membranes, cells were grown in a 10-liter pH-regulated fermentor to an OD₆₆₀ of 2, after which transcription from the *nisA* promoter was switched on by addition of 0.2% (vol/vol) culture supernatant of the nisin A-producing strain NZ9700 (5). The final concentration of nisin A was about 2 ng/ml. The cells were harvested after 1 h of induction, and inside-out membrane vesicles were prepared by lysing the bacteria (20 mg/ml) with a French pressure cell (single passage at 10,000 psi), after (partial) digestion of the cell wall with 10 mg/ml lysozyme for 30 min at 30°C (7). The membrane preparations were stored in liquid nitrogen.

Plasmid Construction. The cloning and sequencing of the *opuA* genes of *L. lactis* IL1403 will be described elsewhere (GenBank accession no. AF234619). For the overexpression of *opuAA* and/or *opuABC*, genes were placed under the control of the *nisA* promoter of *L. lactis* (6). Genes were amplified by PCR by using oligonucleotides

AAfor: 5'-GGGCATGCCATGGCAGTAAAAATAAA-AATTG-3'; AArev: 5'-GGGCGCGGATCCTTTATTCTC-CTCCTC-3'; ABCfor: 5'-GGGCATGCCATGGTTGATT-TAGCTATTGGAC-3', and ABCrev: 5'-GGGCGGGGATC-CTTTAAACCATTTATC-3'.

AAfor plus AArev and ABCfor plus ABCrev were used to amplify *opuAA* and *opuABC*, respectively, whereas AAfor plus ABCrev were used to amplify the *opuA* operon. In each case, a *NcoI* restriction site was engineered at the start of the (first) gene and a *BamHI* restriction site at the end of the (last) gene. After digestion with the corresponding enzymes, the genes were inserted into the *NcoI/BamHI* site of pNZ8048oppAChis (8),

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF234619).

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thereby replacing the native *oppA* gene; the resulting vectors were named pNZ_{oppA}His, pNZ_{oppA}ABHis, and pNZ_{oppA}His. Except for *oppA* in pNZ_{oppA}His, the cloning strategy placed the genes in frame with a sequence specifying a 6-histidine tag at the C terminus of the protein. To engineer a his-tag coding sequence to *oppA*, pNZ_{oppA}His was digested with *NcoI/XbaI*, and the DNA fragment was ligated into the corresponding sites of pNZ_{mleP} (M. Bandell, personal communication). This now placed the *oppA*, the first gene of the *oppA* operon, in frame with a sequence specifying a N-terminal 10-histidine tag. The resulting vector, pNZ_{oppA}2His, is otherwise identical to pNZ_{oppA}His and fuses the *oppA* genes translationally to the *nisA* promoter. Finally, the TTG translation initiation codon in *oppA* was changed into ATG in each of the constructs harboring this gene.

Purification of OpuA. Membranes were resuspended in buffer A (50 mM KP_i, pH 8.0/200 mM KCl/20% glycerol) to a final concentration of 5 mg protein/ml and solubilized with 0.5% *n*-dodecyl β -D-maltoside for 30 min on ice. After centrifugation, the solubilized material was incubated with Ni²⁺-NTA resin (0.5 ml of resin/10 mg of membrane protein) for 1 h at 4°C in the presence of 10 mM imidazole. Subsequently, the resin was washed with 20 column volumes of buffer A supplemented with 0.05% Triton X-100 and 15 mM imidazole. The his-tagged proteins were eluted from the column with 3 column volumes of buffer A supplemented with 0.05% Triton X-100 and 200 mM imidazole.

Immunoblotting. Immunodetection was accomplished by using monoclonal antibodies raised against the 6-histidine tag (Dianova, Hamburg). The proteins were separated by SDS/PAGE (12% polyacrylamide) and transferred to polyvinylidene difluoride membranes by semidry electrophoretic blotting. Detection, by using the Western Light chemiluminescence detection kit with CSPD as a substrate, was performed as recommended by the manufacturer (Tropix, Bedford, MA).

Membrane Reconstitution of OpuA. Liposomes composed of egg phosphatidylcholine and *Escherichia coli* lipids in a 1:3 ratio were prepared, and membrane reconstitution was performed, essentially as described by Knol *et al.* (9). Briefly, preformed liposomes (4 mg/ml) were destabilized by titration with Triton X-100, and the turbidity of the suspension at 540 nm was used to monitor the physical state of the liposomes. Unless stated otherwise, liposomes destabilized just beyond the point of detergent saturation (10) were mixed with the purified OpuA complex in a 100:1 ratio (wt/wt) and incubated for 15 min at 4°C under gentle agitation. To remove the detergent, polystyrene beads (Biobeads SM2; Bio-Rad) were added at a wet weight of 40 mg/ml, and the sample was incubated for another 15 min. Fresh Biobeads SM2 (40 mg/ml) were added to the sample three times and the incubations were continued at 4°C for 30 min, overnight, and 2 h, respectively. Finally, the proteoliposomes were collected by centrifugation, washed twice with 50 mM KP_i, pH 7.0, and stored in liquid nitrogen.

ATP-Energized Uptake in Proteoliposomes. An ATP-regenerating system, consisting of creatine kinase (2.4 mg/ml), ATP (6 mM), MgSO₄ (9 mM), and creatine phosphate (24 mM), was enclosed in the proteoliposomes by two freeze/thaw cycles. After extrusion of the proteoliposomes through a polycarbonate filter (400 nm pore size), the proteoliposomes were washed twice and resuspended in 90 mM KP_i (isoosmolar with the intraliposomal medium), pH 7.0, to a concentration of 80 mg lipid/ml. Before transport, the proteoliposomes were diluted to a lipid concentration of 3.6 mg/ml in 90 mM potassium phosphate, pH 7.0, containing 3 mM MgSO₄ (total volume of 220 μ l). To impose hyperosmotic conditions, additional salt or sugar was added to the medium. Proteoliposomes

were preincubated at 30°C for 2 min, after which transport was initiated by the addition of radiolabeled substrate. At given time intervals, 40- μ l samples were taken and diluted with 2 ml of ice-cold buffer of the same composition and osmolality as the assay medium, except that [¹⁴C]/[³H]glycine betaine was omitted. The samples were filtered rapidly through 0.45 μ m pore-size cellulose nitrate filters (Schleicher & Schuell) and washed once more with 2 ml stop buffer. The radioactivity on the filters was determined by liquid scintillation spectrometry.

Materials. M17 broth was obtained from Difco. Ni²⁺-NTA resin was obtained from Qiagen (Chatsworth, CA), Biobeads SM-2 from Bio-Rad, *n*-dodecyl- β -D-maltoside from Sigma, and Triton-X-100 from Boehringer Mannheim. Total *E. coli* lipid extracts and L- α -phosphatidylcholine from egg yolk were obtained from Avanti Polar Lipids. Radiolabeled [*N*-methyl-¹⁴C] and [*N*-methyl-³H] choline-chloride (55 mCi/mmol and 80 Ci/mmol, respectively) were obtained from Amersham, and these precursors were used to synthesize [*N*-methyl-¹⁴C]glycine betaine and [*N*-methyl-³H]glycine betaine as described (11). Creatine kinase, creatine phosphate, tetracaine, chlorpromazine, and dipyrindole were obtained from Sigma. All other chemicals were of reagent grade and obtained from commercial sources.

Miscellaneous. The osmolalities of media and buffers were measured by freezing point depression with an Osmostat 030 (Gonotec, Berlin). Protein concentration was determined by the method of Lowry *et al.* (12) by using BSA as a standard.

Results

ABC Transporter with Alternative Architecture. The deduced amino acid sequences of OpuAA (408 amino acids) and OpuABC (573 amino acids), comprising the OpuA system of *L. lactis* IL1403, revealed that the proteins are homologous to OpuAA, OpuAB, and OpuAC of *Bacillus subtilis*, but that the domain organization is entirely different. In contrast to the *B. subtilis* system (13), the predicted ligand-binding protein is fused to the C-terminal end of the translocator protein, and the two halves of the binding domain are reversed (3). Importantly, the strong sequence conservation between the binding protein(s) (domains) of *L. lactis* and *B. subtilis* suggests that the receptor domain is directly linked to the last transmembrane segment of the translocator domain. The translocator is predicted to have seven transmembrane segments, with the N terminus at the cytoplasmic face of the membrane and the C-terminal substrate-binding domain located at the extracellular side. There are no indications for an interdomain flexible linker region, which is often observed in membrane proteins composed of two or more functional domains (14).

Overexpression, Purification, and Membrane Reconstitution of OpuA. By using the nisin inducible expression system (6), both subunits of the OpuA system were amplified up to at least 10% of total membrane protein. The subunits could be overexpressed separately or in tandem, and crossreaction with a monoclonal antibody raised against the his-tag confirmed the identity of the components (Fig. 1). The his-tags enabled a one-step purification of OpuAA (45.8 kDa) and OpuABC (62.0 kDa) by using Ni²⁺-NTA affinity chromatography. Both proteins could also be purified as complex (OpuA), provided glycerol was present at a concentration of 20% (vol/vol) (Fig. 1A; lane 4). The low intensity of the OpuAA band as compared with OpuABC (Fig. 1B; lane 1) is because of a lower blotting efficiency and a reduced sensitivity of the antibody for the 10-histidine tag. Purified OpuA was homogeneous, comprised of equal amounts of OpuAA and OpuABC, and had a purity of more than 95%. Although *n*-dodecyl β -D-maltoside was most efficient for solubilization, the purified OpuA complex was reconstituted into Triton X-100-destabilized preformed liposomes, as this gave the highest transport activities of OpuA (data not shown). To drive

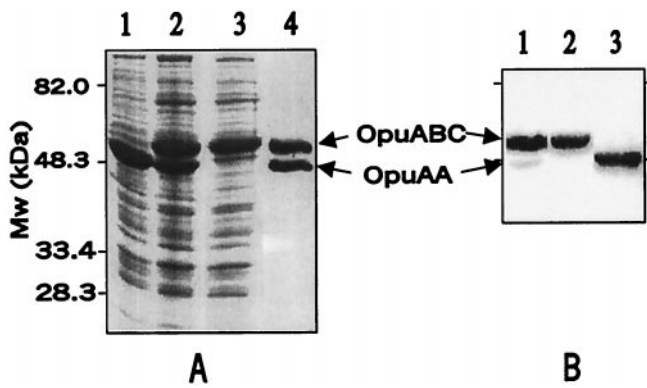


Fig. 1. Amplified expression and purification of OpuA. (A) SDS/PAGE gel (12% polyacrylamide) showing membranes (Coomassie brilliant blue stained) containing OpuAA (lane 1; 120 μ g of protein), OpuA (lane 2; 120 μ g of protein), OpuABC (lane 3; 120 μ g of protein), and purified OpuA (silver stained) (lane 4; 15 μ g of protein). (B) Immunoblot of membranes with overexpressed OpuA (lane 1; 120 μ g protein), OpuABC (lane 2; 120 μ g protein), and OpuAA (lane 3; 250 μ g protein).

the uptake of glycine betaine by OpuA, an ATP-regenerating system, consisting of creatine phosphate, creatine kinase, and ATP, was entrapped in the proteoliposomes. This system ensures that the ATP levels remain high and constant, whereas ADP (inhibitor of most ABC-type of transporters) is not accumulated in time. The established uptake rates of glycine betaine were excellent and highly reproducible.

Osmotic Activation of OpuA. To address the question whether OpuA can act as osmosensor and/or osmoregulator, the system was subjected to hyperosmotic conditions by raising the medium osmolality. A transmembrane osmotic gradient of either ionic (salts) or nonionic compounds (sugars) was able to activate OpuA in the

proteoliposomal system (Fig. 2A). From these experiments, we conclude that, besides OpuAA and OpuABC and the lipid membrane, no other cellular components are needed for the functionality, including osmotic activation, of OpuA. The lipophilic compound glycerol, used at comparable osmolality as the other cosolvents, did not activate the system, apparently because it equilibrated across the lipid membrane within the 2-min preincubation period. Importantly, a transmembrane gradient of KCl fully activated OpuA in the presence of glycerol (Fig. 2A). This shows that the OpuA system senses not external or internal osmolality but rather the transmembrane osmotic gradient.

The kinetic parameters for OpuA-mediated glycine betaine uptake in the proteoliposomal system were determined under iso- and hyperosmotic conditions (Fig. 2B). The apparent maximal rate of glycine betaine uptake (V_{max}) increased from 22.5 ± 0.6 to 108.5 ± 5.0 nmol/min \times (mg of protein) when the outside medium was made hyperosmotic. The affinity constant (K_m) for glycine betaine was not affected (1.5 ± 0.3 μ M). If ionic and nonionic compounds activate OpuA via the same mechanisms, then the threshold osmolality value of the transmembrane osmotic gradient for KCl and sucrose should be similar. Indeed, Fig. 3 shows that KCl and sucrose activate OpuA at equivalent osmolality. The course of the curve is sigmoid, and the activation threshold for the OpuA system is 20 mosmol/kg with a maximal activation reached at 270 mosmol/kg. This low threshold osmolality reflects a high sensitivity of OpuA toward hyperosmotic stress. When the outside medium was made hypoosmotic relative to the inside, the rate of glycine betaine uptake decreased relative to isoosmotic conditions (data not shown). This shows that the basal activity observed under isoosmotic conditions represents a state (conformation) of the protein that is between the inactive and the maximally active one.

Effect of Amphipathic Molecules on OpuA Activity. To determine whether the OpuA system senses the transmembrane osmotic gradient via changes in the physical properties of the phospholipid

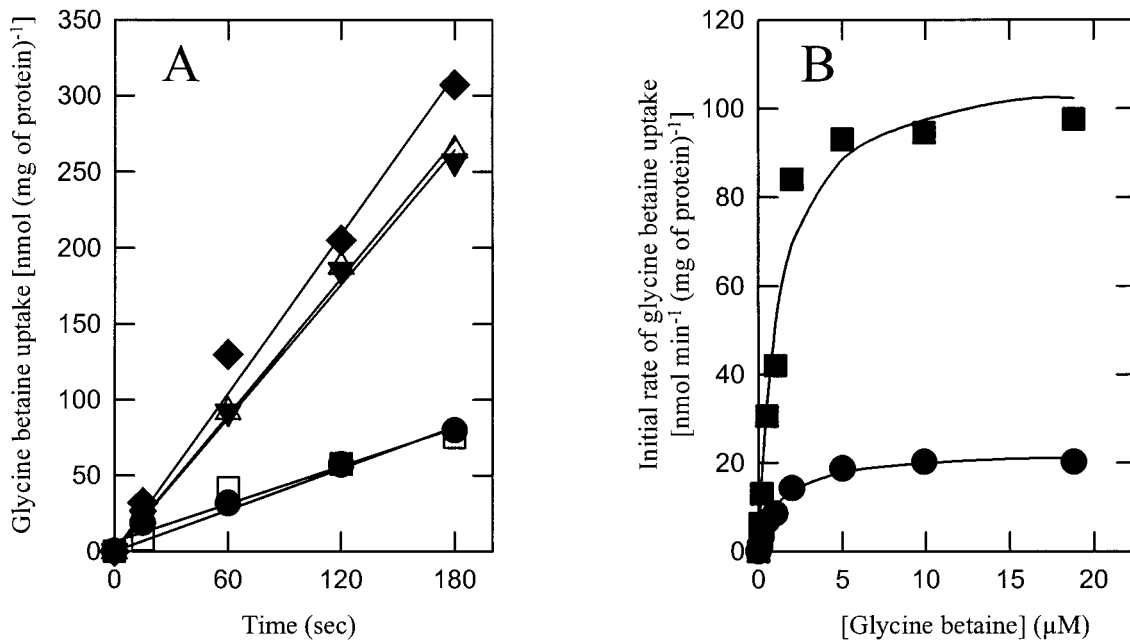


Fig. 2. Osmotic activation of membrane reconstituted OpuA. (A) Uptake of [14 C]glycine betaine (final concentration of 170 μ M) was assayed in 90 mM KP_i , pH 7.0, under isoosmotic (\bullet) and hyperosmotic conditions; the latter were effected by the addition of 200 mM KCl (∇), 10.5% sucrose (\blacklozenge), 2.5% glycerol (\square), or 2.5% glycerol + 200 mM KCl (Δ). The hyperosmotic conditions correspond to 530 mosmol/kg for each of the additives. (B) Uptake of [14 C]glycine betaine was assayed in 90 mM KP_i , pH 7.0, with (\blacksquare) or without (\bullet) 200 mM KCl. The uptake was stopped after 15 seconds, and further handling was as described under *Experimental Procedures*. The curves were fitted with the Michaelis–Menten equation.

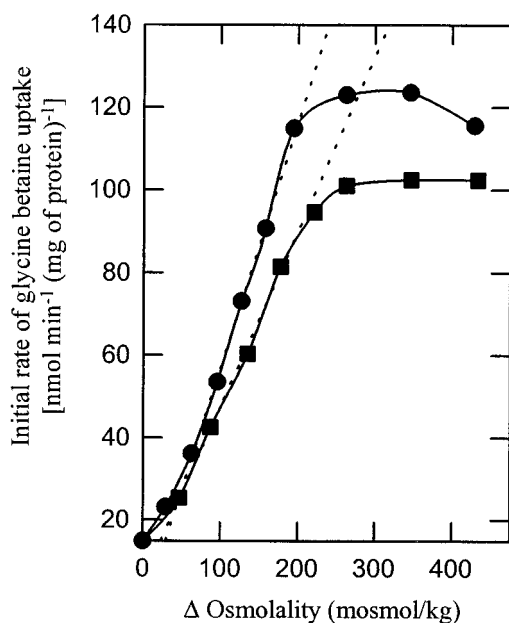


Fig. 3. Threshold value for the transmembrane osmotic gradient needed to activate OpuA. Uptake of [14 C]glycine betaine (final concentration of $80 \mu\text{M}$) was assayed in 90 mM KPi , pH 7.0, in the presence of KCl (■) or sucrose (●). Δ Osmolality refers to the difference in external and internal osmolality.

membrane, experiments were performed with small amphiphilic molecules that partition in the bilayer. These amphiphiles are predicted to alter the curvature stress in the membrane (15, 16), a bilayer property that is also affected by osmotic stress. Fig. 4A shows that low concentrations of the cationic amphipath tetracaine activated the OpuA system under isoosmotic conditions. The activation was maximal at 1 mM tetracaine. Importantly, these concentrations

did not affect the activity of OpuA under hyperosmotic conditions. Concentrations of tetracaine above 1 mM decreased the activation of OpuA both under iso- and hyperosmotic conditions. The amphipaths chlorpromazine (cationic) and dipyrindole (anionic) activated OpuA in the same concentration range as did tetracaine (data not shown). The amphipaths did not increase the leakiness of the proteoliposomes for glycine betaine when used at the low concentrations that activate OpuA. Altogether, these results strongly indicate that the sensing of changes in the physical status of the lipid bilayer via lipid/protein interactions provides the trigger for activation of OpuA.

To further assess the effect of tetracaine on OpuA activity, the influence of this amphiphile was tested at different magnitudes of the transmembrane osmotic gradient. Although the threshold value for the transmembrane osmotic gradient needed for activation of OpuA was clearly lowered, the addition of tetracaine did not result in an overall shift of the hyperosmotic stress dependence toward lower osmolality values (Fig. 4B).

Discussion

The amplified expression, purification, and functional reconstitution of the osmotically activated ABC transport system OpuA is described in this communication. Typical prokaryotic binding protein transporters of the ABC type are composed of five protein(s) (domains), i.e., an extracellular binding protein (receptor), two ATP-binding subunits, and two integral membrane subunits. Except for the substrate-binding protein, the other subunits can be present as distinct polypeptides or fused to one another, but each entity is always present twice (17). In analogy with other ABC transporters, functional OpuA will most likely be composed of two transmembrane subunits and two ATP-binding subunits. Because OpuABC is composed of a translocator fused to the substrate-binding domain, the oligomeric structure implies that two receptor domains are also present. This raises questions about the observations that only a single substrate-binding protein interacts with the dimeric membrane complex and that two lobes of a single substrate-binding protein

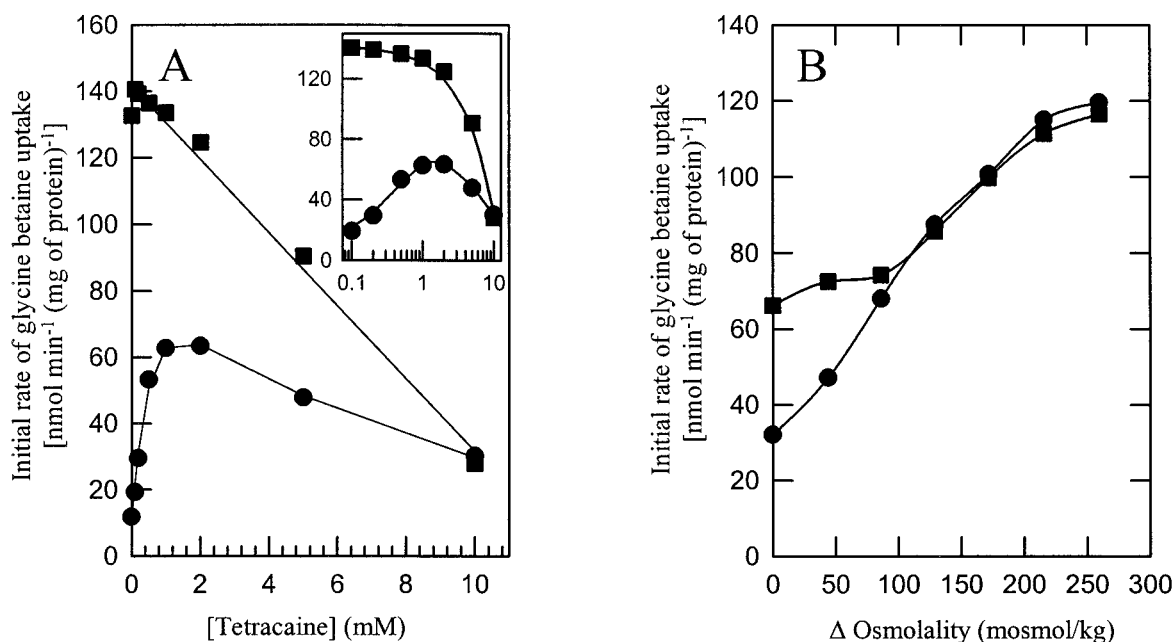


Fig. 4. The effect of tetracaine on OpuA activity. (A) Uptake of [14 C]glycine betaine (final concentration of $80 \mu\text{M}$) was assayed in 90 mM KPi , pH 7.0, with (■) or without (●) 400 mM KCl (680 mosmol/kg). Both curves were fitted with the double exponential decay function $f(x) = a \cdot \exp(-b \cdot x) + c \cdot \exp(-d \cdot x)$. Inset shows the data on a logarithmic scale. (B) Uptake of [14 C]glycine betaine (final concentration of $80 \mu\text{M}$) was assayed in 90 mM KPi , pH 7.0, with (■) or without (●) 1 mM tetracaine. The osmolality of the medium was varied with KCl. Δ Osmolality has the same meaning as in the legend to Fig. 3.

interact with different integral membrane protein(s) (domains) (18, 19). The oligomeric structure of OpuA of *L. lactis* is not unique, as database searches indicate that similar gene clusters, encoding putative glycine betaine ABC transporters, are present in *Streptococcus coelicolor*, *Streptococcus pneumoniae*, *Chlamidia pneumoniae*, and *Helicobacter pylori* (3).

Experiments performed in intact cells have shown that hyperosmotic medium conditions stimulate glycine betaine uptake in *L. lactis* through an increased expression and activity of a transport system (2, 3). At the onset of this work, the molecular nature of the glycine betaine uptake was unknown, and it was unclear which physicochemical parameter(s) affected the transport activity. We can now rigorously rule out the possibilities that either external or internal water activity per se, concentration(s) of specific signal molecule(s), compression of the membrane against the cell wall, or internal hydrostatic pressure regulate the activity. We conclude that OpuAA and OpuABC are essential and sufficient for the osmotic activation of glycine betaine uptake in *L. lactis*, and that the difference between the external and internal potential of all osmotically active solutes is sensed by the system. This transmembrane osmotic gradient is sensed via changes in the membrane lipid bilayer, as amphiphiles such as tetracaine also trigger the activation of the transporter. Thus, OpuA not only acts as osmoregulator but also functions as osmosensor for the cell.

The lipid bilayer used for the membrane reconstitution of OpuA consists predominantly of phosphatidylethanolamine, phosphatidylglycerol (PG), and phosphatidylcholine, in contrast to the PG, cardiolipin, and glycolipids present in the native membrane of *L. lactis* (20). Given the role of the membrane in transducing osmotic signals to OpuA, it is likely that different physical properties associated with a different lipid composition will affect the transport system. In fact, the higher sensitivity of OpuA toward hyperosmotic stress in the *in vitro* system as compared with the *in vivo* situation may relate to these differences (2, 3). How the headgroup composition, acyl chain length, degree of saturation, etc., affect the activity, and activation mechanism of OpuA is currently under investigation.

The use of cationic (e.g., tetracaine, chlorpromazine), anionic (e.g., dipyrimidole), and neutral amphiphiles in patch-clamp experiments on giant *E. coli* spheroplasts suggested that these compounds potentiate the opening of the mechanosensitive channel MscL by selectively expanding one membrane monolayer and thereby altering the membrane bilayer curvature (21). This is consistent with the gating (“activation”) of MscL by hypoosmotic medium conditions (22, 23), as water influx increases the area that is occupied per lipid molecule and thereby alters the curvature stress. OpuA, on the other hand, is activated by hyperosmotic conditions. One would expect that hypo- and hyperosmotic conditions distort the membrane bilayer in a different manner, and that the activation of MscL and OpuA is tuned to these differences. In this regard, it is perhaps surprising

that the same amphiphiles that trigger MscL also activate OpuA. The observation that OpuA is not only activated by hyperosmotic stress but that the basal activity is decreased by hypoosmotic conditions is also relevant in this respect. This suggests that osmotic stress acts on OpuA and MscL via a similar mechanism but with a different (osmotic) setpoint for activation, that is, the sign (hypo- vs. hyperosmotic) and threshold osmolality difference for activation differs for the two systems. Finally, it is worth emphasizing that the experimental setup for testing the effects of the amphiphiles on MscL and OpuA differed. MscL has been studied in native membranes (21), in which the composition of the inner and outer leaflet of the membrane bilayer is different. Cationic and anionic amphiphile are expected to accumulate in either the inner or outer leaflet, depending on the charge/geometric features of the lipids in the membrane bilayer. In the proteoliposomal system used here, the composition of the two leaflets of the bilayer is by definition the same, and the uneven distribution of tetracaine will be transient as the amphiphile flop-flips from the outer to the inner leaflet. At present, it is not known which fraction of the amphiphiles flop-flip to the inner leaflet of the membrane on the time scale of the experiments.

Recent theoretical studies suggest that the distribution of the lateral pressure in a lipid bilayer is strongly affected by the incorporation of interfacially active solutes (amphiphiles) as well as by an altered lipid composition (15). In these studies, lateral pressure or curvature stress is a function of the depth normal to the protein in the membrane plane, and it is speculated that variations in this stress may be coupled to conformational changes in proteins (15). Future work is aimed at specifying the osmotic activation of OpuA in more detail by correlating threshold values for activation to changes in curvature stress and identifying the portions in OpuA that actually sense changes in the physical state of the membrane.

While this manuscript was in preparation, a paper was published on the osmotic activation of the sodium-dependent glycine betaine carrier BetP from *Corynebacterium glutamicum* (24). Because there is a fundamental difference in the transport mechanism of the secondary transport system BetP and the ABC transporter OpuA, it will be interesting to find out whether osmoregulatory properties of both systems are similar. In contrast to ion-linked transporters such as BetP, OpuA translocates unidirectionally, and only those molecules that are reconstituted in the *in vivo* orientation contribute to the observed uptake. This will facilitate identification of the region(s) involved in osmosensing when the properties of the inner and outer leaflet of the membrane bilayer are affected differently through either the application of osmotic gradients or specific amphiphiles.

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