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Glycine Betaine Uptake after Hyperosmotic Shift in *Corynebacterium glutamicum*

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Osmoregulatory uptake of glycine betaine in whole cells of *Corynebacterium glutamicum* ATCC 13032 (wild type) was studied. The cells actively take up glycine betaine when they are osmotically shocked. The total accumulation and uptake rate were dependent on the osmotic strength of the medium. Kinetic analysis revealed a high-affinity transport system (K_m, 8.6 ± 0.4 μM) with high maximum velocity (110 nmol · min^{-1} · mg [dry weight]$^{-1}$). Glycine betaine functioned as a compatible solute when added to the medium and allowed growth at an otherwise inhibitory osmotic strength of 1.5 M NaCl. Proline and ectoine could also be used as osmoprotectants. Glycine betaine is neither synthesized nor metabolized by *C. glutamicum*. The glycine betaine transport system is constitutively expressed at a basal level of activity. It can be induced up to eightfold by osmotic stress and is strongly regulated at the level of activity. The transport system is highly specific and has its pH optimum in the slightly alkaline range at about pH 8. The uptake of the zwitterionic glycine betaine is mediated by a secondary symport system coupled to cotransport of at least two Na^+ ions. It is thus driven both by the membrane potential and the Na^+ gradient. An extremely high accumulation (internal/external) ratio of up to 4 × 10^6 was measured, which represents the highest accumulation ratio observed for any transport system.

A strategy of bacterial, fungal, animal, and plant cells to overcome hyperosmotic stress conditions is the intracellular accumulation of inorganic or organic solutes (34). Since these solutes may reach very high cytosolic concentrations, they have to be compatible with metabolic processes and cellular structures and are thus termed compatible solutes (5). Examples of eubacterial compatible solutes are glycine betaine, proline, ectoine, glutamic acid, trehalose, and potassium (14, 31). Among them, the most widespread is glycine betaine. In response to hyperosmotic stress *Escherichia coli* and *Salmonella typhimurium* first accumulate potassium glutamate (3). This leads to the activation and induction of enzymes and transport systems specific for osmoregulation (8, 9). Enterobacteria possess two glycine betaine carriers, ProU (7, 24) and ProP (6, 33). ProU is encoded by an operon, consisting of the genes *proV*, *proW*, and *proX*, coding for a primary binding protein-dependent system. It is mainly regulated on the level of transcription. ProP is a secondary, sodium-dependent uptake system that is constitutively expressed and mainly regulated on the activity level. Both systems also reveal transport activity for proline, ectoine, and taurine (16, 26, 33). The situation in gram-positive bacteria is not as clear. *Staphylococcus aureus* also possesses two glycine betaine uptake systems (30). Both transport systems are secondary carriers and function in symport with sodium. The so-called low-affinity system also accepts proline; the high-affinity system is specific for glycine betaine.

Several transport proteins of coryneform bacteria have been described previously (19). In this paper we present data identifying an osmoregulatory glycine betaine uptake system in the gram-positive bacterium *Corynebacterium glutamicum*. This bacterium is of great industrial interest, because of its ability to produce large quantities of amino acids such as glutamate and lysine. Industrial culture media contain high concentrations of carbohydrates, e.g., molasses. This results in high osmolarity of the production media. As glycine betaine is also one of the major compatible solutes in plants, it is commonly present in these production media, and use of this compound as a compatible by a *C. glutamicum* solute had to be expected.

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MATERIALS AND METHODS

Growth of organisms. *C. glutamicum* ATCC 13032 (wild type) was grown aerobically at 30°C on a rotary shaker (130 rpm) overnight in the following media: complex medium (brain heart infusion [BHI]; Difco, Detroit, Mich.) and minimal medium, as previously described by Keilhauer et al. (18). In most experiments, 0.5 M NaCl was added for hyperosmotic growth conditions, and BHI was diluted 1:1 or 1:2 with distilled water for low-osmolarity medium.

Chemicals. Radiochemicals were purchased from Amersham International (Buckinghamshire, United Kingdom). The following labelled compounds were used: [^{14}C]choline, [^{14}C]carboxyinulin, [^{14}C]tetraphenylphosphonium bromide, and ^3H_2O. All chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany), Fluka (Neu-Ulm, Germany), and Sigma (St. Louis, Mo.); glass-fiber discs (type F) were acquired from Millipore (Eschborn, Germany).

Synthesis of [^{14}C]glycine betaine. Synthesis of [^{14}C]glycine betaine by oxidation of [^{14}C]choline (specific activity, 52 μCi/μmol) with choline oxidase (from an *Alcaligenes* sp.; Sigma) was performed basically as described by Landfald and Strøm (22), with slight modifications. In order to dilute the ethanolic [^{14}C]choline solution, the volume was increased to 500 μl; therefore, 30 U of choline oxidase was used. More than 99% of the [^{14}C]choline was converted to [^{14}C]glycine betaine. [^{14}C]glycine betaine was identified by the method of Blunden et al. (2). After purification, no other radioactive substances could be detected by thin-layer chromatography.

Determination of initial rate of glycine betaine uptake. In order to optimally induce the glycine betaine uptake system, in most experiments cells were grown in the presence of 0.5 M NaCl. Early-stationary-phase cells were harvested by centrifugation and washed in buffer containing 50 mM potassium phosphate (pH 7.5) and 10 mM NaCl. For the measurement of glycine betaine uptake, the final pellet was suspended in the same buffer to a cell density between 0.04 and 7.2 mg (dry weight)/ml. The cells were stored on ice until used. Before the uptake experiment, the cells were transferred to test tubes, energized by 10 mM glucose, and osmotically stressed by adding 625 mM sodium chloride for 3 min at 30°C (if not indicated otherwise). In order to measure the dependence of glycine betaine uptake activity on the external pH, the cells were resuspended to an optical density at 600 nm of 100 and diluted 30-fold in 50 mM MES (morpholineethanesulfonic acid)-Tris buffer containing 625 mM NaCl and 10 mM KCl at different pH values. Uptake was initiated by adding [^{14}C]glycine betaine (0.2 to 200 μM, final concentration; approximately 0.1 μCi/ml of reaction mix). Uptake was

measured by rapid filtration of 200-μl aliquots on glass-fiber filters (Millipore) at time intervals of 10 or 15 s. The filters were dried for 2 h, and the radioactivity was measured by liquid scintillation counting. The rate of glycine betaine uptake was linear for at least 1 min depending on the applied glycine betaine concentration and the cell density. For the calculation of uptake rates, at least four kinetic points were used. In experiments with ionophores, valinomycin (20 μM), CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) (50 μM), and nigericin (2 μM) were used in cell suspensions containing approximately 1 mg (dry weight)/ml.

Determination of cell volume and membrane potential. The cell volume and membrane potential were determined as described earlier (10, 20). Separation of the cells from the surrounding medium was achieved by silicone oil centrifugation. The cytoplasmic volume was determined by using [^{14}C]taurine as a nonpenetrating marker for the extracellular space and ^3H_2O as the marker for the total space. The difference between the total and the taurine space, i.e., the cytosolic volume, decreased from 1.9 μl/mg (dry weight) to 1.37 μl/mg (dry weight) after the addition of 1 M sorbitol, to 1.45 μl/mg (dry weight) after the addition of 550 mM NaCl, and to 1.42 μl/mg (dry weight) after the addition of 625 mM NaCl. These values were determined 5 min after the osmotic shock. The internal volume after osmotic shock with 625 mM NaCl remained constant for about 1 h. The membrane potential was determined from the distribution of the permeant cation [^{14}C]tetraphenylphosphonium bromide. The values obtained for membrane potential were corrected according to the method of Zaritzky et al. (12, 35).

Determination of steady-state level of glycine betaine accumulation. In order to determine the maximum glycine betaine accumulation, conditions had to be found such that (i) the total amount of externally added glycine betaine was low enough not to exceed the maximum internal concentration of glycine betaine when the steady-state accumulation ratio had been reached and (ii) the total cytoplasmic volume was low enough to limit the amount of radioactivity to be added in view of the extremely high internal accumulation. Thus, an aliquot of 0.55 ml of cell suspension with 0.03 to 0.06 mg (dry weight)/ml was incubated for 1 h with 2 to 10 μM tracer-free [^{14}C]glycine betaine. The cells were spun for 4 min by centrifugation in reaction vessels. The amount of labelled glycine betaine in the supernatant was determined by liquid scintillation counting. This value was subtracted from the total radioactivity added to obtain the glycine betaine accumulated internally.

Other methods. For determination of internal sodium concentrations, 1 ml of cells was directly centrifuged in reaction vessels into 300 μl of silicone oil. After the supernatant buffer was carefully removed, the reaction vessels were washed two times by adding water on top of the silicone layer. After removal of the silicone oil, the cell pellet was suspended in 500 μl of 5% (wt/wt) trichloroacetic acid and measured by flame spectrometry (Eppendorf, Hamburg, Germany). The (internal) cytoplasmic and the (external) buffer volume of the cell pellet was determined essentially as described above. Osmolarity was measured by cryoscopy in an Osmomat 030 (Gonotec, Berlin, Germany). For thin-layer chromatography, cells were incubated for 2 h under conditions of osmotic stress and extracted by the addition of 0.2% cetyltrimethylammonium bromide. Cell extract (5 μl) was applied to Merck thin-layer chromatography plates (Kieselgel 60, 250 μm) in 50:50 methanol-H_2O. Radioactivity was analyzed by using a thin-layer chromatography scanner.

RESULTS

Growth of *C. glutamicum* under salt stress. The addition of NaCl in concentrations of 1 and 1.5 M, respectively, significantly reduced the growth rate and growth yield of *C. glutamicum*. This inhibitory effect could be alleviated by the addition of glycine betaine, especially with 1.5 M NaCl, for which the growth rate without glycine betaine was extremely low. Maximal protection against osmotic stress was achieved with glycine betaine concentrations of at least 1.5 mM. The addition of higher concentrations showed no further protection. Other putative osmoprotective substances were also tested in *C. glutamicum* in the presence of 1.5 M NaCl. Ectoine was nearly as effective as glycine betaine, whereas proline led to a less pronounced improvement of growth. The addition of choline, alanine, and glycine showed no osmoprotecting effect. A slight but significant protection was observed with glutamine and glutamic acid.

Metabolic role of glycine betaine. We did not observe uptake of [^{14}C]choline under any conditions. As choline did not serve as an osmoprotectant either, this may indicate that there is either no choline uptake system or no pathway for glycine betaine synthesis from choline in *C. glutamicum* (22). Glycine betaine was not degraded either. Cells that were osmotically shocked by the addition of 625 mM NaCl and which had taken up labelled glycine betaine were extracted after 2 h of incubation. The extract was analyzed by thin-layer chromatography. Only a single peak, which had the same R_f value as the glycine betaine reference, occurred. We analyzed in detail the finding that *C. glutamicum* cannot synthesize or degrade glycine betaine, regardless of whether the cells are subjected to osmotic stress (data not shown).

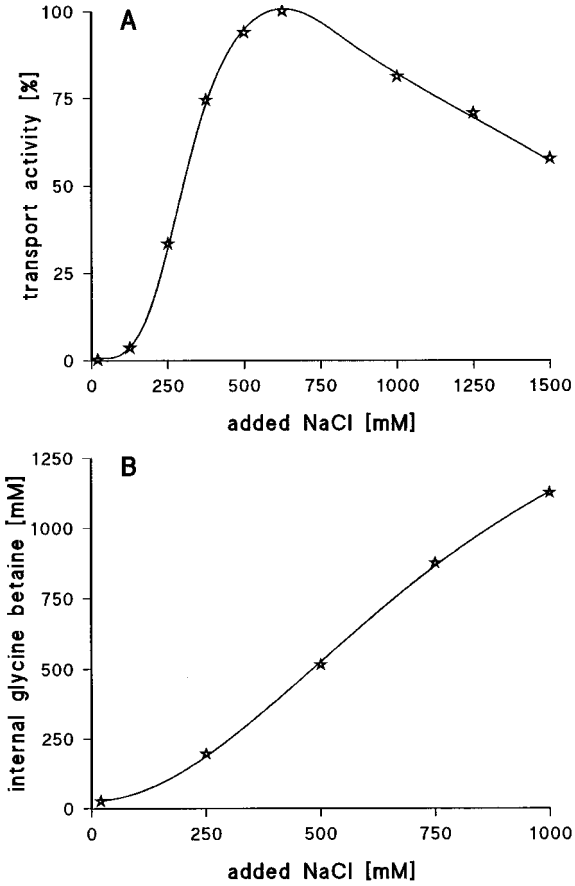

FIG. 1. Dependence of glycine betaine uptake on NaCl. (A) Activation of the glycine betaine transport system by the addition of different amounts of NaCl to the uptake buffer. Cells were grown in complex medium in the presence of 0.5 M NaCl and washed in cold hypoosmotic medium (see Materials and Methods). Uptake of labelled glycine betaine was measured essentially as described in Materials and Methods, in the presence of different NaCl concentrations in the transport assay. The 100% value of the uptake rate was 74 nmol/min/mg (dry weight). (B) Internal concentration of glycine betaine as a function of the external NaCl concentration in the uptake buffer under steady-state conditions and the conditions described for panel A.

Factors affecting glycine betaine uptake system. The activity of glycine betaine uptake did not change during different growth phases (data not shown) but depended on the growth media, its osmotic strength, and the solute used for the osmotic upshift (see below). The glycine betaine uptake system turned out to be strongly affected by the osmolarity of the medium used for the uptake measurement (Fig. 1A). In 50 mM potassium phosphate buffer (pH 7.5) containing 20 mM NaCl (no osmotic shock), the uptake rate was negligible; it increased with inclusion of NaCl to a maximum rate of 110 nmol · min^{-1} · mg (dry weight)$^{-1}$ at 625 mM NaCl (the concentration used in the standard uptake assay) and decreased at higher osmolarities. Activation was very fast, 90% of maximum rate being reached at 30 s after the addition of 625 mM NaCl (data not shown).

Effects on the expression level were less dramatic. The ad-

TABLE 1. Influence of different growth conditions on induction of glycine betaine transport system[a]

Growth medium additions	Osmolarity (mosM)	OD_{600}	pH	Uptake rate (nmol/min/mg [dry wt])
None	120[b]	3.0	8.1	16
None	180[c]	5.5	8.1	19
None	350	10.0	7.2	27
NaCl	1,230	8.3	7.3	58
K-Ac	380[c]	4.6	8.2	14
K-Ac	550	11.3	7.9	24
K-Ac, NaCl	1,420	8.9	7.5	75
NaCl, Ac-induced[d]	1,420	12.3	8.0	106
Glc	230[c]	7.8	7.3	6
Glc	400	17.9	7.2	11
Glc	1,260	17.5	7.3	28

[a] The basic growth medium was complex medium. Where indicated, 1% (wt/vol) of the respective carbon sources (glucose [Glc] and potassium acetate [K-Ac]) or 0.5 M NaCl was added. The optical density at 600 nm (OD_{600}) and pH were determined after overnight growth. The pH of cultures grown in the presence of glucose was kept around pH 7.2 by the addition of KOH.

[b] In order to achieve low osmolarities, the growth medium was diluted 1:2 with distilled water.

[c] The growth medium was diluted 1:1 with distilled water.

[d] For induction by potassium acetate, cells were grown overnight in complex medium plus 0.5 M NaCl and were induced for 4 h by the addition of potassium acetate.

dition of 500 mM NaCl to the complex growth medium induced the expression of the uptake system four- to eightfold, depending on the carbon source (Table 1). Maximum induction was found when cells grown overnight in BHI medium containing 0.5 M NaCl were incubated with 1% potassium acetate for 4 h. In the presence of glucose in the growth medium, the maximum activity was significantly less than that in cells grown without glucose. In minimal medium, the addition of different nitrogen sources, such as urea, ammonium sulfate, or glutamine, had no effect on the induction level of the carrier (data not shown).

Basic properties of glycine betaine uptake. The highest glycine betaine uptake rate in *C. glutamicum* was achieved when the cells were washed with ice-cold hypotonic buffer and then shocked by the addition of >500 mM NaCl. By the use of wash buffer isoosmotic to the growth medium, the maximum uptake rate decreased by about 60%. The uptake rate depended on the presence of an appropriate energy source, such as glucose (data not shown). Total accumulation of glycine betaine depended on the osmotic strength of the uptake buffer (Fig. 1B), e.g., at 1 M external sodium chloride, the internal glycine betaine concentration was 1.2 M. The optimal uptake rate occurred in the range between pH 7.5 and 8.5 (Fig. 2). The pK_a value of the carboxy group of glycine betaine is about 4.0; thus, at pH 8.0 the zwitterionic form is available almost exclusively.

Since many glycine betaine carriers also transport proline or ectoine (13, 15, 30), glycine betaine uptake was measured with the following unlabelled substrates in 50-fold excess: proline, ectoine, choline, glycine, *N*-methyl glycine, glutamic acid, glutamine, γ-amino butyric acid, alanine, and taurine. Only proline showed a slight inhibition of about 20%. Ectoine, also shown to be an effective osmoprotectant in *C. glutamicum* (see above), had no effect.

Kinetic parameters and transport mechanism. Kinetic analysis revealed a single saturable uptake system with a K_m of 8.6 ± 0.4 μM and a V_{max} of 110 ± 1.1 nmol · min^{-1} · mg (dry weight)$^{-1}$ in cells in which the uptake was fully induced and activated. Glycine betaine is taken up against a concentration gradient. It therefore needs an energy source as a driving force. The uptake was completely abolished by the uncoupler CCCP

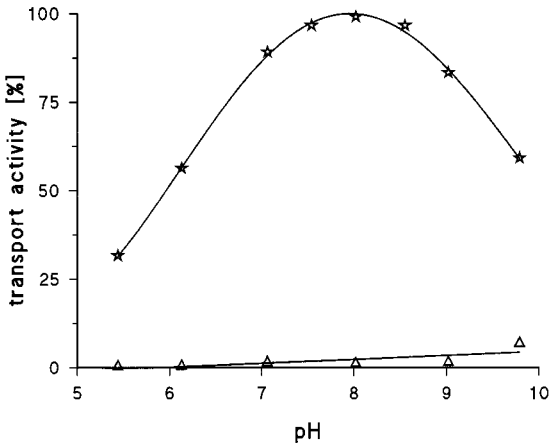

FIG. 2. Dependence of glycine betaine uptake rate on external pH in the absence (stars) and presence of 50 μM CCCP (triangles). Further conditions were as described in the legend to Fig. 1; uptake was measured at 625 mM NaCl, by using 50 μM [^{14}C]glycine betaine.

(Fig. 2) and to a different extent by the ionophores valinomycin and nigericin, which catalyze an electrogenic K^+ transport or an electroneutral K^+/H^+ exchange, respectively. By varying the external K^+ concentration in the presence of valinomycin, defined K^+ diffusion potentials were established in *C. glutamicum* (4, 20) (Fig. 3). The measured glycine betaine uptake rates were correlated to the experimentally determined membrane potential over a certain range of −80 to −50 mV, which indicates a ΔΨ-driven secondary system. Under these conditions, the cytosolic ATP concentration did not change significantly (data not shown). Transport was inhibited by nigericin at a low external potassium concentration of 10 mM. This inhibitory effect could be completely overcome by the addition of higher external K^+ concentrations (400 mM) (data not shown). The glycine betaine uptake system is fully reversible. This was

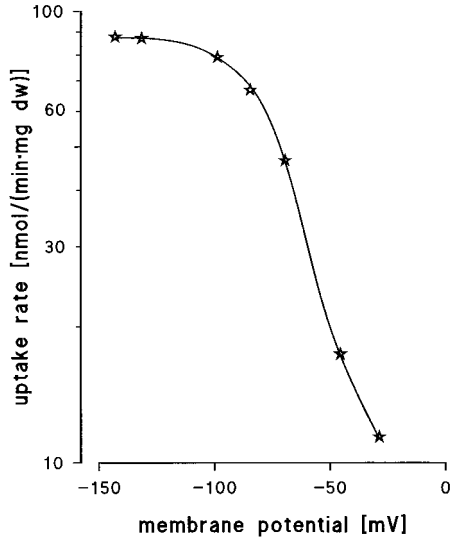

FIG. 3. Dependence of glycine betaine uptake rate on membrane potential. Diffusion potentials were created by varying the external K^+ concentration in the presence of 20 μM valinomycin and measured as described in Materials and Methods. In this experiment, NaCl and KCl were present in varying concentrations, always to a total of 625 mM, according to the desired diffusion potentials. [^{14}C]glycine betaine at a concentration of 50 μM was used.

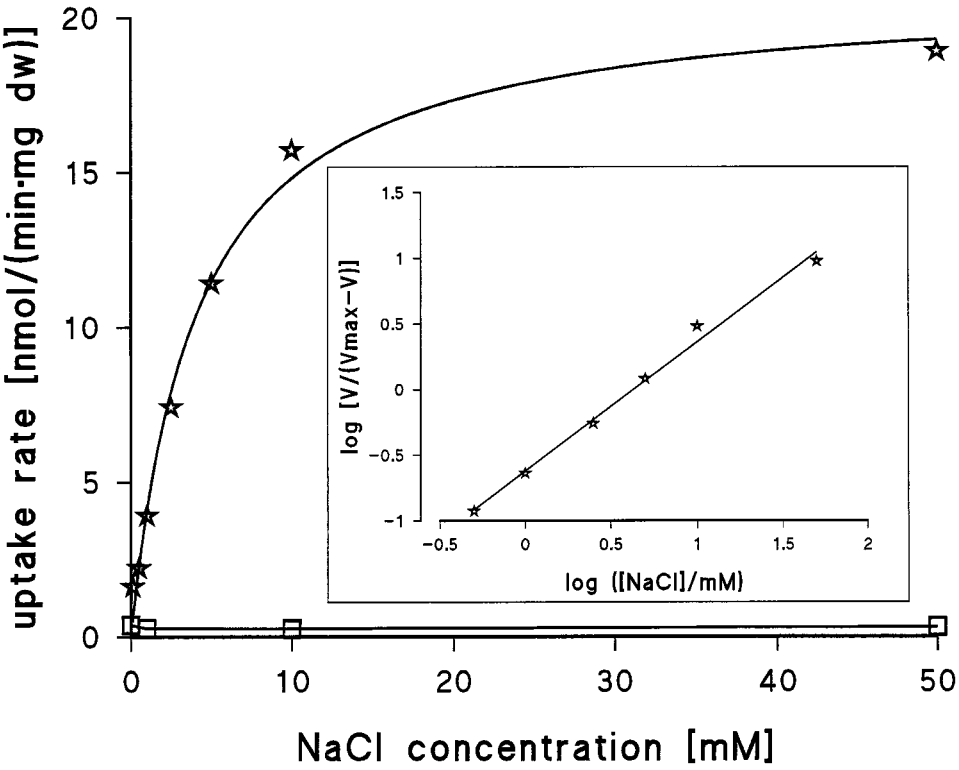

FIG. 4. Sodium dependence of glycine betaine transport system. Osmotic upshift was carried out by the addition of 1 M sorbitol (stars). In a control experiment, only NaCl (squares) was added. Except for sodium, standard conditions were used in the uptake assay (see Materials and Methods), and the concentration of [^{14}C]glycine betaine was 50 μM. The apparent K_m for Na^+ was 4.1 ± 0.5 mM. (Inset) Hill plot of the kinetics data, with a Hill coefficient (n) of 0.98.

shown by adding a nigericin-CCCP mixture to cells that had taken up [^{14}C]glycine betaine. The immediate efflux of more than 90% of the label was observed (results not shown).

As glycine betaine is transported in the uncharged zwitterionic form and the carrier is obviously a secondary transport system, translocation across the membrane has to be coupled to the transport of ions. We therefore investigated whether glycine betaine transport in *C. glutamicum* is coupled to symport of protons and/or sodium ions. By varying the external NaCl concentration in the presence of 1 M sorbitol for osmotic shock, an apparent K_m value for sodium of 4.1 ± 0.4 mM with respect to uptake stimulation was obtained (Fig. 4). In order to exclude direct activation of the carrier by the added NaCl, in a control experiment only sodium chloride was added. Up to concentrations of 50 mM NaCl, which were too low for osmotic activation of the carrier (cf. Fig. 1A), no activation of uptake was observed. For a further kinetic characterization in terms of binding interaction, the data were transformed according to the Hill equation. A Hill coefficient (n) of 0.98 was obtained (Fig. 4, inset).

Energetic parameters of glycine betaine transport. As glycine betaine is not metabolized in *C. glutamicum*, the cotransport stoichiometry can be derived from the measured steady-state accumulation ratio. Therefore, the chemical potential of glycine betaine, the membrane potential, and the chemical potentials of the putative coupling ions were measured under

TABLE 2. Determination of gradients under equilibrium conditions in glycine betaine transport system[a]

Supplement(s)	pH	Accumulation ratio	Membrane potential (mV)	$[Na^+]_{in}$ (mM)	n
600 mM NaCl	7.5	$(3.9 \pm 0.6) \times 10^6$	−136 ± 4	10–50[b]	1.5–2.0
60 mM NaCl, 540 mM KCl	7.5	$(6.4 \pm 0.9) \times 10^5$	−133 ± 5	7.5 ± 3.3	1.8 ± 0.4
6 mM NaCl, 594 mM KCl	7.5	$(6.8 \pm 1.2) \times 10^5$	−137 ± 6	2.7 ± 1.6	2.2 ± 0.4
6 mM NaCl, 594 mM KCl	6.5	5.0×10^4	−117	2.4 ± 1.2	2.0
6 mM NaCl, 594 mM KCl	8.2	1.6×10^6	−144	2.1 ± 1.0	2.1

[a] Cells were grown in complex medium at 0.6 M NaCl, washed, and resuspended in 50 mM potassium phosphate buffer plus the indicated additions of NaCl and KCl. The determinations of the corresponding values (means ± standard deviations) of glycine betaine and sodium accumulations (internal/external), as well as the membrane potentials, were done in the presence of the same amounts of NaCl and glycine betaine. The experiments for which the results are given in lines 1 to 3 were carried out at a minimum of three different concentrations of glycine betaine (see Materials and Methods); for typical values of absolute concentrations, see the Discussion. The values for the membrane potential and the internal Na^+ concentration were calculated on the basis of at least four independent experiments; the scatter is mainly due to the standard error of the volume determinations (see Materials and Methods). The value for the internal Na^+ concentration ($[Na^+]_{in}$) also includes the error of subtracting the external Na^+ contamination. The cotransport stoichiometry (n) value of (Na^+/glycine betaine) was calculated by the following equation: $n = Z \log ([bet_{in}]/[bet_{ex}])/(-\Delta\Psi + Z \log ([Na^+_{ex}]/[Na^+_{in}])$.

[b] The internal Na^+ concentration for this experiment was only estimated (see the text).

different conditions (Table 2). In secondary transport systems, the electrochemical potential of the accumulated substrate equals the driving forces in thermodynamic equilibrium. For uptake of glycine betaine together with monovalent cations, this is described as follows:

$$Z \log ([bet_{in}]/[bet_{ex}]) = n(-\Delta\Psi + Z \log ([X^+_{ex}]/[X^+_{in}])) \quad (1)$$

where Z is 2.3 RT/F, bet_{in} and bet_{ex} are the internal and external amounts of betaine, n is the value for coupling stochiometry, X^+ is the cotransported monovalent cation (internal [in]; external [ex]), and $\Delta\Psi$ is the membrane potential.

In order to elucidate the kind of coupling ion, we determined the glycine betaine accumulation and $\Delta\Psi$ and varied the concentration and the chemical potentials of protons and sodium ions. A symport with protons should lead to increasing betaine accumulation ratios at a higher ΔpH (positive outside) and vice versa. As bacteria tend to keep the proton motive force constant (1), the variation of ΔpH concomitantly leads to variations in $\Delta\Psi$. At pH 7.5, the internal and external pH values are equal (20), and the glycine betaine accumulation was determined as 3.9×10^6. In the presence of a ΔpH (positive outside), resulting in a lowered $\Delta\Psi$, the accumulation decreased. An inverse ΔpH (positive inside) led to an increase in the accumulation. Thus, especially in comparison of the results presented in Table 2 (lines 3 and 5), participation of protons appears to be very unlikely. In order to estimate the transport stoichiometry (sodium/glycine betaine), the observed data were transformed according to equation (1). Since it was impossible to accurately measure the internal Na^+ concentration at 600 mM external NaCl, the range of internal concentration used for these conditions (10 to 50 mM) represents an rough estimation based on the measured values. The data in Table 2 revealed values around 2 for the coupling stochiometry under very different external concentrations of glycine betaine, Na^+, and H^+.

DISCUSSION

Previous studies (16, 17) have suggested that the accumulation of glycine betaine in response to osmotic stress is important for *Brevibacterium lactofermentum*, which is closely related to *C. glutamicum*. Osmoprotectants can be accumulated in the cytosol either via synthesis or by uptake (9). Accumulation of osmoprotectants other than glycine betaine by biosynthesis has been observed previously in *C. glutamicum* (11). Glycine betaine was identified as a minor internal osmoregulatory compound in *C. glutamicum*, which, however, presumably originated from the small amount of yeast extract in the medium used (14). Our results indicate that in *C. glutamicum* glycine betaine is the most effective osmoprotectant, when added to the growth medium, followed by ectoine and proline. This situation is similar to that in *E. coli*, in which glycine betaine and ectoine led to about the same protective effect in media of higher osmolarity (15) and proline showed less protection (6). The effect of glycine betaine being more effective than proline has been described for *S. aureus* as well (27). In contrast to that in *E. coli*, inhibition of uptake by proline and ectoine was absent, indicating that the glycine betaine carrier in *C. glutamicum* is very specific. The osmoprotecting effects of ectoine and proline furthermore indicate that there must be at least one additional uptake system for the transport of these substances.

Besides other facts, the observed direct dependence of the uptake rate on the membrane potential over a wide range suggests that glycine betaine uptake is mediated by a secondary transport system. We did not observe any correlation of transport activity with the cytosolic ATP content. Furthermore, the fact that an equilibrium with the membrane potential and the sodium gradient was obtained with a variety of external buffer conditions argues against a primary mechanism. So far, we have not found any indication for a second glycine betaine uptake system in *C. glutamicum*. Transport was totally inhibited by the uncoupler CCCP, and the Eadie-Hofstee plot resulted in a straight line over a wide range of concentrations. Since we found that glycine betaine is transported as a zwitterion, cotransport ions are needed to explain the dependence on membrane potential. Na^+ was identified as the coupling ion, binding with a relatively low affinity of 4.1 mM. The Hill coefficient, indicating the number (n) of cooperatively interacting binding sites, as calculated from these data was 0.98. This is in agreement with either binding of one sodium ion or the noncooperative binding of sodium ions to two or more binding sites.

The maximum steady-state accumulation of glycine betaine, measured under appropriate conditions, was determined as about 4×10^6. This means that the cytosolic concentration of glycine betaine was around 400 mM, whereas the concentration in the medium was only 100 nM. This is, to our knowledge, the highest steady-state accumulation described for any transport system, including primary systems. The values for glycine betaine accumulation in *E. coli* vary between 10^5 and 10^3 (23, 33). The essential prerequisite for such extremely high accumulation is a low level of passive membrane permeability of the transported solute, no metabolic conversion, and a tightly coupled carrier. Under a variety of conditions, we measured a coupling stoichiometry for Na^+ ions of around 2 (Na^+/glycine betaine). We cannot strictly exclude the presence of a glycine betaine efflux system as observed in *E. coli* and *S. typhimurium* (21, 25), since, on the one hand, a deletion mutant of the uptake system is not available and *C. glutamicum* cannot be loaded with betaine by biosynthesis from choline. We thus conclude that glycine betaine is transported in symport with at least two sodium ions, and a higher Na^+/betaine stoichiometry cannot be ruled out.

We observed inhibition of betaine uptake by nigericin at low external K^+ concentrations. As found in other cases, this could be explained by modulation of carrier activity by a decrease in the cytoplasmic pH (20, 29). Thus, it is not necessary to argue for a particular regulation mechanism of the betaine uptake system with respect to K^+ and/or H^+ gradients in *C. glutamicum*. Glycine betaine uptake is affected by external factors both at the activity level and at the level of expression, the former being extremely effective. Without osmotic stress the system is completely switched off, irrespective of the level of induction. The time necessary to activate the system to a level of more than 90% of its maximum transport activity after osmotic upshift with 625 mM NaCl was less than 30 s, indicating a half-time of activation in the range of a few seconds. This is fast compared with *E. coli* ProP, for which a half-time of activation of about 1 min was determined (28). In summary, the glycine betaine uptake system of *C. glutamicum* seems to be similar to the ProP system in *E. coli*. Both are secondary systems and sodium dependent (32), mainly with regulation at the level of activity, and both are very fast (V_{max} of ProP is about 75 nmol/min/mg [dry weight]) (6, 33).

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REFERENCES

1. **Bakker, E. P., and W. E. Mangerich.** 1981. Interconversion of components of the bacterial proton motive force by electrogenic potassium transport. J. Bacteriol. **147:**820–827.
2. **Blunden, G., M. M. El Baroundi, S. M. Gordon, W. F. H. McLean, and D. J. Rogers.** 1981. Extraction, purification and characterisation of Dragendorff-positive compounds from some British marine algae. Bot. Mar. **23:**451–456.
3. **Booth, I. R., and C. F. Higgins.** 1990. Enteric bacteria and osmotic stress: intracellular potassium glutamate as a secondary signal of osmotic stress? FEMS Microbiol. Rev. **75:**239–246.
4. **Bröer, S., and R. Krämer.** 1991. Lysine secretion by *Corynebacterium glutamicum*. 2. Energetics and mechanism of the transport system. Eur. J. Biochem. **202:**137–143.
5. **Brown, A. D.** 1976. Microbial water stress. Bacteriol. Rev. **40:**803–846.
6. **Cairney, J., I. R. Booth, and C. F. Higgins.** 1985. *Salmonella typhimurium proP* gene encodes a transport system for the osmoprotectant betaine. J. Bacteriol. **164:**1218–1223.
7. **Cairney, J., I. R. Booth, and C. F. Higgins.** 1985. Osmoregulation of gene expression in *Salmonella typhimurium*: *proU* encodes an osmotically induced betaine transport system. J. Bacteriol. **164:**1224–1232.
8. **Csonka, L. N.** 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. **53:**121–147.
9. **Csonka, L. N., and A. D. Hanson.** 1991. Procaryotic osmoregulation: genetics and physiology. Annu. Rev. Microbiol. **45:**569–606.
10. **Ebbighausen, H., B. Weil, and R. Krämer.** 1991. Carrier mediated acetate uptake in *Corynebacterium glutamicum*. Arch. Microbiol. **155:**505–510.
11. **Frings, E., H. J. Kunte, and E. A. Galinski.** 1993. Compatible solutes in representatives of the genera *Brevibacterium* and *Corynebacterium*: occurrence of tetrahydropyrimidines and glutamine. FEMS Microbiol. Lett. **109:** 25–32.
12. **Giessen, S.** 1993. Diploma thesis. University of Düsseldorf, Düsseldorf, Germany.
13. **Gowrishankar, J.** 1985. *proP* mediated proline transport also plays a role in *Escherichia coli* osmoregulation. J. Bacteriol. **166:**331–333.
14. **Imhoff, J. F.** 1986. Osmoregulation and compatible solutes in eubacteria. FEMS Microbiol. Rev. **39:**57–66.
15. **Jebbar, M., R. Talibart, K. Gloux, T. Bernard, and C. Blanco.** 1992. Osmoprotection of *Escherichia coli* by ectoine: uptake and accumulation characteristics. J. Bacteriol. **174:**5027–5035.
16. **Kawahara, Y., Y. Yoshihara, S. Ikeda, and Y. Hirose.** 1990. Effect of glycine betaine, an osmoprotective compound on the growth of *Brevibacterium lactofermentum*. Appl. Microbiol. Biotechnol. **33:**574–577.
17. **Kawahara, Y., Y. Yoshihara, S. Ikeda, H. Yoshii, and Y. Hirose.** 1990. Stimulatory effect of glycine betaine on L-lysine fermentation Appl. Microbiol. Biotechnol. **34:**87–90.
18. **Keilhauer, C., L. Eggeling, and H. Sahm.** 1993. Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. J. Bacteriol. **175:**5595–5603.
19. **Krämer, R., E. Boles, L. Eggeling, A. Erdmann, M. Gutmann, W. Kronemeyer, L. Palmieri, and H. Sahm.** 1994. Mechanism and energetics of amino-acid transport in coryneform bacteria. Biochim. Biophys. Acta **1187:**245–249.
20. **Krämer, R., C. Lambert, C. Hoischen, and H. Ebbighausen.** 1990. Uptake of glutamate in *Corynebacterium glutamicum*. 1. Kinetic properties and regulation by internal pH and potassium. Eur. J. Biochem. **194:**929–935.
21. **Lamark, T., O. B. Styrvold, and A. R. Strøm.** 1992. Efflux of choline and glycine betaine from osmoregulating cells of *Escherichia coli*. FEMS Microbiol. Lett. **96:**149–154.
22. **Landfald, B., and A. R. Strøm.** 1986. Choline-glycine betaine pathway confers a high level of osmotic tolerance in *Escherichia coli*. J. Bacteriol. **165:** 849–855.
23. **Le Rudulier, D., A. R. Strøm, A. M. Dandekar, L. T. Smith, and R. C. Valentine.** Molecular biology of osmoregulation. Science **224:**1064–1068.
24. **Lucht, J. M., and E. Bremer.** 1994. Adaptation of *Escherichia coli* to high osmolarity environments: osmoregulation of the high-affinity glycine betaine transport system ProU. FEMS Microbiol. Rev. **14:**3–20.
25. **Koo, S-P., C. F. Higgins, and I. R. Booth.** 1991. Regulation of compatible solute accumulation in *Salmonella typhimurium*: evidence for a glycine betaine efflux system. J. Gen. Microbiol. **137:**2617–2625.
26. **McLaggan, D., and W. Epstein.** 1991. *Escherichia coli* accumulates the eucaryotic osmolyte taurine at high osmolarity. FEMS Microbiol. Lett. **81:**209–214.
27. **Miller, K. J., S. C. Zelt, and J. Bae.** 1991. Glycine betaine and proline are the principal compatible solutes of *Staphylococcus aureus*. Curr. Microbiol. **23:** 131–137.
28. **Milner, J. L., S. Grothe, and J. M. Wood.** 1988. Proline porter II is activated by a hyperosmotic shift in both whole cells and membrane vesicles of *Escherichia coli* K12. J. Biol. Chem. **263:**268–276.
29. **Poolman, B., K. J. Hellingwerf, and W. N. Konings.** 1987. Regulation of the glutamate-glutamine transport system by intracellular pH in *Streptococcus lactis*. J. Bacteriol. **169:**2272–2276.
30. **Pourkmailian, B., and I. R. Booth.** 1992. Glycine betaine transport by *Staphylococcus aureus*: evidence for two transport systems and their possible roles in osmoregulation. J. Gen. Microbiol. **138:**2515–2518.
31. **Severin, J., A. Wohlfarth, and E. A. Galinski.** 1992. The predominant role of recently discovered tetrahydropyrimidines for osmoadaptation of halophilic eubacteria. J. Gen. Microbiol. **138:**1629–1638.
32. **Stewart, L. M. D., and I. R. Booth.** 1983. Na^+ involvement in proline transport in Escherichia coli. FEMS Microbiol. Lett. **19:**161–164.
33. **Wood, J. M.** 1989. Proline porters effect the utilisation of proline as nutrient or osmoprotectant for bacteria. J. Membr. Biol. **106:**183–202.
34. **Yancey, P. H., M. E. Clark, S. C. Hand, R. D. Bowlus, and G. N. Somero.** 1982. Living with water stress: evolution of osmolyte systems. Science **217:** 1214–1222.
35. **Zaritzky, A., M. Kihara, and R. M. Macnab.** 1981. Measurement of membrane potential in *Bacillus subtilis*: a comparison of lipophilic cations, rubidium ion and a cyanine dye, as probes. J. Membr. Biol. **63:**215–231.