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Osmotic Regulation of Intracellular Solute Pools in *Lactobacillus plantarum*

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Bacteria respond to changes in medium osmolarity by varying the concentrations of specific solutes in order to maintain constant turgor pressure. The cytoplasmic pools of K^+, proline, glutamate, alanine, and glycine of *Lactobacillus plantarum* ATCC 14917 increased when the osmolarity of the growth media was raised from 0.20 to 1.51 osmol/kg by KCl. When glycine-betaine was present in a high-osmolarity chemically defined medium, it was accumulated to a high cytoplasmic concentration, while the concentrations of most other osmotically important solutes decreased. These observations, together with the effects of glycine-betaine on the specific growth rate under high-osmolarity conditions, suggest that glycine-betaine is preferentially accumulated in *L. plantarum*. Uptake of glycine-betaine, proline, glutamate, and alanine was studied in cells that were alternately exposed to hyper- and hypo-osmotic stresses. The rate of uptake of proline and glycine-betaine increased instantaneously upon increasing the osmolarity, whereas that of other amino acids did not. This activation occurred also under conditions in which protein synthesis was inhibited and was most pronounced when cells were pregrown at high osmolarity. The duration of net transport was a function of the osmotic strength of the assay medium. Glutamate uptake was not activated by an osmotic upshock, and the uptake of alanine was low under all conditions tested. When cells were subjected to osmotic downshock, a rapid efflux of accumulated glycine-betaine, proline, and alanine occurred whereas the pools of other amino acids remained unaffected. The results indicate that osmolyte efflux is, at least to some extent, mediated via specific osmotically regulated efflux systems and not via nonspecific mechanisms as has been suggested previously.

Biological membranes are readily permeable to water molecules, but they present an effective barrier to most other solutes. Under conditions of high external osmolarity, water will move out of the cell, causing changes in cell volume, intracellular solute concentration, and turgor pressure. Bacteria protect themselves against these deleterious osmotic effects by the uptake or synthesis of a limited number of solutes. These so-called compatible solutes are not inhibitory to most cellular processes, even at submolar concentrations. In fact, these molecules may even stabilize the native state of polypeptides and lipids (32). Compatible solutes include amino acids (glutamate, glutamine, and proline), amino acid derivatives (betaines, peptides, and N-acetylated amino acids), polyols, and sugars (trehalose and sucrose) (2, 3). A wide range of halophilic bacteria synthesize tetrahydropyrimidines (ectoines) which are accumulated to high intracellular levels in response to osmotic stress (23). Recently, it was discovered that ectoines taken up from the medium not only provide direct protection against osmotic stress but also act as chemical mediators that affect the synthesis of other osmolytes (8, 28).

Betaines are fully N-methylated amino acid derivatives. Glycine-betaine is the predominant member of this family of compatible solutes and is known to protect a wide range of different organisms against a high external osmolarity. Thus far, only cyanobacteria and some CO_2-fixing bacteria have been shown to carry out de novo synthesis of glycine-betaine. Some bacteria, including both gram-negative and gram-positive organisms, are able to synthesize glycine-betaine from choline by a two-step oxidation process, catalyzed by a membrane-bound dehydrogenase (3). Other microorganisms, however, are dependent on transport of this compound from the medium. Glycine-betaine uptake is observed in several lactic acid bacteria (7, 18).

In *Escherichia coli* and *Salmonella typhimurium*, the primary response towards an increase in medium osmolarity involves the accumulation of potassium-glutamate (6, 16). Other early responses include the uptake of proline and glycine-betaine via activation of the constitutive ProP carrier (17). At later times the proline and glycine-betaine accumulation levels are raised further following expression of the inducible ProU system (14). When glycine-betaine is absent from the medium, synthesis of trehalose takes place in *E. coli* (25).

Not only hyper- but also hypo-osmotic conditions impose stress to microorganisms. To adapt to osmotic downshock, cells need to release part of their cytoplasmic solutes. Stretch-activated channels have been implicated in facilitating the nonspecific exit of a wide variety of compounds (1, 27). Compatible solutes like glycine-betaine and proline are thought to be excreted via such osmotically regulated efflux systems (9). Furthermore, studies with *E. coli* have shown that the release of K^+, glutamate, and trehalose is mediated via similar efflux mechanisms (22, 26). The specificity of the efflux process is unclear. It has been suggested that efflux is nonspecific when the downshock is more severe and specific when less severe hypo-osmotic conditions are applied (22).

In this study the pool sizes of compatible solutes in *Lactobacillus plantarum* were determined by atomic absorption spectrophotometry and high-performance liquid chromatography (HPLC) analysis. The activation of the osmolyte transport

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systems in response to osmotic upshock and the release of solutes following osmotic downshock were studied.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *L. plantarum* ATCC 14917 was obtained from the American Type Culture Collection, Rockville, Md. Single colonies on MRS agar were transferred to MRS medium (4) containing 4 mM glucose. Exponentially growing cultures were supplemented with 10% (vol/vol) glycerol, rapidly frozen, and stored at −80°C. For all experiments cells were inoculated from a frozen stock into a chemically defined medium (CDM) at pH 6.7 containing 0.5% (wt/vol) glucose. The CDM was prepared according to the method of McFeeters and Chen (15) with minor modifications. The concentrations of vitamin B_{12}, *p*-aminobenzoic acid, folic acid, and biotin were increased to 10 mg/liter in the vitamin stock solution. High-osmolarity media were obtained by addition of 0.8 M KCl or 35% sucrose to the CDM. Unless indicated otherwise, cells were grown at 30°C.

Determination of intracellular K^+ and amino acid pools. Measurements of amino acid and K^+ pools were performed essentially as described by Kunji et al. (10). Exponentially grown cells were rapidly separated from the medium through cellulose nitrate filters with a 0.45-µm pore size (Schleicher & Schuell GmbH, Dassel, Germany) by using a manifold filtration apparatus (Hoefer, San Francisco, Calif.) and a DIVAC 2.4 L pump (Labelled AG, Cologne, Germany). Because of exopolysaccharide formation, dilute cell suspensions had to be used to collect the cells by filtration, which increased the variations in the K^+ measurements somewhat. Approximately 1 mg of total cell protein was retained on the filter. The cells were washed twice with 2 ml of ice-cold 100 mM sodium phosphate, pH 6.5, with or without 0.8 M NaCl (depending on the osmolarity of the medium). Filters were transferred to vials containing 0.3 ml of 5% (vol/vol) perchloric acid and 125 µM γ-aminobutyric acid (internal standard). After 30 min of incubation, the cellular extracts were centrifuged and the supernatant was used to determine the pool sizes. The K^+ concentrations were measured on an atomic absorption spectrophotometer (Perkin-Elmer 3030). For the analysis of the amino acids, 200 µl of extract was pipetted into an Eppendorf tube containing 200 µl of 1 M KOH–1 M KHCO₃ and samples were stored at −20°C. Subsequent derivatization with dansylchloride was performed as described by Tapuhi et al. (29) and Wiedmeier et al. (31). Dansylated amino acids were separated by reversed-phase HPLC as described previously (21).

Transport assays. (i) Radiolabelled compounds. Cells were harvested by centrifugation, washed twice with 50 mM potassium phosphate (pH 6.5), and resuspended to a protein concentration of approximately 10 mg/ml. Prior to transport, cells were diluted to a protein concentration of 0.02 to 0.04 mg/ml in 50 mM potassium phosphate, pH 6.5, containing 10 mM glucose. The pH dependence of transport was determined in 30 mM citric acid–30 mM K_2HPO_4–30 mM CHES [2-(*N*-cyclohexylamino)ethanesulfonic acid] (CKC buffer). The pH was adjusted to the appropriate values with KOH or HCl. After 5 min of preenergization at 30°C, transport was initiated by adding [*N-methyl*-¹⁴C]glycine-betaine, L-[U-¹⁴C]proline, L-[U-¹⁴C]glutamate, L-[U-¹⁴C]alanine, L-[U-¹⁴C]lysine, or L-[U-¹⁴C]valine. The osmolarity was varied by diluting the cells with assay buffers of identical composition with various concentrations of KCl, NaCl, sucrose, or lactulose. Osmotic downshock was performed by dilution with buffer containing 10 mM glucose and radiolabelled substrate. At given time intervals, samples of 100 or 200 µl were taken and diluted into 2 ml of ice-cold LiCl (0.1 to 0.8 M, depending on the osmolarity of the buffer). The samples were rapidly filtered through 0.45-µm-pore-size cellulose nitrate filters (Schleicher & Schuell) and washed with 2 ml of LiCl (0.1 to 0.8 M). The radioactivity on the filter was determined by liquid scintillation spectrometry.

(ii) Unlabelled amino acids. Experimental conditions for unlabelled amino acids were similar to those used for radiolabelled compounds, except that the filters were washed twice with 2 ml of ice-cold 100 mM sodium phosphate (pH 6.5) (containing 0 to 0.8 M Nacl, depending on the osmolarity of the buffer), and then the cells were extracted and amino acids were derivatized as described above.

Miscellaneous. Protein was measured by the method of Lowry et al. (13) with bovine serum albumin as a standard. The osmolarities of media and buffers were measured by freezing-point depression with an Osmomat 030 (Gonotec, Berlin, Germany). Growth experiments were performed in enzyme-linked immunosorbent plate assays. Plate wells containing 200 µl of culture were sealed by adding 75 µl of sterile silicone oil (1.03 g/ml), and growth rates were determined from A_{620} increases with a Multiscan MCC/340 MKII (Flow Laboratories, Lugano, Switzerland). Aerobic growth was monitored in flasks that were continuously shaken. Modelling of growth and calculation of specific growth rates were performed by using the modified Gompertz equation as described by Zwietering et al. (33). Intracellular concentrations were calculated by using the water-accessible cytoplasmic volume of *L. plantarum* as reported by Tseng et al. (30). Cytoplasmic volumes were shown to be constant under the different culture and assay conditions (low and high osmolarities) as inferred from flow cytometry measurements with a National Institute of Coastal and Marine Management optical plankton analyzer (5, 20). It was observed that forward light scatter, perpendicular light scatter, and time of flight were not affected by osmolarity (data not shown).
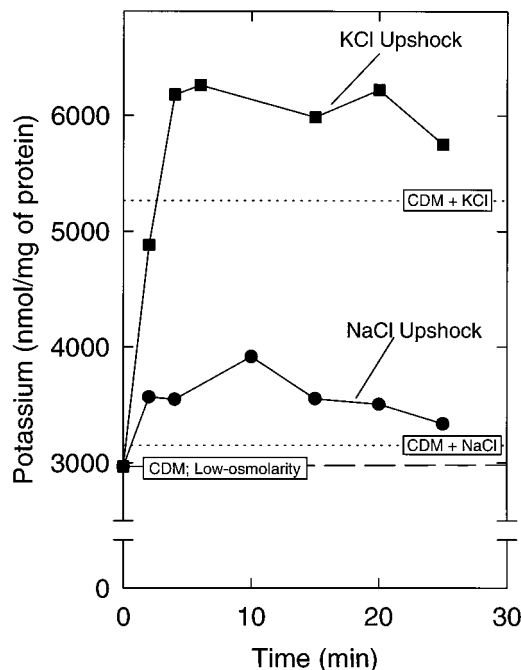

FIG. 1. Effect of hyperosmotic shock on the uptake of potassium in *L. plantarum* ATCC 14917. Cells were grown in a CDM until reaching an A_{660} of about 0.25 (exponential growth). Potassium uptake was assayed following the addition of KCl (squares) or NaCl (circles) to a final concentration of 0.8 M; the standard CDM contains 35 mM K^+. The steady-state intracellular K^+ concentrations of cells growing at high (dotted lines; CDM plus 0.8 M NaCl and CDM plus 0.8 M KCl) and low (dashed line) osmolarities are also shown. Potassium concentrations of cellular extracts were measured by atomic absorption spectrometry.

Synthesis of [*N-methyl*-¹⁴C]glycine-betaine. [*N-methyl*-¹⁴C]glycine-betaine was synthesized enzymatically from [*N-methyl*-¹⁴C]choline (55 mCi/mmol) as described elsewhere (11). The purity of the glycine-betaine was tested by thin-layer chromatographic analysis as described by Speed and Richardson (24). Radioactivity on the thin-layer chromatography plates showed a 100% conversion of choline to a product that comigrated with radiolabelled genuine glycine-betaine (made on request by Amersham, Buckinghamshire, England; 55 mCi/mmol).

Chemicals. The radiolabelled compounds [*N-methyl*-¹⁴C]choline (55 mCi/mmol), L-[U-¹⁴C]proline (260 mCi/mmol), L-[U-¹⁴C]alanine (174 mCi/mmol), L-[U-¹⁴C]glutamate (266 mCi/mmol), L-[U-¹⁴C]lysine (282 mCi/mmol), and L-[U-¹⁴C]valine (280 mCi/mmol) were obtained from Amersham. All other chemicals were of reagent grade and obtained from commercial sources.

RESULTS

Effects of osmotic stress on intracellular solute pools. (i) K^+ ions. To establish whether the response of *L. plantarum* towards increased medium osmolarity involves the accumulation of K^+, the ion content of cytoplasmic extracts of exponentially growing *L. plantarum* cells was analyzed by atomic absorption spectrometry. The steady-state K^+ concentrations of the cytoplasm increased from $3,000 \pm 500$ nmol/mg of protein in standard CDM to $5,300 \pm 900$ nmol/mg of protein in high-osmolarity medium (CDM plus 0.8 M KCl). Addition of 2.5 mM glycine-betaine to the low- as well as to the high-osmolarity medium did not affect the steady-state K^+ concentrations ($5,300 \pm 100$ nmol/mg). Uptake of K^+ is a primary event in the adaptation of many bacteria to hyperosmotic conditions (2). To establish whether this is also the case for *L. plantarum*, transport experiments in which K^+ uptake was measured directly after an increase in osmolarity were performed by adding 0.8 M NaCl or 0.8 M KCl to cells exponentially growing in CDM. In these cells K^+ transport was not significantly affected by an osmotic upshock with NaCl (Fig. 1). However, when
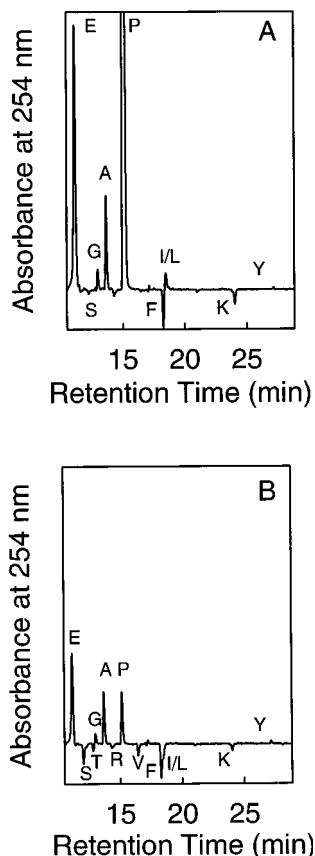
FIG. 2. HPLC elution profiles of dansylated amino acids in perchloric acid extracts from exponentially growing *L. plantarum* ATCC 14917. (A) Difference chromatogram from cells cultured in CDM and cells cultured in CDM supplemented with 0.8 M KCl; (B) the same as panel A except that 2.5 mM glycine-betaine was present in both media.

these cells were subjected to an osmotic upshock with KCl, the K^+ pools increased in the first 5 min after the upshock. After this increase, the cytoplasmic K^+ levels slowly decreased towards the steady-state values that were observed for cells growing in high-osmolarity CDM plus 0.8 M KCl (Fig. 1). Assuming that the increased K^+ concentrations upon addition of 0.8 M KCl indeed reflect the intracellular pool, the uptake must be due to a system with a very low affinity, because the standard CDM already contained 35 mM potassium. Since 0.8 M NaCl does not enhance the cytoplasmic K^+ concentration in exponentially growing cells, the experiments are taken as an indication that K^+ uptake has no role in the response of *L. plantarum* towards an increase in osmolarity.

(ii) **Amino acids.** In osmotically stressed bacteria several amino acids are known to accumulate to high intracellular concentrations (2). In *L. plantarum* the levels of glutamate, proline, alanine, and glycine increased 6-, 35-, 3-, and 48-fold, respectively, upon growth in CDM plus 0.8 M KCl relative to levels in CDM (Fig. 2). The increase in glutamate levels is quantitatively most important (Table 1). Glutamate is an essential amino acid for *L. plantarum* that has to be taken up (12, 19). Since glycine was not present in the medium, the increase in glycine concentrations must be due to de novo synthesis. The increase in proline and alanine pools can be due to either increased synthesis and/or increased transport rates.

Pool sizes in the presence of glycine-betaine. When glycine-betaine was added to high-osmolarity media, decreases in the steady-state concentrations of proline, glutamate, alanine, and glycine were observed. The pool sizes, however, remained greater than in cells that were precultured in medium with a low osmolarity (Table 1; Fig. 2). The K^+ ion concentration of the cytoplasm was not affected by glycine-betaine; however, the expected drop in intracellular potassium could have been masked by the relatively large error in the K^+ ion measurements. In transport experiments, glycine-betaine accumulated to approximately 1,500 nmol/mg of protein in both 0.8 M KCl- and 35% sucrose-stressed cells. Osmotically nonstressed cells of *L. plantarum*, which were grown in the presence of glycine-betaine, accumulated this osmolyte to approximately 300 nmol/mg of protein. Thus, the addition of glycine-betaine to a chemically defined growth medium with a high osmolarity resulted in large intracellular pools of glycine-betaine, while the concentrations of most other osmotically important solutes were lowered. Glycine-betaine was accumulated only when the compound was added to the growth medium, indicating that *L. plantarum* cannot synthesize this osmolyte (data not shown).

Growth rates of *L. plantarum* under different osmotic stresses. The effects of the major osmolytes glycine-betaine and proline on the osmolarity dependence of the growth rate were investigated next. Under conditions in which growth was limited by high concentrations of KCl (Fig. 3A) or NaCl (data not shown), addition of 2.5 mM glycine-betaine or proline increased the specific growth rates of *L. plantarum*. The increase in growth rate was observed both aerobically (data not shown) and anaerobically (Fig. 3A). Stimulation of growth by glycine-betaine was achieved within 60 min after the addition of the osmolyte (Fig. 3B). Addition of glycine-betaine or proline to cells cultured under sucrose stress did not significantly increase the specific growth rate (data not shown). In fact, the specific growth rates under sucrose stress were similar to those under KCl or NaCl stress in the presence of glycine-betaine. The specific growth rates of *L. plantarum* were also not significantly affected by glycerol up to 2.5 osmol/kg (data not shown). Moreover, glycine-betaine had no effect on the growth of glycerol-stressed cells. The results overall indicate that osmotic stress due to sucrose (or other sugars) is less deleterious to the cells than osmotic stress due to salts, possibly because the cells are able to use the sugars (or derived compounds) as osmoprotectants.

TABLE 1. Effect of osmotic stress on the intracellular amino acid pools in *L. plantarum*[a]

Amino acid	External concn (mM)	Intracellular concn (nmol/mg of protein) in:			
		CDM	CDM + betaine[b]	CDM + KCl[c]	CDM + KCl + betaine[b,c]
Glu	1.2	57	36	280	110
Gln	0.0	6.0	9.9	48	15
Lys	1.0	12	7.2	4.5	4.2
His	1.0	1.5	0.9	<0.3	0.3
Arg	0.6	11	8.1	3.6	3.3
Val (+Met)	1.3	14	9.0	11	8.7
Ala	0.7	23	18	63	39
Gly	0.0	<0.3	<0.3	14	8.4
Pro	0.6	7.2	4.2	250	9.9
Ser	0.5	19	22	23	7.8
Thr	0.3	23	19	16	9.9
Tyr	0.1	2.4	1.2	2.7	2.4

[a] The data are the means for duplicate experiments; the differences between results of individual experiments were less than 10%. Trp, Phe, Leu, and Ile concentrations were not determined.
[b] Betaine (2.5 mM) was added to the medium.
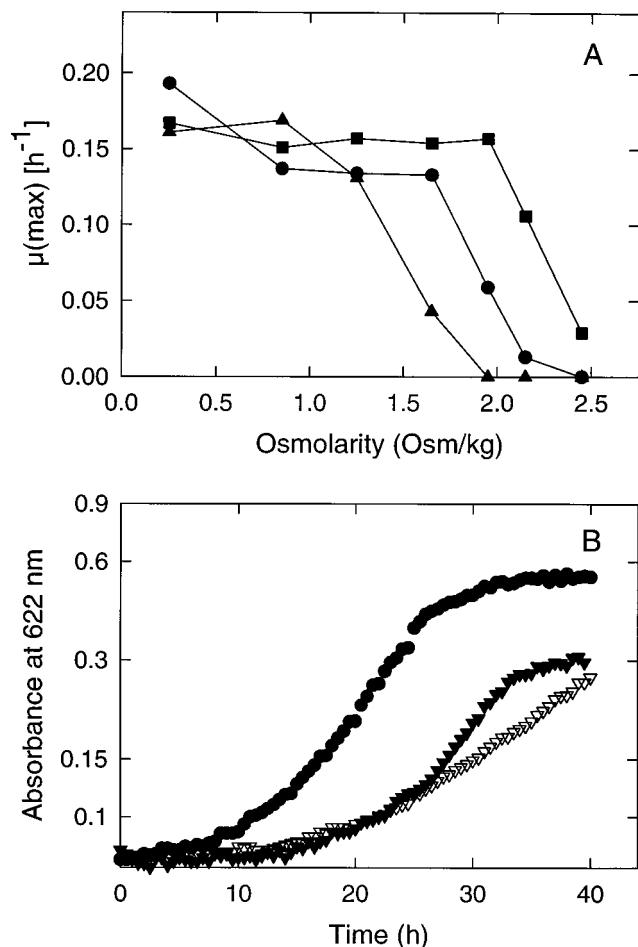[c] KCl (0.8 M) was added to the medium.

FIG. 3. Effects of medium osmolarity, glycine-betaine, and choline on the growth rate of *L. plantarum* ATCC 14917 under anaerobic conditions. (A) The osmolarities were raised by adding KCl at the concentration indicated. Growth was determined in CDM without (circles) and with 2.5 mM glycine-betaine (squares) or 2.5 mM choline (triangles). (B) Effect of glycine-betaine (added at 25 h) on the growth rate. Cells were grown in CDM (circles) and CDM supplemented with 1.0 M KCl (closed and open triangles); the effect of 2.5 mM sterile glycine-betaine added at 25 h is shown by closed triangles.

In some bacteria choline is a precursor for aerobic synthesis of glycine-betaine. Choline, however, was not able to alleviate growth inhibition in aerobically grown cells of *L. plantarum* under KCl and NaCl stress (data not shown).

Effects of osmolarity on uptake of amino acids and compatible solutes. On the basis of the pool sizes for glycine-betaine and its growth-stimulating effect under conditions of high medium osmolarity, glycine-betaine seems to be preferentially accumulated in *L. plantarum*. The protection against osmotic stress by glycine-betaine required the addition of the compatible solute to the medium. Transport experiments were performed to establish if, and at which level, glycine-betaine uptake was regulated by osmolarity. In order to be able to raise the osmolarity of the assay buffer, the cells were washed and resuspended in 50 mM potassium phosphate, pH 6.5, irrespective of whether the cells were grown in low- or high-osmolarity media. Control experiments indicated that the washing with hypotonic media did not affect the glycine-betaine transport rates compared with rates after washing with isotonic media (data not shown); notice that the standard CDM does not contain glycine-betaine, and therefore the intracellular glycine-

betaine concentrations are always zero at the start of the experiment.

The capacity of *L. plantarum* to transport glycine-betaine was increased threefold when cells were cultured in high-osmolarity media relative to that in low osmolarity media (Fig. 4). This increase most likely reflects an enhanced expression of the transport system. The transport rates and accumulation levels of glycine-betaine rapidly increased when the osmolarity of the assay buffer was raised (Fig. 4), even when protein synthesis was inhibited by chloramphenicol (100 μg/ml) (data not shown). This suggest that osmotic regulation of the glycine-betaine transport system occurs not only at the genetic level but also at the protein level (activation). A similar activation of transport was observed for proline (data not shown). Transport of alanine could not be detected. Glutamate uptake, on the other hand, was not activated when the osmolarity of the assay buffer was increased (Fig. 4), but the expression level of the glutamate uptake system was higher in cells precultured on high-osmolarity medium compared with that in cells precultured in low-osmolarity medium. Neither activation of transport nor changes in expression as a result of increased medium osmolarity were observed for the uptake of valine and lysine (Fig. 4). In fact, the rates of transport were lower when a high concentration of KCl was present in the assay buffer, which is consistent with the observation that the pool sizes of these amino acids are somewhat decreased under osmotic stress (Table 1).

Activation of glycine-betaine transport. Activation of glycine-betaine transport was observed in the pH range of 4.5 to
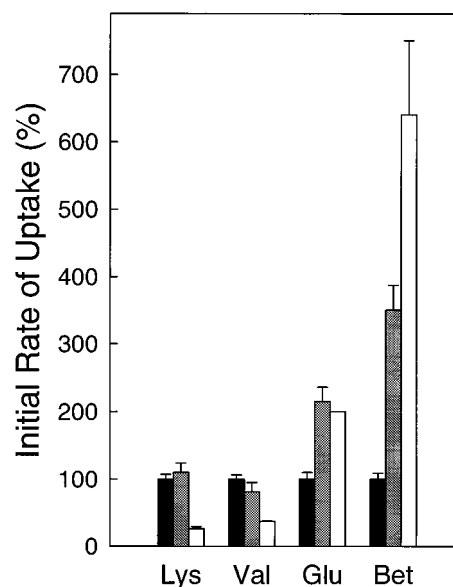

FIG. 4. Effect of hyperosmotic shock on the uptake of glycine-betaine and amino acids. Cells were grown on CDM without (solid bars) and with (open bars and cross-hatched bars) 0.8 M KCl. Uptake of L-[U-14C]lysine (3.2 μM final concentration), L-[U-14C]valine (3.6 μM final concentration), L-[U-14C]glutamate (1.3 mM final concentration), and [N-methyl-14C]glycine-betaine (1.3 mM final concentration; Bet) was assayed in 50 mM potassium phosphate, pH 6.5, at a final protein concentration of 0.02 mg/ml. After 6 min of preenergization with 10 mM glucose, uptake was initiated ($t = 0$) by adding labelled substrate. Cross-hatched and solid bars represent uptake without 0.85 M KCl added to the assay buffer. Open bars represent uptake with 0.85 M KCl added to the assay buffer. Open bars represent uptake with 0.85 M KCl added to the assay buffer. The 100% values correspond with uptake rates of 0.53 nmol/min/mg of protein for lysine, 0.46 nmol/min/mg of protein for valine, 16 nmol/min/mg of protein for glutamate, and 11 nmol/min/mg of protein for glycine-betaine. Data represent the means for triplicate experiments. Standard deviations are given.
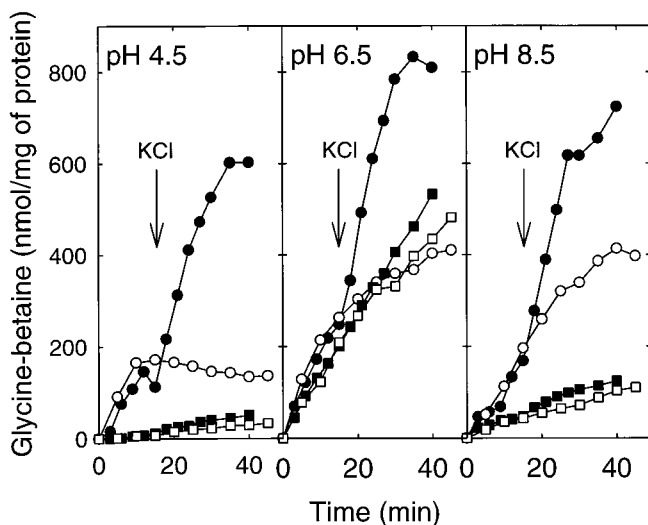
FIG. 5. Effect of external pH and preculture conditions on the activation of glycine-betaine transport. Cells were grown on CDM without (squares) and with (circles) 0.8 M KCl. After 5 min of preenergization with 10 mM glucose, uptake was initiated ($t = 0$) by the addition of [^{14}C]glycine-betaine (1.3 mM final concentration). Uptake was assayed in CKC buffer at different pHs at final protein concentrations of 0.31 to 0.42 mg/ml. Open and closed symbols represent uptake without and with, respectively, 0.85 M KCl added at 15.5 min (indicated by the arrows).

8.5 (Fig. 5). The activation was most prominent in cells that were grown under osmotic stress, i.e., 35% sucrose (data not shown) or 0.8 M KCl (Fig. 5). This suggests that at high medium osmolarity a second transport system that requires the high osmolarity for activity is expressed or, alternatively, that some inducible factor stimulates the constitutive glycine-betaine transport activity.

The final level of accumulation of glycine-betaine was proportional to the increase in medium osmolarity, but the initial rates of uptake were similar irrespective of whether the osmotic strength was raised by 0.1, 0.2, 0.4, 0.8, or 1.2 M KCl (Fig. 6). Iso-osmotic concentrations of sucrose, lactulose, NaCl, and KCl had similar effects on the transport rates and accumulation levels for glycine-betaine (data not shown). Thus, the final internal concentrations of glycine-betaine are solely determined by the osmotic strength of the assay buffer, indicating that activation of transport is caused by a disturbance of osmostasis. In the absence of glycine-betaine, the turgor pressure might be restored by the uptake of ions from the buffer (K$^+$, Cl$^-$, or P$_i$) and/or synthesis of osmolytes in the cell. To test whether the cell is able to restore osmostasis through any of these mechanisms, the uptake of glycine-betaine uptake was assayed 0, 10, and 30 min after the upshock. These experiments clearly showed that the initial rate of glycine-betaine uptake is not affected when the osmotic upshock is given up to 30 min prior to the initiation of transport (Fig. 7). The accumulation levels, on the other hand, decreased when the time period between upshock and initiation of transport was elongated (Fig. 7). These experiments indicate that *L. plantarum* is unable to restore osmostasis rapidly when glycine-betaine is absent, even though K$^+$ plus some anions and glucose are present in the medium. The data overall also suggest that glycine-betaine transport rates are increased (activated transport) when the difference between internal and external osmolarities exceeds a certain threshold value (Fig. 6 and 7). When the external and internal osmolarities are balanced (e.g., because of glycine-betaine

uptake), the signal causing activated transport disappears and net glycine-betaine transports stops. The final accumulation level of glycine-betaine seems to be determined by the extent of the osmotic shift.

Effect of osmotic downshock on glycine-betaine and proline transport. Osmotic downshock caused a rapid efflux of both glycine-betaine and proline at a rate that was much higher than the respective uptake rates (Fig. 8). Furthermore, the drop in intracellular concentration of glycine-betaine after an osmotic downshock depended on the dilution factor, indicating that efflux, like uptake, is dependent on the osmolarity of the medium. The effects of an osmotic downshock on the amino acid pools were analyzed in cells that were grown in CDM with a high osmolarity (1.51 osmol/kg). It was observed that the cytoplasmic pools of proline, alanine, and glutamate decreased rapidly when the cells were subjected to an osmotic downshock. The glutamate pools, however, recovered to normal values within 10 min following the osmotic downshock (not shown), whereas proline and alanine pools remained substantially lower (Fig. 9). Cytoplasmic lysine and valine concentrations increased somewhat upon osmotic downshock, while the levels of tyrosine, glycine, and others (not shown) were not affected. From these experiments it is concluded that solute efflux upon osmotic downshock occurs for only a limited number of compatible solutes.

Finally, the pools of glycine-betaine were also lowered rapidly when the osmotic downshock followed depletion of the cells from metabolic energy by incubation with 500 μM iodoac-
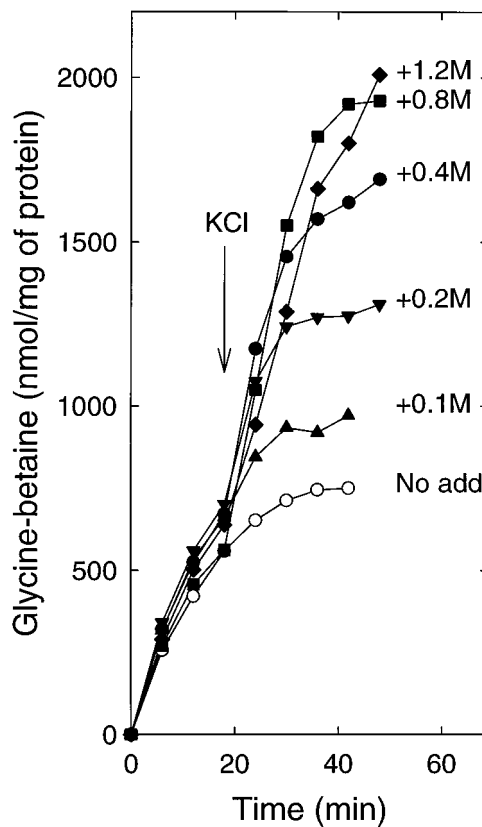

FIG. 6. Glycine-betaine transport at varying medium osmolarities. Cells were grown on CDM containing 0.8 M KCl, washed, and resuspended in potassium phosphate, pH 6.5. After 6 min of preenergization with 10 mM glucose, uptake was initiated ($t = 0$) by the addition of [^{14}C]glycine-betaine (1.3 mM final concentration). The final protein concentration was 0.24 mg/ml. The medium osmolarity was varied by adding KCl at 18.5 min (indicated by the arrow).
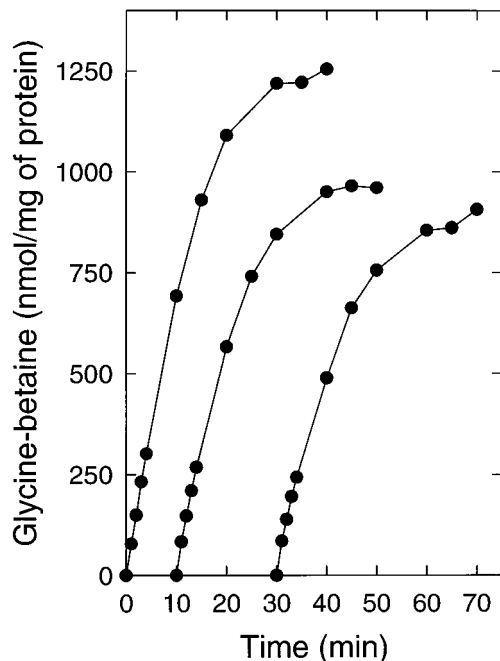
FIG. 7. Glycine-betaine uptake in cells in which the period between osmotic upshock and initiation of glycine-betaine uptake was varied. Cells were grown in a CDM containing 0.8 M KCl. Uptake of [14C]glycine-betaine (1.3 mM final concentration) was assayed in 50 mM potassium phosphate, pH 6.5, at a final protein concentration of 0.20 mg/ml. After 4.5 min of preenergization with 10 mM glucose, a hyperosmotic shock was given by adding KCl to a final concentration of 0.85 M ($t = 0$). Labelled glycine-betaine was added after 0, 10, or 30 min as indicated in the graph.

etate for 15 min (data not shown). The iodoacetate treatment resulted in a complete inhibition of glycine-betaine and leucine uptake. These data suggest that efflux does not require metabolic energy.

DISCUSSION

Compatible solutes are accumulated by microorganisms in response to osmotic stress and allow the organisms to grow over a wide range of osmolarities. Intracellular pools of compatible solutes can be raised by increased synthesis or uptake from the medium. In this study the pools of compatible solutes (K^+, amino acids, and glycine-betaine) and the mechanisms of osmostasis in *L. plantarum* have been investigated. *L. plantarum* was cultured on a CDM in order to be able to relate changes in pool sizes to synthesis and/or transport.

When glycine-betaine was added to the medium, a significant increase in the growth rate was observed, especially at higher osmolarities. Growth stimulation by glycine-betaine at high osmolarities was most pronounced when KCl and NaCl were added to the medium and was not observed under sucrose or glycerol stress. The growth rates of sucrose-stressed cells were higher than those of KCl- or NaCl-stressed cells, indicating that the cytoplasmic osmolarity was probably balanced (partly) through accumulation of this sugar or a compound derived from sucrose. Alternatively, osmolytes that protect the cells equally well as glycine-betaine might be synthesized when the cells are sucrose stressed. Nuclear magnetic resonance spectroscopy measurements have confirmed that when glycine-betaine is present in the medium, it is accumulated in the cytoplasm of salt-stressed cells and that this

compound is the main (organic) osmolyte in *L. plantarum* under these conditions (unpublished results).

When glycine-betaine is omitted from a high-osmolarity medium, other organic osmolytes are expected to accumulate under hyperosmotic stress. Indeed, analysis of the cytoplasmic amino acid pools in *L. plantarum* showed that the internal concentrations of alanine, glycine, proline, and glutamate are elevated under these conditions. From these, only glutamate and proline contribute significantly to the total cytoplasmic osmolarity (280 and 260 nmol/mg of protein, respectively). However, the sum of the concentrations of these amino acids is much smaller than the amount of glycine-betaine that is accumulated in cells growing in high-osmolarity medium. Addition of glycine-betaine to the high-osmolarity CDM resulted in its accumulation to about 1,500 nmol/min/mg of protein, while the increases in glutamate, proline, alanine, and glycine were substantially lower (Table 1). In the absence of glycine-betaine, the increases in osmolytes identified can only partly compensate for the increase in external osmolarity. We hope to detect and identify possible other organic osmolytes in cells growing in the absence of glycine-betaine by natural-abundance ^{13}C nuclear magnetic resonance spectroscopy.

Not only are organic osmolytes important for the response of bacteria to hyperosmotic conditions, but also potassium is known to be a key molecule for osmotic adaptation in many (especially gram-negative) bacteria. The steady-state intracellular concentration of K^+ increased from 3,000 to 5,300 nmol/mg of protein when *L. plantarum* was cultured in CDM to which 0.8 M KCl was added. When *L. plantarum* was cultured in CDM containing 0.8 M NaCl, the K^+ pools were hardly increased (Fig. 1). An apparent uptake of K^+ from the CDM is observed only when 0.8 M KCl is used to raise the osmolarity of the medium; addition of 0.8 M NaCl does not
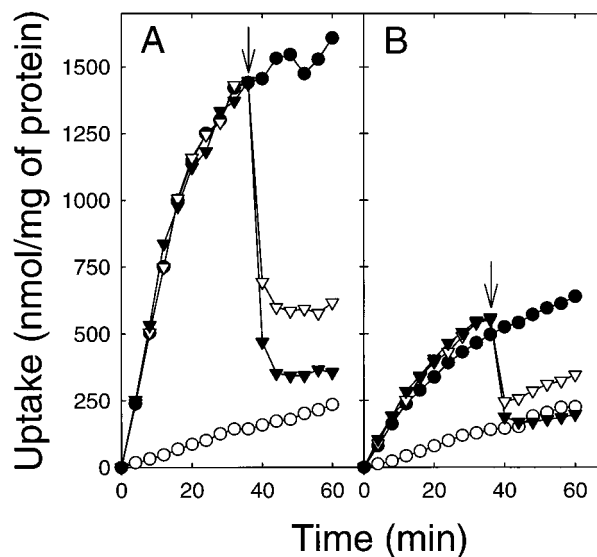

FIG. 8. Glycine-betaine and proline efflux in response to hypo-osmotic shock. Cells were grown in CDM containing 0.8 M KCl. Uptake of [14C]glycine-betaine (1.3 mM final concentration) (A) and L-[14C]proline (1.3 mM final concentration) (B) was assayed in 50 mM potassium phosphate, pH 6.5, at a final protein concentration of 0.20 mg/ml. After 5 min of preenergization (10 mM glucose), uptake was initiated ($t = 0$) by the addition of labelled substrate (open circles) or by the addition labelled substrate together with 0.85 M KCl (closed circles and open and closed triangles). After 36.5 min the samples with KCl were diluted 3-fold (open triangles) or 5.5-fold (closed triangles) with potassium phosphate, pH 6.5, containing 10 mM glucose and 1.3 mM radioactively labelled substrate (indicated by the arrows).
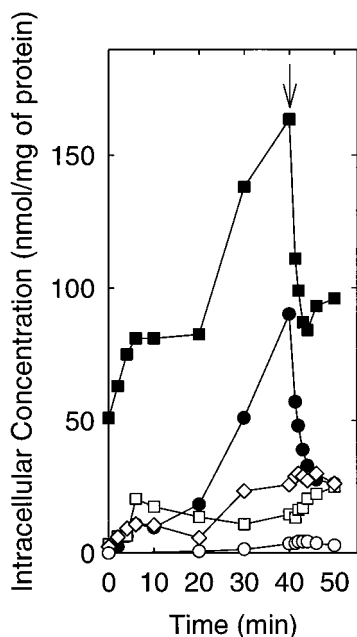
FIG. 9. Effect of hypo-osmotic shock on the internal pools of alanine (closed squares), proline (closed circles), glycine (diamonds), valine (open squares), and tyrosine (open circles). Cells were grown on CDM containing 0.8 M KCl and washed twice with 50 mM potassium phosphate (pH 6.5), and 1 min prior to energization with 10 mM glucose ($t = 0$), the cells were resuspended to a final protein concentration of 0.67 mg/ml in CDM containing all amino acids plus 0.8 M KCl (pH 6.5). After 40.5 min, the samples were diluted fivefold with CDM supplemented with 10 mM glucose but without KCl (indicated by the arrows). Total amino acid pools were quantified by HPLC analysis of perchloric acid extracts.

result in an increased uptake of K^+ (Fig. 1). At present we cannot exclude the possibility that the increase in K^+ concentration upon increasing the medium osmolarity with 0.8 M KCl is due to nonspecific binding of the cation to the cell surface. On the other hand, the increase in K^+ occurs over a period of 4 min (Fig. 1) and the cells are washed extensively with K^+-free media (iso-osmotic) prior to the determination of the K^+ contents. Since an increase in medium osmolarity does not per se result in significant changes in the K^+ concentrations (see NaCl upshock), the experiments overall suggest that the primary response towards hyperosmotic conditions in *L. plantarum* does not involve a rapid uptake of potassium. This notion is confirmed by the experiment in which the time between osmotic upshock imposed by KCl and the addition of glycine-betaine was varied (Fig. 7). If one assumes that the period of activated glycine-betaine uptake lasts only when there is an osmotic inbalance, the cell is still far from osmostasis 30 min after the upshock. The decreased glycine-betaine levels in the assays initiated 30 min after the upshock (about 30% decrease relative to the zero time assays) could reflect a partial restoration of turgor pressure (Fig. 7).

The increased pool sizes for proline and glycine-betaine in high-osmolarity media correlate with an activation of the corresponding transport system(s) due to osmolarity changes (stress imposed by lactulose, sucrose, KCl, and NaCl). The mechanism of this activation is not known, but it is possible that a disturbance of osmostasis is sensed by the transporter(s) through changes in membrane tension. Glycine-betaine and proline uptake in *L. plantarum* cells, grown in CDM plus 0.8 M KCl, exhibits monophasic kinetics under assay conditions of low and high osmolarity. The apparent V_{max} and K_m of trans-

port are increased seven- and fivefold, respectively (unpublished results). The activation of glycine-betaine uptake is most pronounced (largest V_{max} effect) when cells are pregrown in the presence of high salt (or sucrose). This suggests that high medium osmolarity turns a high-affinity (low-apparent-K_m), low-capacity (low-V_{max}) system into a low-affinity, high-capacity system. However, the possibility that activation of the low-affinity, high-capacity system masks the kinetics of the high-affinity, low-capacity system cannot yet be excluded. Currently, we are trying to isolate glycine-betaine and proline transport mutants in order to discriminate between these two possibilities. Interestingly, those solutes of which the accumulation is stimulated upon osmotic upshock (e.g., glycine-betaine, alanine, and glutamate) are rapidly released upon osmotic downshock. Large stretch-activated channels are thought to be responsible for this efflux (1). By isolating the appropriate transport mutants, we hope to establish whether regulation of transport of compatible solutes takes place at the level of influx or efflux or both.

Transport of glutamate is not directly affected by a high osmolarity of the assay medium, irrespective of how the cells are grown. The glutamate transport activities, however, are higher in cells grown at high than at low osmolarity, indicating that glutamate transport is affected by the medium osmolarity at the level of protein synthesis. Since protein synthesis is relatively slow, it is unlikely that accumulation of glutamate is crucial in the primary response towards a hyperosmotic shock. On the contrary, glycine-betaine and proline are accumulated immediately via activation of their respective transport systems when the medium osmolarity is raised. At a later stage glycine-betaine and proline concentrations are raised further via induction of the transport system(s) in a way similar to that observed for glutamate.

Glycine-betaine accumulation was not only observed when the medium osmolarity was high (1.51 osmol/kg), but significant amounts of glycine-betaine were also accumulated when the cells were grown without addition of KCl, NaCl, or sucrose (0.20 osmol/kg). Similar observations have been made for other gram-positive bacteria, but this is not observed for gram-negative species. This difference may be related to the fact that, in general, gram-positive bacteria maintain a higher turgor pressure (20 to 25 atm [ca. 2,000 to 2,500 kPa]) than gram negative strains (3 to 5 atm [ca. 300 to 500 kPa]). Compatible solutes, which are not interfering with essential cellular functions, are therefore crucial to regulate the high turgor pressure, in particular in gram-positive bacteria.

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