

# Glycine Betaine Fluxes in *Lactobacillus plantarum* during Osmostasis and Hyper- and Hypo-osmotic Shock\*

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**Bacteria respond to changes in medium osmolarity by varying the concentrations of specific solutes in order to maintain constant turgor. The primary response of *Lactobacillus plantarum* to an osmotic upshock involves the accumulation of compatible solutes such as glycine betaine, proline, and glutamate. We have studied the osmotic regulation of glycine betaine transport in *L. plantarum* by measuring the overall and unidirectional rates of glycine betaine uptake and exit at osmostasis, and under conditions of osmotic upshock and downshock. At steady state conditions, a basal flux of glycine betaine (but no net uptake or efflux) is observed that amounts to about 20% of the rate of "activated" uptake (uptake at high osmolarity). No direct exchange of <sup>14</sup>C-labeled glycine betaine in the medium for unlabeled glycine betaine in the cytoplasm was observed in glucose metabolizing and resting cells, indicating that a separate glycine betaine efflux system is responsible for the exit of glycine betaine. Upon osmotic upshock, the uptake system for glycine betaine is rapidly activated (within seconds), whereas the basal efflux is inhibited. These two responses account for a rapid accumulation of glycine betaine until osmostasis is reached. Upon osmotic downshock, glycine betaine is rapidly released by the cells in a process that has two kinetic components, *i.e.* one with a half-life of less than 2 s which is unaffected by the metabolic status of the cells, the other with a half-life of 4–5 min in glucose-metabolizing cells which is dependent on internal pH or a related parameter. We speculate that the former activity corresponds to a stretch-activated channel, whereas the latter may be facilitated by a carrier protein. Glycine betaine uptake is strongly inhibited immediately after an osmotic downshock, but slowly recovers in time. These studies demonstrate that in *L. plantarum* osmostasis is maintained through positive and negative regulation of both glycine betaine uptake and efflux, of which activation of uptake upon osmotic upshock and activation of a "channel-like" activity upon osmotic downshock are quantitatively most important.**

In bacteria the intracellular concentration of compatible solutes is regulated by the osmolarity of the environment, which involves changes in transport activities as well as synthesis and/or degradation of these compounds (1). The regulation occurs both at the genetic (transcription) and enzyme (activa-

tion) level. For instance, in *Escherichia coli* and *Salmonella typhimurium* glycine betaine and proline are taken up via the constitutive, low affinity ProP system and the inducible, high affinity ProU system (1). The transport of proline and glycine betaine via ProP and ProU is stimulated by an increase in external osmolarity (2, 3). In addition, the ProP and ProU expression levels are also increased when the medium osmolarity is raised (2). Exit of glycine betaine and proline in *E. coli* and *S. typhimurium* is thought to be mediated by specific efflux systems, but the molecular evidence is limited (4–6).

Upon moderate osmotic downshock (from 0.5–0.8 to 0.2–0.3 M NaCl or KCl), bacteria specifically release compatible solutes such as K<sup>+</sup>, trehalose, glutamate, proline, and glycine betaine, whereas other low molecular weight compounds are retained by the cells (4–7). It has been suggested that a severe osmotic downshock leads to a more aspecific efflux of solutes (6, 8). Experiments with artificial membranes that were loaded with carboxyfluorescein indicated that low molecular weight solutes are able to cross (aspecifically) the lipid bilayer under conditions of osmotic downshock (9, 10), but no data are available about the significance of such events *in vivo*. Mechanosensitive channels that respond to changes in turgor pressure are thought to be involved in the specific release of solutes upon osmotic downshock (4, 6, 11). The activity of these channels has been shown in patch clamp experiments using giant azolectin liposomes that were fused with bacterial membranes or spheroplasts. In both Gram-negative and -positive bacteria, the opening and closing of channels with different conductivities can be triggered by suction in patch clamp experiments (12–14). Recently, the gene encoding one of the channels, *MscL*, of *E. coli* was cloned and sequenced, and the channel activity of the purified protein was demonstrated after reconstitution into artificial liposomes (8, 15).

A number of inhibitors of mechanosensitive channels have been described in eukaryotic cells (16–18). Some of these inhibitors have been used to modulate channel activity in bacteria, of which only gadolinium was found to inhibit the release of specific solutes upon osmotic downshock (6, 11). In patch clamp experiments with giant liposomes fused with membranes of *E. coli*, gadolinium (Gd<sup>3+</sup>) specifically inhibited the mechanosensitive channel activities, although about 10-fold higher concentrations of Gd<sup>3+</sup> were needed to inhibit the bacterial channels than the eukaryotic ones (11). Gadolinium also inhibited the purified and reconstituted *MscL* channel of *E. coli* in liposomes (15) and the ion is therefore considered to be a relatively specific inhibitor of mechanosensitive channels in bacteria.

In *Lactobacillus plantarum*, the intracellular concentration of glycine betaine, proline, glutamate, and alanine are specifically affected under conditions of osmotic imbalance (7). In this study, we report on the fluxes of glycine betaine in *L. plantarum* using dual label experiments. This allows us to follow the inward and outward fluxes simultaneously, under osmostasis

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as well as under conditions of changing osmolarity (osmotic upshock and downshock). These studies provide, for the first time, a rather complete picture of the intricacies of the regulation of transport of compatible solutes in bacteria.

#### MATERIALS AND METHODS

##### Bacterial Strains and Culture Conditions

*L. plantarum* was grown in chemically defined medium at pH 6.7, containing 0.5% (w/v) glucose at 30 °C as described previously (7). High osmolarity media were obtained by adding 0.8 M KCl to the chemically defined medium.

##### Transport Assays

**Uptake and Efflux in Glucose-metabolizing Cells**—Unless specified otherwise, 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 0.1–0.6 mg/ml in 50 mM potassium phosphate, pH 6.5. Cells were preenergized at 30 °C for 5 min by the addition of 10 mM glucose, after which transport was initiated by the addition of radiolabeled substrate. To increase the medium osmolarity aliquots of 3 M KCl or buffer were added simultaneously with the radiolabeled substrate. Osmotic downshock was performed by diluting the assay medium with buffer (preheated at 30 °C) containing 10 mM glucose and radiolabeled substrate(s). Differential labeling (*[N-methyl-<sup>3</sup>H]* glycine betaine and *[N-methyl-<sup>14</sup>C]* glycine betaine) was used to follow the unidirectional rates of uptake and efflux. The pH profile of glycine betaine efflux was determined in 30 mM citric acid, 30 mM K<sub>2</sub>HPO<sub>4</sub>, and 30 mM CHES,<sup>1</sup> adjusted to the appropriate pH with KOH (CKC buffer). At given time intervals, samples of 100–500 µl were taken and diluted into 2 ml of ice-cold LiCl (0.1–0.8 M, identical to the osmolarity of the buffer). The samples were rapidly filtered through 0.45-µm pore-size cellulose-nitrate filters (Schleicher & Schuell GmbH, Dassel, Germany) and washed with 2 ml of LiCl (0.1–0.8 M).

**Exchange and Efflux in Resting Cells**—Cells (1 mg of protein/ml) were loaded with *[N-methyl-<sup>14</sup>C]* glycine betaine in the presence of 10 mM glucose under conditions of high osmolarity (50 mM potassium phosphate, pH 6.5, plus 0.8 M KCl). After 60 min of uptake, the cells were centrifuged, washed twice with potassium phosphate, pH 6.5, containing 0.8 M KCl, and finally resuspended to a protein concentration of 6–10 mg/ml. To monitor exchange and/or efflux, the cells were diluted 40-fold into potassium phosphate, pH 6.5, and of varying osmolarity with or without 100 mM unlabeled glycine betaine. All experiments were carried out at 30 °C. Further handlings were the same as described under "Uptake and Efflux in Glucose-metabolizing Cells."

##### Preparation and Loading of Membrane Vesicles and (Proteo)liposomes

Membrane vesicles of *L. plantarum* were isolated as described by Otto *et al.* (19) using 0.3 mg of deoxyribonuclease and ribonuclease/ml (final concentration). Liposomes were prepared from egg phosphatidylcholine and purified *E. coli* phospholipids (Sigma) in a ratio of 1:3 (20). Cytochrome *c* oxidase-containing proteoliposomes were prepared according to Driessen *et al.* (20). Fusion of liposomes with membrane vesicles and loading with *[<sup>14</sup>C]* glycine betaine was done by freeze-thaw-extrusion. The mixture of liposomes and membrane vesicles was rapidly frozen in liquid nitrogen, thawed slowly at room temperature, and subsequently extruded 11 times through 400-nm pore-size polycarbonate filters (Avestin) (21). Transport was assayed as described above.

##### Miscellaneous

Protein was determined by the method of Lowry *et al.* (22) 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).

##### Synthesis of *[N-methyl-<sup>14</sup>C]* Glycine Betaine and *[N-methyl-<sup>3</sup>H]* Glycine Betaine

Radiolabeled glycine betaine was synthesized enzymatically from *[N-methyl-<sup>14</sup>C]*- or *[N-methyl-<sup>3</sup>H]* choline chloride (40–60 mCi/mmol and 60–90 Ci/mmol, respectively) as described by Landfald and Strøhm (23). The purity of the glycine betaine was checked by thin layer chromatography according to Speed and Richardson (24). Radioactivity on

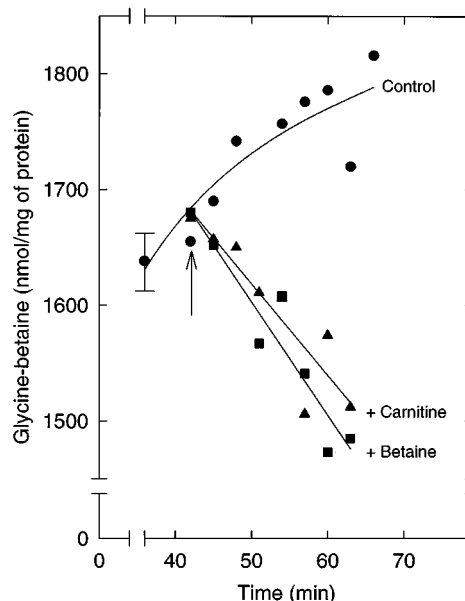


FIG. 1. Glycine betaine exit in glucose metabolizing cells of *L. plantarum*. Cells were grown on chemically defined medium containing 0.8 M KCl, washed, and resuspended in potassium phosphate, pH 6.5. After 6 min of preenergization with 10 mM glucose, uptake was initiated at zero time by the addition of *[<sup>14</sup>C]* glycine betaine (final concentration of 1.3 mM) and KCl (final concentration of 0.8 M). After 46 min of uptake (indicated by the arrow), a 77-fold excess of unlabeled glycine betaine (squares), carnitine (triangles), or an equivalent volume of water (circles) was added. The final protein concentration was 0.15 mg/ml.

the TLC plates showed a 100% conversion of choline to a product that co-migrated with <sup>14</sup>C-labeled genuine glycine betaine (made on request by Amersham Corp., 55 mCi/mmol).

##### Chemicals

Radiolabeled *[N-methyl-<sup>14</sup>C]* choline-chloride and *[N-methyl-<sup>3</sup>H]* choline-chloride (55 mCi/mmol and 80 Ci/mmol, respectively) were obtained from DuPont NEN. Radiolabeled L-*[U-<sup>14</sup>C]* lysine (282 mCi/mmol), L-*[U-<sup>14</sup>C]* leucine (312 mCi/mmol), and L-*[U-<sup>14</sup>C]* glutamic acid (266 mCi/mmol) were obtained from Amersham Corp. (Buckinghamshire, UK). All other chemicals were of reagent grade and obtained from commercial sources.

##### RESULTS

**Characterization of Glycine Betaine Exit**—Since ATP synthesis, but not the proton motive force, was required for glycine betaine uptake,<sup>2</sup> we speculate that this osmolyte is taken up by an ATP-dependent transporter rather than a secondary transport system. ATP-dependent solute uptake systems generally operate unidirectionally, whereas secondary transporters are able to catalyze efflux of solutes down the concentration gradient as well as exchange of solutes (25). Nevertheless, when unlabeled glycine betaine or the substrate analogue carnitine was added to cells that had accumulated *[<sup>14</sup>C]* glycine betaine, a slow exit of label was observed (Fig. 1). To characterize the glycine betaine exit activity, glucose-metabolizing cells were preloaded with *[<sup>14</sup>C]* glycine betaine at high osmolarity, followed by washing with equiosmolar buffer to remove external glucose and label. This washing procedure took about 15 min and resulted in a depletion of cells from (endogenous) metabolic energy as the ATP levels fell to "zero" and uptake of glycine betaine or proline was no longer observed (data not shown). When *[<sup>14</sup>C]* glycine betaine-loaded cells were diluted iso-osmotically, a slow but significant efflux of glycine betaine was observed (Fig. 2, filled circles). Since efflux is similar with or

<sup>1</sup> The abbreviation used is: CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid.

<sup>2</sup> E. Glaasker, W. N. Konings, and B. Poolman, unpublished results.

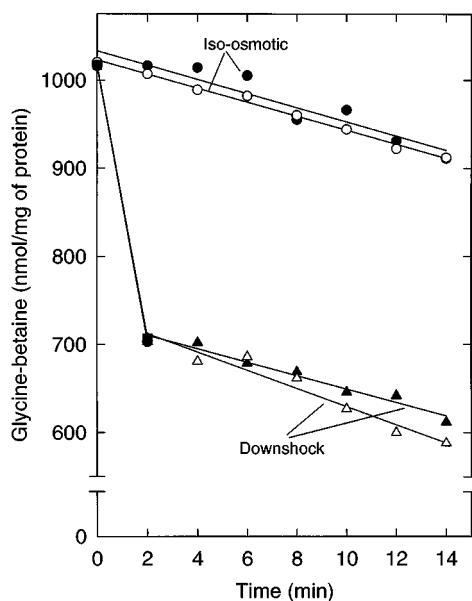


FIG. 2. **Glycine betaine exit in resting cells under iso-osmotic and hypo-osmotic conditions.** Cells were grown on chemically defined medium containing 0.8 M KCl, washed, and resuspended in potassium phosphate, pH 6.5, to a final protein concentration of 0.61 mg/ml. Cells were energized with 10 mM glucose and allowed to take up [ $^{14}\text{C}$ ]glycine betaine (final concentration of 1.3 mM) at high osmolarity (0.8 M KCl) for 60 min. The loaded cells were washed thoroughly with potassium phosphate, pH 6.5, containing 0.8 M KCl. The experiment was initiated by diluting [ $^{14}\text{C}$ ]glycine betaine loaded cells into potassium phosphate with (circles) or without (triangles) 0.8 M KCl. Open and closed symbols represent the exit of [ $^{14}\text{C}$ ]glycine betaine in the presence of 100 mM unlabeled glycine betaine or 100 mM KCl, respectively. The final protein concentration was 0.15 mg/ml.

without external glycine betaine, it is unlikely that [ $^{14}\text{C}$ ]glycine betaine exit is caused by direct exchange for external glycine betaine. These results suggest that glycine betaine is transported out of the cells by a separate efflux system. The observed exit rates at osmolarity are similar for glucose metabolizing cells (Fig. 1) and resting cells (Fig. 2), suggesting that efflux of glycine betaine takes place either passively via the lipid bilayer or protein-mediated via a system that catalyzes downhill glycine betaine export without the use of metabolic energy. Upon osmotic downshock, however, a much more rapid release of label was observed both in resting (Fig. 2) and in glucose metabolizing cells (see section "fluxes of glycine betaine upon osmotic downshock"). The presence of glycine betaine in the medium also did not affect this rapid efflux process.

**Fluxes of Glycine Betaine under Osmostatic Conditions**—To establish how the pools of glycine betaine are precisely regulated, the net fluxes under osmotic (steady state) conditions were determined first. Cells in high osmolarity media were allowed to take up [ $^{14}\text{C}$ ]glycine betaine until the steady state was reached. At this point, a low amount of [ $^3\text{H}$ ]glycine betaine was added that did not significantly affect the overall concentration of glycine betaine. Since uptake of glycine betaine had reached steady state no net accumulation of glycine betaine is observed under these conditions. The accumulation of [ $^3\text{H}$ ]label (Fig. 3, filled squares) now reflects the unidirectional rate of glycine betaine uptake (and efflux!). Since a direct exchange of the differently labeled pools of glycine betaine is unlikely (see above, Fig. 2), these results suggest that the steady state reflects an equilibrium between uptake and efflux which are facilitated by separate systems. Importantly, the unidirectional rates of [ $^3\text{H}$ ]glycine betaine uptake were similar in cells that had reached osmolarity at different external osmolarities (data not shown).

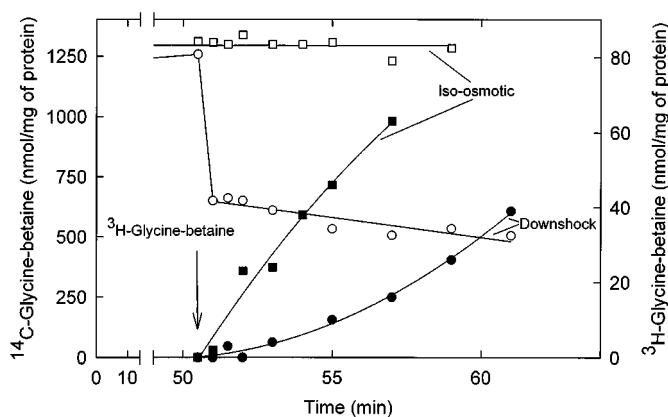


FIG. 3. **Unidirectional rates of [ $^3\text{H}$ ]glycine betaine uptake at osmolarity and upon osmotic downshock.** Cells were grown on chemically defined medium containing 0.8 M KCl, washed and resuspended in potassium phosphate, pH 6.5. After 5 min of preenergization with 10 mM glucose, uptake was initiated by the addition of [ $^{14}\text{C}$ ]glycine betaine (final concentration of 1.3 mM) and KCl (final concentration of 0.8 M). After 50.5 min, the samples were diluted 5-fold with potassium phosphate with (squares) or without (circles) 0.8 M KCl, but containing 10 mM glucose, 1.3 mM [ $^{14}\text{C}$ ]glycine betaine, and 1  $\mu\text{M}$  [ $^3\text{H}$ ]glycine betaine. Transport of [ $^{14}\text{C}$ ] and [ $^3\text{H}$ ]glycine betaine was monitored (open and closed symbols, respectively). The final protein concentration was 0.60 mg/ml.

**Fluxes of Glycine Betaine upon Osmotic Upshock**—In a previous study, we have shown that an osmotic upshock increases glycine betaine and proline uptake rates as well as the final accumulation levels (7). Since the steady state glycine betaine pools are the resultant of uptake and efflux (see above), we studied both activities separately and under conditions that osmolarity is disturbed. It is possible that the observed activation of glycine betaine uptake is due to an inhibition of efflux and/or to a stimulation of uptake. To discriminate between these two possibilities, uptake of glycine betaine was followed by using two different labels ( $^{14}\text{C}$  and  $^3\text{H}$ ) to monitor the uptake of glycine betaine. Uptake of [ $^{14}\text{C}$ ]glycine betaine was followed at low osmolarity (Fig. 4; closed squares). At different time intervals, the osmolarity was raised by adding KCl together with tracer amounts of [ $^3\text{H}$ ]glycine betaine and the initial uptake rates of both [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ]glycine betaine were measured. Since [ $^{14}\text{C}$ ]glycine betaine is also present intracellularly, it is a substrate for both the efflux and the uptake systems. When the efflux of glycine betaine is inhibited under conditions of osmotic upshock, the rates deduced from the uptake of [ $^3\text{H}$ ] and [ $^{14}\text{C}$ ]glycine betaine should be identical. The data indicate that the initial uptake rates of [ $^{14}\text{C}$ ] and [ $^3\text{H}$ ]glycine betaine are indeed similar (Fig. 4, open symbols), suggesting that the efflux of glycine betaine is inhibited during an osmotic upshock. The rate constants of glycine betaine exit under high osmolarity conditions (buffer plus 0.8 M KCl) were determined in separate experiments (e.g. Fig. 3, filled squares) and the corresponding rates were subtracted from the unidirectional rate of [ $^3\text{H}$ ]glycine betaine uptake. These hypothetical lines (Fig. 4, dotted lines) represent uptake of glycine betaine when efflux would not be inhibited. The difference between this theoretical line and the actual uptake curves indicates that even though efflux is inhibited upon osmotic upshock, the effect of this (negative) regulation on the overall glycine betaine flux is limited.

**Fluxes of Glycine Betaine upon Osmotic Downshock**—A rapid exit of glycine betaine was observed when an osmotic downshock was given to glucose metabolizing cells that had accumulated glycine betaine to steady state levels at high osmolarity (Fig. 3). The extent of the release of glycine betaine was dependent on the drop in external osmolarity, i.e. 300, 450, and

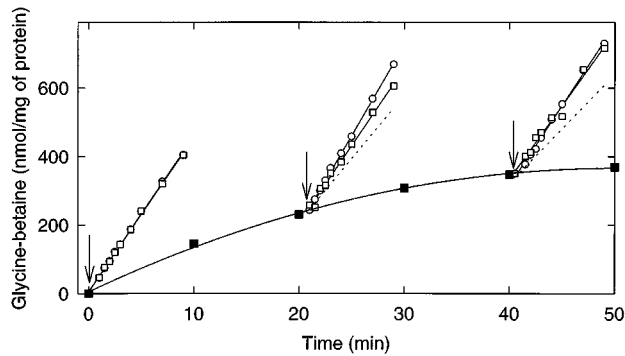


FIG. 4. Unidirectional rates of [ $^3\text{H}$ ]glycine betaine uptake upon hyperosmotic shock. Cells were grown on chemically defined medium containing 0.8 M KCl, washed and resuspended in potassium phosphate, pH 6.5. After 5 min of preenergization with 10 mM glucose, uptake was initiated at zero time by the addition of [ $^{14}\text{C}$ ]glycine betaine (final concentration of 1.3 mM). [ $^3\text{H}$ ]Glycine betaine (final concentration of 1  $\mu\text{M}$ ) and KCl (final concentration of 0.8 M) were added after 0, 20.5, and 40.5 min of [ $^{14}\text{C}$ ]glycine betaine uptake (closed squares). Open squares and circles represent the uptake of  $^{14}\text{C}$ - and  $^3\text{H}$ -labeled glycine betaine upon hyperosmotic shock, respectively. The final protein concentration was 0.39 mg/ml. The dotted lines represent the unidirectional rate of glycine betaine uptake from which the steady state efflux rate was subtracted.

700 nmol/mg of protein was released when the external KCl concentration was lowered from 0.8 to 0.48, 0.32, and 0.16 M KCl, respectively. The efflux was less pronounced in cells that were cultured at low osmolarity; only 100 nmol of glycine betaine/mg of protein were released when the external KCl concentration was lowered from 0.8 to 0.16 M KCl. However, these cells also accumulated glycine betaine to much lower concentrations than cells cultured at high osmolarity. Therefore, the dependence of efflux on the internal concentration of glycine betaine was investigated next. Cells that had taken up glycine betaine for 5, 10, 15, 20, and 25 min, resulting in internal concentrations of glycine betaine of 300, 550, 750, 900, and 1050 nmol/mg of protein (Fig. 5), respectively, were subjected to the same osmotic downshock. It was observed that efflux of glycine betaine was biphasic, as was observed in resting cells (Fig. 2), and that the extent of efflux was more pronounced when more glycine betaine had accumulated (Fig. 5). Since the fast efflux was completed within 1 s (data not shown), which is the time resolution of the experiment, it was not possible to estimate the rate(s) (constants) of the rapid phase. The rates of efflux in the second phase appear to be dependent on the internal glycine betaine concentration (Fig. 5, inset).

In analogy with the observed inhibition of glycine betaine efflux upon osmotic upshock, we have tested whether the uptake of glycine betaine is inhibited upon osmotic downshock. Cells were allowed to take up [ $^{14}\text{C}$ ]glycine betaine at high osmolarity. When the steady state was reached, an osmotic downshock was given by diluting with buffer containing [ $^3\text{H}$ ]glycine betaine. The unidirectional rate of [ $^3\text{H}$ ]glycine betaine uptake was almost zero in the first 2 min following the osmotic downshock (Fig. 3, closed circles). After this period, the uptake slowly recovered in time, probably as a result of restoration of the turgor pressure. The internal amounts of [ $^{14}\text{C}$ ]glycine betaine under iso-osmotic conditions and following osmotic downshock are shown for comparison (Fig. 3, open symbols).

**Characterization of Glycine Betaine Efflux**—glycine betaine efflux has at least two kinetic components (Figs. 2, 3, and 5). To separate the two events, attempts were made to specifically inhibit one of the two phases of efflux. When an osmotic downshock was performed at pH 4.5, 5.5, 7.5, and 8.5, the rates of the second (slow) phase of efflux were 25, 50, 88, and 112

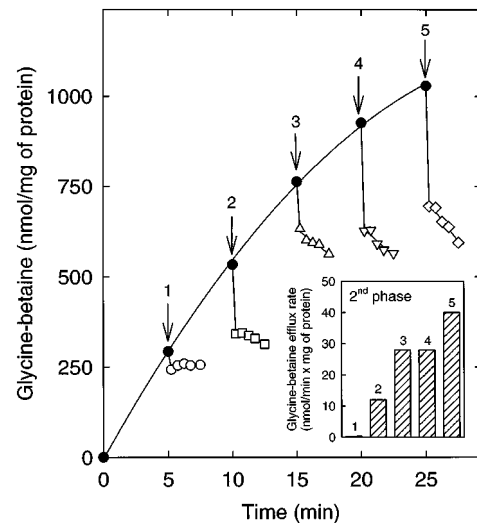


FIG. 5. Efflux of [ $^{14}\text{C}$ ]glycine betaine upon hypo-osmotic shock. Cells were grown on chemically defined medium containing 0.8 M KCl, washed, and resuspended in CKC buffer, pH 6.5. After 5 min of preenergization with 10 mM glucose, uptake was initiated at zero time by the addition of [ $^{14}\text{C}$ ]glycine betaine (final concentration of 1.3 mM) and KCl (final concentration of 0.8 M). After 5.5, 10.5, 15.5, 20.5, and 25.5 min the samples were diluted 5-fold with CKC buffer, pH 6.5, containing 10 mM glucose and 1.3 mM [ $^{14}\text{C}$ ]glycine betaine, but no KCl (indicated by the arrows and numbers). The efflux rates of the second phase of efflux are presented in the inset (the numbers in the inset correspond to the numbers in the graph). The final protein concentration was 0.28 mg/ml.

nmol/mg  $\times$  min, respectively, whereas the first (rapid) phase of efflux was not affected (data not shown). Since *L. plantarum* is unable to maintain the internal pH constant over this pH range (the internal pH decreased from 7.4 to 5.0 when the external pH was varied from 7.0 to 4.5),<sup>2</sup> it is possible that the inhibition of the slow phase of efflux was caused by the decrease of the internal rather than the external pH. Evidence for this suggestion was obtained from experiments in which the external pH was kept constant at pH 6.5, and the internal pH was lowered by the  $\text{K}^+/\text{H}^+$  ionophore nigericin. Since nigericin alone was able to inhibit the slow phase of efflux, whereas dissipation of the membrane potential by valinomycin did not affect glycine betaine efflux (data not shown), the data are most consistent with an inhibition of efflux caused by changes in cytoplasmic pH rather than by the dissipation of the proton motive force or lowering of the external pH. Biphasic exit of glycine betaine upon osmotic downshock was also observed in resting cells that were loaded with [ $^{14}\text{C}$ ]glycine betaine (Fig. 2), confirming that the efflux is indeed independent of the proton motive force. The data of the slow phase of efflux are consistent with carrier (uniporter) mediated transport of glycine betaine.

To test whether or not the rapid phase of efflux was caused by lysis of cells in the suspension, the integrity of the cells was monitored with the membrane impermeant fluorescent dye ethidiumbromide homodimer. Ethidiumbromide homodimer becomes fluorescent upon binding to DNA and can be used as an indicator of cell lysis (26, 27). When the indicator was added to cells that were subjected to osmotic downshock, no increase in fluorescence was observed (data not shown). However, an increase in fluorescence was observed upon disruption of the cells by sonication. These results show that cell lysis does not occur to an extent that can explain the rapid phase of efflux.

**Inhibitor Studies**—To investigate a possible role of mechanosensitive channels in the rapid and/or slow phase of glycine betaine efflux from *L. plantarum*, several known inhibitors of these channels were studied for their effect on glycine betaine efflux upon osmotic downshock. The following inhibitors were tested: 1 mM  $\text{Gd}^{3+}$ , 100 mM tetraethylamine, 2 mM quinidine,

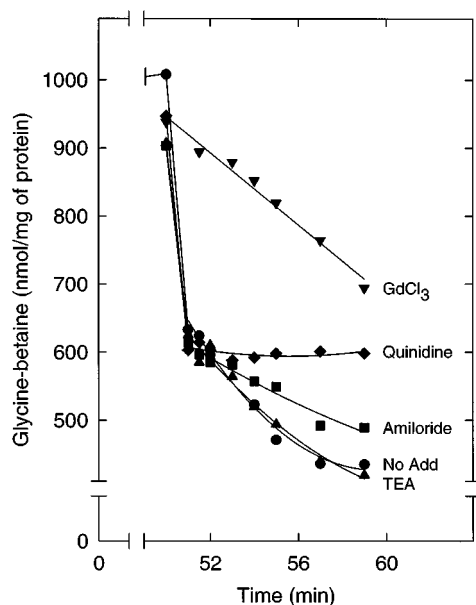


FIG. 6. Effect of inhibitors of mechanosensitive channels on [<sup>14</sup>C]glycine betaine efflux. Cells were grown on chemically defined medium containing 0.8 M KCl, washed, and resuspended in HEPES, pH 6.5. After 5 min of preenergization with 10 mM glucose, uptake was initiated at zero time by the addition of [<sup>14</sup>C]glycine betaine (final concentration of 1.3 mM) and KCl (final concentration of 0.8 M). After 50.5 min of uptake the samples were diluted 5-fold with HEPES, pH 6.5, containing 10 mM glucose and 1.3 mM [<sup>14</sup>C]glycine betaine (circles) with 1 mM GdCl<sub>3</sub> (▼), 2 mM quinidinechloride (◆), 1 mM amiloride (■), or 100 mM tetraethylammonium chloride (▲). The final protein concentration was 0.34 mg/ml.

and 1 mM amiloride. It was found that Gd<sup>3+</sup> specifically inhibited the rapid phase of efflux, whereas quinidine inhibited the slow phase (Fig. 6). The inhibition of the rapid phase of efflux by gadolinium was specific as both 5 mM Ca<sup>2+</sup> and 5 mM Mg<sup>2+</sup>, which also interact with lipid head groups (28), did not elicit any inhibition of efflux (data not shown). Control experiments showed that the uptake rates of glycine betaine, glutamate, lysine, and leucine were only somewhat inhibited in the presence of 1 mM Gd<sup>3+</sup>. Quinidine, on the other hand, abolished the uptake of lysine and leucine (proton motive force-driven), whereas the uptake of glutamate and glycine betaine (ATP-driven) was not affected (data not shown). Therefore the inhibition by quinidine is not necessary specific, but might be caused by the lowering of the internal pH. Furthermore, it cannot be excluded that partitioning of quinidine into the lipid bilayer affects the efflux activity (12, 29).

#### DISCUSSION

When bacteria are faced with changes in external osmolarity, they respond by raising (upon hyperosmotic shock) or lowering (upon hypo-osmotic shock) the cytoplasmic pools of specific molecules, termed compatible solutes. Glycine betaine is not only used as a compatible solute by many prokaryotic and eukaryotic (micro)organisms, it is also the *main* osmolyte in the cytoplasm of most bacteria that are grown at high osmolarities in the presence of glycine betaine (1). Some bacteria are able to synthesize glycine betaine from choline via a two step oxidation pathway (1, 23, 30), but *L. plantarum* cannot synthesize this compound (7). Therefore, the cytoplasmic pools of glycine betaine in *L. plantarum* are solely determined by the net transport rates (uptake and efflux) of glycine betaine. We have studied the regulation of the glycine betaine pools by dissecting the uptake and efflux activities at osmotic, and hyperosmotic and hypo-osmotic shock.

When the osmolarity is raised by adding 0.8 M KCl to glucose-

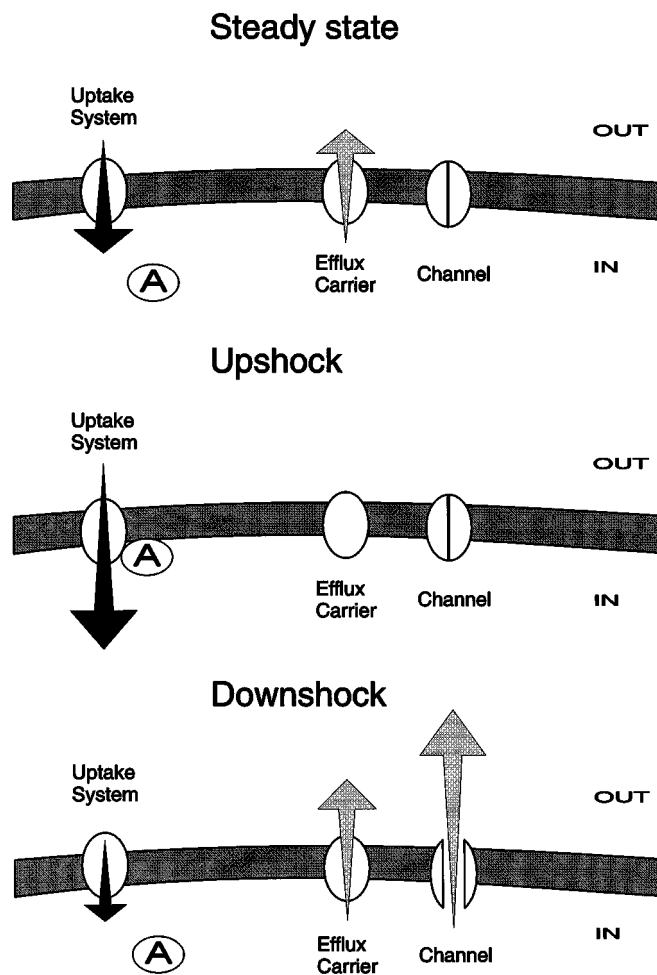


FIG. 7. Schematic representation of the glycine betaine fluxes in *L. plantarum*. A denotes the factor that activates the glycine betaine uptake system(s) upon osmotic upshock. The relative fluxes are indicated by the size of the arrows.

metabolizing cells in 50 mM potassium phosphate, pH 6.5, the glycine betaine uptake rates increase instantaneously from 15 to about 80 nmol/min × mg of protein, and the final accumulation levels rise from 400 to about 1500 nmol/mg of protein (7). Since this increase in transport rates and final accumulation levels was observed in the presence of chloramphenicol, it must be related to changes in activity rather than expression levels of glycine betaine transport system(s). When [<sup>14</sup>C]glycine betaine was taken up to steady state levels, the addition of tracer amounts of [<sup>3</sup>H]glycine betaine allowed us to estimate the steady state fluxes of uptake and efflux. The stationary exit flux is inhibited directly after osmotic upshock, but this "constitutive" efflux activity is not the major site of regulation; the increased glycine betaine uptake is more than can be explained by the inhibition of efflux. This notion is confirmed by experiments with cells grown on chemically defined medium containing 0.8 M KCl (but no glycine betaine), which were washed and resuspended in potassium phosphate (low osmolarity), and subsequently, at different moments in time, subjected to an osmotic upshock. When the osmotic upshock was given at *t* = 0, simultaneously with the addition of [<sup>14</sup>C]glycine betaine (so no internal glycine betaine is present), a large increase in the initial rate of glycine betaine uptake is already observed (Fig. 4).

When the osmolarity of glucose metabolizing cells in potassium phosphate containing 0.8 M KCl is lowered, whereas the external concentration [<sup>14</sup>C]glycine betaine is kept constant, a rapid loss (within 1 s) of accumulated glycine betaine is ob-

served. After this rapid initial loss, a second slower phase of glycine betaine efflux is observed (Fig. 5). The rapid phase of efflux is independent of pH and the metabolic energy status of the cell, and is inhibited by gadolinium. This rapid efflux is therefore reminiscent of that of mechanosensitive channels (11, 16–18).

The slow phase of glycine betaine efflux is observed under conditions of osmotic stress as well as under conditions of osmotic downshock, *i.e.* the efflux under osmotic conditions is kinetically similar to the second phase of efflux that follows an osmotic downshock. The following observations pertinent to the slow phase of efflux are relevant. (i) The pH dependence of the process and the inhibition by nigericin suggest that the slow phase of efflux is inhibited by a low internal pH and therefore protein mediated. Since no direct exchange of [<sup>14</sup>C]glycine betaine (in the cytoplasm) for unlabeled glycine betaine (in the medium) is observed, this efflux of glycine betaine is driven by the concentration gradient (downhill efflux). (ii) Upon osmotic upshock this efflux is inhibited as shown by the experiment presented in Fig. 4. (iii) Upon osmotic downshock, the kinetics of the second phase of efflux is dependent on the metabolic status of the cells. In both glucose-metabolizing and resting cells at a medium pH of 6.5, a constitutive glycine betaine efflux of about 10 nmol/min × mg of protein is observed under iso-osmotic conditions (Figs. 1–3). Upon osmotic downshock, the slow phase of glycine betaine efflux remains 10 nmol/min × mg of protein in resting cells (Fig. 2), whereas in glucose-metabolizing cells at pH 6.5 the overall efflux rates can be as high as 40 nmol/min × mg of protein (Figs. 5 and 6). Although in the latter experiments overall fluxes are measured, the increased rates can only partly be accounted for by the inhibition of uptake (~10 nmol/min × mg of protein). The additional increase in efflux rate seems therefore to be a consequence of the osmotic downshock. (iv) The rate of efflux in the second phase, following an osmotic downshock in glucose metabolizing cells, is dependent on the intracellular glycine betaine concentration (Fig. 5).

When an osmotic downshock is given, uptake of [<sup>3</sup>H]glycine betaine is inhibited in the first 2 min following osmotic downshock (Fig. 3). Subsequently, the rate of glycine betaine uptake gradually increases to normal values in parallel with the restoration of the osmotic imbalance. Overall, the experiments indicate that the glycine betaine uptake system(s) are not only activated upon osmotic upshock (see above), but also inhibited upon osmotic downshock. Similar to the stimulation of glycine betaine uptake upon osmotic upshock, the inhibition of glycine betaine uptake upon osmotic downshock is instantaneous. The quick response of the glycine betaine uptake system(s) to changes in osmolarity (upshock as well as downshock) suggests that membrane tension or turgor pressure is sensed by the transporter. Since the regulation of glycine betaine uptake occurs over a wide range of osmolarities, *i.e.* the “activated” rate of uptake is independent of the extent of the osmotic upshock, we speculate that the regulation involves an on/off mechanism, rather than one in which the activity varies gradually with the external osmolarity.

The main findings of this study are summarized in Fig. 7, in which the putative glycine betaine transport systems and the corresponding fluxes at iso-osmotic, hyperosmotic, and hypo-osmotic conditions are shown. The sizes of the arrows reflect the magnitude of the corresponding fluxes through the uptake system(s) (black arrows) and efflux systems (gray arrows). The

steady state of glycine betaine uptake represents an equilibrium between uptake and efflux of glycine betaine that is mediated by independent systems. The steady state fluxes of glycine betaine uptake and exit are similar at high and low osmolarities. Upon a hyperosmotic shock the uptake of glycine betaine is increased, whereas efflux is inhibited. When an osmotic downshock is given to the cells, an overall exit of glycine betaine is observed. Glycine betaine efflux consists of a rapid initial phase and a slower second phase; the unidirectional rate of uptake is lowered upon osmotic downshock. We speculate that the rapid efflux takes place via a mechanosensitive channel-like activity in the first second following the osmotic downshock, whereas the second phase of efflux is due to downhill efflux via a carrier-like mechanism (uniporter). The channel closes when the turgor pressure has decreased sufficiently. In later stages, the slower phase of glycine betaine efflux may serve to fine-tune the turgor pressure. It has a component that is regulated by the medium osmolarity, but also occurs to some extent under conditions of osmotic stress (constitutive efflux activity, see above).

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