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Betaine Transport Imparts Osmotolerance on a Strain of *Lactobacillus acidophilus*

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Unlike most *Lactobacillus acidophilus* strains, a specific strain, *L. acidophilus* IFO 3532, was found to grow in rich medium containing 1 M sodium acetate, KCl, or NaCl. This strain could also grow with up to 1.8 M NaCl or 3 M nonelectrolytes (fructose, xylose, or sorbitol) added. Thus, this strain was tolerant to osmotic pressures up to 2.8 osM. A search for an intracellular solute which conferred osmoprotection led to the identification of glycine betaine (betaine). Betaine was accumulated to high concentrations in cells growing in MRS medium supplemented with 1 M KCl or NaCl. Uptake of [^{14}C]betaine by *L. acidophilus* 3532 cells suspended in buffer was stimulated by increasing the medium osmotic pressure with 1 M KCl or NaCl. The accumulated betaine was not metabolized further; transport was relatively specific for betaine and was dependent on an energy source. Other lactobacilli, more osmosensitive than strain 3532, including *L. acidophilus* strain E4356, *L. bulgaricus* 8144, and *L. delbrueckii* 9649, showed lower betaine transport rates in response to an osmotic challenge than *L. acidophilus* 3532. Experiments with chloramphenicol-treated *L. acidophilus* 3532 cells indicated that the transport system was not induced but appeared to be activated by an increase in osmotic pressure.

Many species of bacteria respond to high medium osmotic pressures by accumulating low-molecular-weight solutes to high intracellular concentrations (for reviews, see references 5, 21, 33, and 52). The resulting increase in the cytoplasmic osmolarity acts to maintain the osmotic equilibrium between the intracellular and extracellular environments, that is, to prevent the decrease in turgor pressure which would arise when the extracellular osmotic pressure is increased. The osmotically active solutes accumulated by microorganisms include amino acids and amino acid derivatives (1, 9, 33, 36, 45, 47, 48), carbohydrates (3), and potassium (13). The quaternary amine *N,N,N*-trimethylglycine (betaine) has been reported to be an important osmoprotective molecule in several groups of gram-negative eubacteria, including halotolerant bacteria (28), extreme (14) and moderate halophiles (22), and nonhalophilic bacteria (5, 6, 30–32). Betaine is a metabolically inert compound, and because of its dipolar nature, no counterions need to be accumulated to maintain electroneutrality. Thus, betaine appears to be particularly suitable as a compatible solute (3), as it does not inhibit cytoplasmic enzyme activity even when present at high concentrations and can protect the cell against the deleterious effects of high salt concentrations (40, 52). Betaine accumulation in response to osmotic stress has been demonstrated primarily in gram-negative bacteria.

In this communication we report that *Lactobacillus acidophilus* IAM 3532 is significantly more resistant to osmotic stress than most strains of *L. acidophilus* (25). The osmoresistance can be attributed to a betaine-specific active transport system which is activated, but not induced, by high medium osmotic pressures.

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MATERIALS AND METHODS

Bacteria and growth conditions. *L. acidophilus* IAM 3532 (IFO 3532) was obtained from the Institute of Applied Microbiology, University of Tokyo, Bunkyo-Ku, Tokyo, Japan. *L. acidophilus* ATCC E4356, *Lactobacillus delbrueckii* ATCC 9649, and *Lactobacillus bulgaricus* ATCC 8144 were obtained from the American Type Culture Collection, Rockville, Md. All strains were grown aerobically without agitation at 37°C in commercially available MRS medium (Difco Laboratories, Detroit, Mich.), and inoculum cultures were transferred weekly. Growth was monitored by optical density determinations at 625 nm of appropriately diluted cultures.

Identification of intracellular betaine. *L. acidophilus* 3532 was grown in MRS medium containing 1.0 M NaCl or 1.0 M KCl and harvested during exponential phase by centrifugation at 10,000 × *g* for 10 min at 4°C. The cells were washed twice in 50 mM citric acid adjusted to pH 6.2 with Na$_2$HPO$_4$ (citrate-phosphate buffer) containing 1.0 M KCl, where indicated. The intracellular material was extracted by incubating for 1 h at 25°C with 5% (vol/vol) *n*-butanol and stored at −20°C. Extracts prepared from cells disrupted by sonication, by extraction with 10% perchloric acid followed by neutralization with KOH, or by boiling cells suspended in water and then centrifuging to remove cell debris, gave results similar to butanol extracts. The extracts and MRS medium were chromatographed on silica gel thin-layer chromatography plates (J. T. Baker Chemical Co., Philipsburg, N.J.) using 75% methanol–25% NH$_4$OH (vol/vol) as the solvent (10). Two other solvent systems used were 75% isopropanol–25% H$_2$O (vol/vol) and 84% NH$_4$OH–8% ethanol–8% H$_2$O (vol/vol/vol). The plates were exposed to I$_2$ vapor to visualize quaternary amines.

The betaine concentrations of the cell extracts were determined by the periodide method (43). MRS medium was

chromatographed on silica gel plates as described above; the material with the same mobility as genuine [^{14}C]betaine was eluted with 1.0 N NaOH and assayed by the periodide method.

NMR spectroscopy. Perchloric acid extracts were prepared from 200-ml cultures of *L. acidophilus* 3532 cells grown for 21 h in either MRS medium or MRS medium supplemented with 1.0 M NaCl. The cultures were cooled to 4°C, and the cells were harvested by centrifugation for 5 min at 3,000 × *g*. The cell pellets were suspended in 3 ml of 9.2 M ice-cold HClO$_4$ per mg (dry weight) of cells (final HClO$_4$ concentration, 0.25 M) and kept on ice for 10 min. The cell debris was removed by centrifugation, the supernatants were lyophilized, the residue was suspended in one-fifth volume of D$_2$O, and the insoluble material was removed by centrifugation. The supernatant was examined by ^{13}C nuclear magnetic resonance (NMR) spectroscopy.

Broad-band decoupled natural-abundance ^{13}C NMR spectra of the cell extracts and authentic glycine betaine were obtained at 100.62 MHz on a Bruker AM-400 WB spectrometer (USA Bruker Instruments, Inc., Manning Park, Billerica, Mass.) operating in a Fourier transform mode. Carbon-13 chemical shift assignments were made by using internal standards (glucose and lactic acid), spectra obtained with purified, authentic reference compounds, and the NMR reference literature (2, 23).

For ^{1}H NMR spectroscopy, butanol extracts of cells grown in MRS medium with 1 M NaCl were purified further. HCl was added to 1 N, and the solution was percolated through Dowex 50W-X4 cation exchange resin (Dow Chemical Co., Midland, Mich.) and eluted with 1.5 N HCl. The fractions containing periodide-reactive material were pooled, lyophilized, and purified further by reverse-phase chromatography on C$_{18}$ Bond Elut columns (Analytichem International, Harbor City, Calif.) prepared as specified by the manufacturer. The eluent was lyophilized, the dry material was dissolved in 0.5 to 1.0 ml of D$_2$O, treated with Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.), and centrifuged, and the supernatant was examined by ^{1}H NMR spectroscopy at 400 MHz in the Bruker spectrometer.

Betaine transport assays. Cells were grown in MRS medium, with or without 1.0 M KCl or NaCl, harvested during late exponential phase, and washed twice in citrate-phosphate buffer. The cells were suspended in the same buffer, glucose (40 mM final) was added as an energy source, and, where indicated, KCl or NaCl was added. The experiment was started by addition of 2.0 mM [methyl-^{14}C]glycine betaine ([^{14}C]betaine) at a 2.0 mM final concentration (4.5 mCi/mol). The reaction mixtures were incubated at 28°C with gentle agitation in a water bath shaker (New Brunswick Scientific Co., New Brunswick, N.J.). Samples (1 ml) were removed at intervals and centrifuged through a mixture of silicone oils, as described previously (26). When the reaction mixtures contained more than 0.8 M KCl or NaCl, a denser oil (100% Fluid 550, Dow-Corning, Midland, Mich.) was required. The radioactivity in the cell pellets, supernatants, and reaction mixtures was determined by liquid scintillation counting.

To determine the betaine content of *L. acidophilus* 3532 cells during growth, sterile [^{14}C]betaine (2.5 nmol, 0.125 μCi) was added together with the inoculum (50 μl of a stationary-phase culture) to 12.5 ml of 80% MRS medium supplemented with 1.0 M KCl. Growth at 37°C without agitation was followed by determinations of optical density at 625 nm at intervals. Samples (1.0 ml) were taken 18 to 41 h later, and cells were centrifuged for radioactivity counting. Portions of
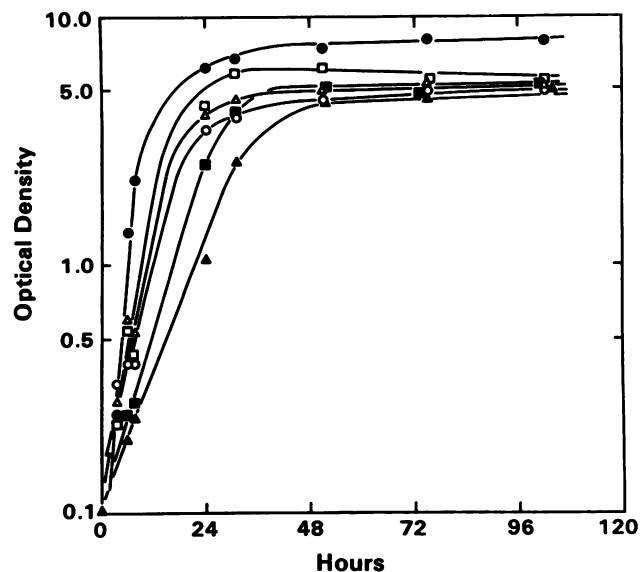

FIG. 1. Growth of *L. acidophilus* 3532 at high osmotic pressures. Cells were grown in MRS medium (●) or in MRS medium supplemented with 1 M KCl (○), 1 M NaCl (△), 1 M sodium acetate (□), 3 M fructose (▲), or 3 M sorbitol (■).

the supernatants and of the cultures were counted also. The cellular betaine content was calculated from the internal/external ratio of ^{14}C radioactivity and a betaine concentration in the medium of 0.5 mM.

Other measurements. The total amino acids in the cell extracts were estimated from the ninhydrin-reactive material (44). Standard methods were used to determine glycerol (4) and reducing sugars (19). Sodium and potassium were measured by flame photometry, and the osmolality of the medium was measured with an osmometer (Advanced Instruments Inc., Needham Heights, Mass.). Intracellular aqueous volumes were determined by using ^{3}H$_2$O to measure total pellet aqueous volumes and [^{14}C]polyethylene glycol (M_r, ca. 900) for the extracellular aqueous volumes, as described previously (26). The intracellular volume values were 1.68 and 1.37 μl/mg of cells (dry weight) for cells growing in MRS medium and MRS medium containing 1.0 M NaCl, respectively.

Chemicals. ^{3}H$_2$O, [^{14}C]polyethylene glycol, and [^{14}C]betaine were obtained from New England Nuclear Corp., Boston, Mass.; [^{14}C]betaine was purified by extraction from silica gel thin-layer chromatography plates after development in 75% methanol–25% ammonium hydroxide (vol/vol) (10). All other chemicals were of reagent quality.

RESULTS

Growth of *L. acidophilus* 3532 at high osmotic pressure. A specific strain of *L. acidophilus*, strain 3532, was able to grow to high cell densities under conditions that did not support growth of other lactobacilli, that is, in the presence of 1 M sodium acetate. This strain could also grow in MRS medium supplemented with other electrolytes, including 1.0 M NaCl and KCl (Fig. 1). Salt addition raised the medium osmolality from 0.44 to approximately 2.35 osM. *L. acidophilus* 3532 could grow in MRS broth containing up to 1.8 M NaCl. Although the growth rate decreased as the NaCl concentration varied from 0.2 to 1.8 M, the cells achieved
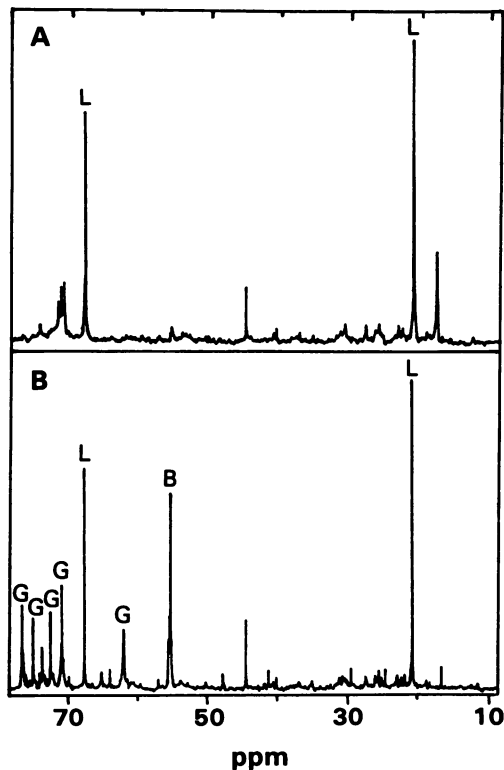
FIG. 2. Tracings of ^{13}C NMR spectra of perchloric acid extracts of *L. acidophilus* 3532 cells grown in MRS medium (A) or MRS medium supplemented with 1 M NaCl (B). The peaks were identified from spectra obtained with authentic compounds and are lactic acid (L), glucose (G), and betaine (B).

final densities >60% of those of cells in unsupplemented medium. These cells also grew when the osmotic pressure was increased with nonelectrolytes. On addition of 1.0 M sorbitol, fructose, xylose, or arabinose (approximately 1.2 osM) the cells grew somewhat more slowly than in MRS medium, with 20 to 30% longer culture doubling times, but to comparable cell densities. Growth was seen at 2.6 to 2.8 osM in medium containing 3 M fructose, sorbitol (Fig. 1), xylose, or glucose (not shown). At osmolalities exceeding 3.1 osM, reached by adding 4 M sugars, there was no growth.

Effect of medium osmolality on growth of other lactobacilli. In contrast, the other strains of *Lactobacillus* tested grew only under conditions of lower osmotic pressure. *L. bulgaricus* ATCC 8144 and a typical strain of *L. acidophilus*, ATCC E4356, grew in MRS medium supplemented with up to 0.3 M NaCl. *L. delbrueckii* ATCC 9649 was slightly more osmoresistant, tolerating up to 0.6 M NaCl added to the medium. *L. delbrueckii* 9649 also grew in MRS medium supplemented with 0.5 M sodium acetate, sorbitol, arabinose, and KCl; these cells did not grow in the presence of 1 M electrolytes or arabinose.

Identification of the intracellular osmolyte of osmotically stressed *L. acidophilus* 3532. The internal aqueous volume of cells suspended in 50 mM citrate-phosphate buffer, pH 6.2, decreased from 1.68 to 1.37 μl/mg of cells (dry weight) when 1 M NaCl was added. This decrease showed that cells exposed to high salt concentrations did not shrink sufficiently to concentrate the cytoplasm in response to the increase in external solute concentration. Therefore, the

marked resistance to osmotic stress of *L. acidophilus* 3532 was believed to be due to the accumulation of an osmotically active solute. To identify a potential osmoprotective compound, extracts prepared from cells grown in MRS medium with or without 1.0 M NaCl or KCl were analyzed by standard chemical tests. There were no significant differences between the extracts in total amino acid content (ninhydrin-positive material), carbohydrates (reducing sugars), glycerol, K^{+}, or Na^{+}. Although cells grown with 1 M NaCl added had a five- to sixfold increase in proline, the cellular proline was, at most, only 20 mM.

The concentration of quaternary amines, however, was significantly greater in extracts of 1 M KCl-grown cells than in cells grown in unsupplemented MRS medium. This suggested that betaine or a similar compound may be accumulated by osmotically stressed cells. Thin-layer chromatography of cell extracts with three solvent systems showed one prominent spot which cochromatographed with authentic betaine.

The natural-abundance ^{13}C NMR spectrum obtained from perchlorate extracts of cells grown in the presence of 1 M NaCl revealed several peaks not seen in extracts from cells grown in unsupplemented MRS medium (Fig. 2). Many of these peaks represent residual glucose trapped in the cell pellet and presumably are due to the slower rate of growth and fermentation in the NaCl-containing medium. In addition, there was a major peak at 55 ppm in the NaCl-grown cell extract spectrum. The compound responsible for this peak was identified as glycine betaine on the basis of published spectra (2, 10, 23) and comparisons with spectra obtained with authentic betaine. Furthermore, the ^{1}H NMR spectrum of purified material was identical to that of authentic betaine (not shown).

Betaine uptake by *L. acidophilus* 3532. The source of intracellular betaine in *L. acidophilus* 3532 cells growing under high osmotic pressure conditions is probably the yeast extract component (8) of medium MRS. We therefore tested the ability of cells to transport radioactive betaine against a concentration gradient. Strain 3532 cells accumulated [^{14}C]betaine when the osmolality was raised with 0.5 and 1.0 M NaCl (Fig. 3). The rate of betaine transport was proportional to the medium osmolality (Fig. 3, Table 1). The radioactive material taken up by the cells was extracted, and more than 90% was found to cochromatograph with genuine betaine. Thus, betaine was not metabolized after transport into the cells. The nature of the salt used to increase the medium osmolality had a minor effect, as addition of 1.0 M NaCl or KCl resulted in similar betaine transport activity. Supplementing MRS medium with additional betaine did not render the cells resistant to higher salt concentrations.

Betaine uptake by other lactobacilli. If the ability to accumulate betaine to high intracellular levels results in the osmotolerance of *L. acidophilus* 3532, one would expect other, less osmotolerant lactobacilli not to accumulate the osmolyte as rapidly or to equally high levels. This was found to be the case (Table 1). *L. acidophilus* E4356 and *L. bulgaricus* 8144, which are both more osmosensitive than *L. acidophilus* 3532, transported betaine at significantly lower rates.

Properties of the betaine transport system of *L. acidophilus* 3532. The betaine transport system was found to be relatively specific for glycine betaine, as related amino acids were not inhibitory. Proline α-aminoisobutyric acid, glycine, and choline at 100-fold-higher concentrations had no effect on the transport rate of 0.2 mM betaine (Table 2). Dimethylglycine, however, decreased betaine transport by
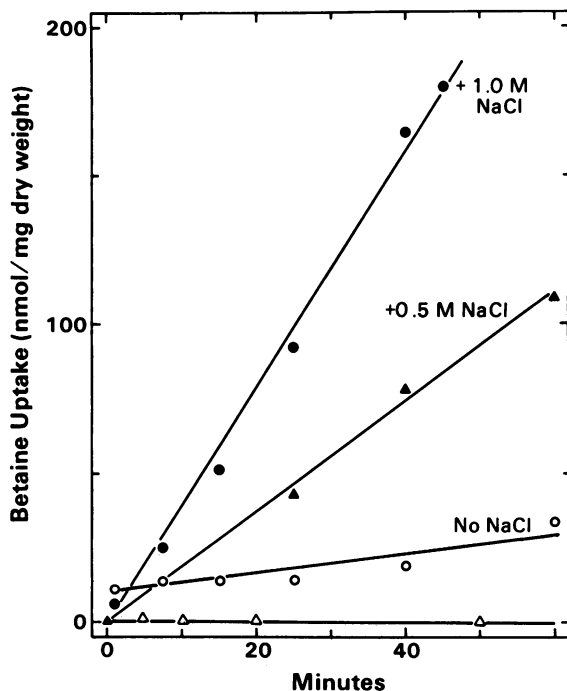
FIG. 3. Effect of NaCl on betaine uptake by *L. acidophilus* 3532. Cells were assayed in buffer supplemented with 40 mM glucose plus 0.5 or 1.0 M NaCl; △, no additions.

TABLE 2. Effect of analogs on betaine transport[a]

Addition	Betaine transport (nmol/mg of cells [dry wt] per min)
None	8.9
Proline	7.9
α-Aminoisobutyric acid	9.4
Glycine	8.6
Choline	8.8
Dimethylglycine	4.1

[a] The cells were harvested after overnight growth in MRS medium containing 1.0 M KCl and prepared for betaine uptake assays in the presence of 1.0 M KCl as described in the text. The [14C]betaine concentration was 0.2 mM, and the various analogs were added to 20 mM.

50%. The K_m for betaine, measured in the presence of 1 M KCl, was 50 μM.

As expected of transport against a concentration gradient, the accumulation of betaine required metabolic energy, that is, a fermentable carbon source, such as glucose (Fig. 3, Table 3). All the metabolic inhibitors tested reduced betaine uptake by *L. acidophilus* 3532 (Table 3). Omission of the energy source, glucose, or addition of the glycolytic inhibitor iodoacetate abolished betaine uptake. Uptake was reduced by protonophores such as the classical uncoupling agents 2,4-dinitrophenol, carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone, and pentachlorophenol. Addition of carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone (25 μM) or pentachlorophenol (50 μM) to cells that had accumulated [14C]betaine over 40 min of incubation stopped further uptake, but did not cause efflux over the following 7 to 10 min (not shown). Antibiotic ionophores (41) were also inhibitory, including the K^+-specific ionophore valinomycin (tested in the presence of 50 mM KCl) and the K^+ or Na^+/H^+ exchangers monensin and nigericin. The membrane potential-dissipating anion SCN^- (not shown) and arsenate, which

inhibits reactions involving high-energy phosphate bonds, also reduced betaine transport, as did the H^+-ATPase inhibitor N',N'-dicyclohexylcarbodiimide (DCCD) (Table 3). The DCCD effect would suggest that ATP, or a related high-energy phosphate bond compound, is not sufficient for betaine transport, since DCCD would not be expected to decrease the intracellular ATP concentration, as has been seen in *Streptococcus lactis* (35). To test whether betaine accumulation could be driven by a proton motive force, valinomycin was added to cells in low K^+ medium in the absence of glucose to generate a K^+ diffusion potential (27). However, no accumulation of betaine was seen.

Betaine content of growing *L. acidophilus* 3532 cells. Cells growing in MRS medium without osmotic supplements were found to contain approximately 4 mM betaine during the exponential and stationary phases. In cells growing in 1.0 M KCl-supplemented MRS medium, however, the accumulated betaine reached approximately 430 mM during exponential phase and decreased to approximately 160 mM as the cells entered the stationary phase. Similar values were obtained when [14C]betaine was used to assay the betaine accumulated in growing cells. Medium MRS contained approximately 16 mM of periodide-reactive material, most of which is not betaine, as thin-layer chromatography showed a number of yellow spots after exposure to I_2 vapor. Elution of material with the same mobility as authentic radioactive

TABLE 1. Effect of KCl concentration on betaine transport by three *Lactobacillus* strains[a]

Strain	Betaine transport (nmol/mg of cells [dry wt] per min) at KCl concn in medium:		
	0	0.3 M	1.0 M
L. acidophilus 3532	1.48	1.94	7.93
L. acidophilus E4356	0.05	0.99	1.74
L. bulgaricus 8144	0	0	0.86

[a] The cells were grown in MRS medium supplemented with 1.0 M NaCl for *L. acidophilus* 3532 and 0.3 M NaCl for the other two strains. The cells were harvested, washed twice in buffer, and assayed in buffer supplemented with glucose and KCl as described in the text.

TABLE 3. Effect of metabolic inhibitors on betaine transport by *L. acidophilus* 3532[a]

Addition (concn)	% of uninhibited rate[b]
Iodoacetate (1 mM)	0
Arsenate[c] (10 mM)	52
Arsenate[c] (100 mM)	40
2,4-Dinitrophenol (1 mM)	18
PCP[d] (50 μM)	20
CCFP[d] (25 μM)	32
Nigericin (10 μg/ml)	23
Monensin (10 μg/ml)	20
DCCD (0.1 mM)	11
Valinomycin (10 μg/ml)	48
Valinomycin (glucose omitted) (10 μg/ml)	0
Glucose omitted	0

[a] The cells were prepared as described in the text and tested in the presence of 40 mM glucose and 1.0 M KCl or NaCl. The metabolic inhibitors were added just before [14C]betaine; the cells were preincubated with DCCD for 20 min.

[b] The uninhibited betaine transport rates were 7.2 and 3.3 nmol/min per mg of cells (dry weight) in the presence of 1 M KCl and NaCl, respectively.

[c] Assayed in MES buffer, pH 6.2.

[d] PCP, Pentachlorophenol; CCFP, carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone.
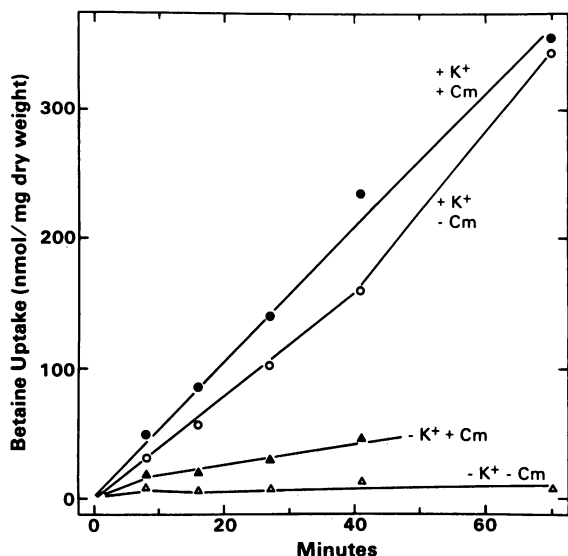
FIG. 4. Effect of chloramphenicol (Cm) on betaine uptake by *L. acidophilus* 3532. The cells were grown for 5.5 h to an optical density at 625 nm of 1.3 (0.5 mg of cells, dry weight). The culture was divided into two portions, and 100 μg of chloramphenicol per ml of 95% ethanol or an equivalent volume of 95% ethanol was added. After 2 h of further incubation, when the culture without drug had grown to an optical density at 625 nm of 3.0 and the cells with drug added had stopped growing, each flask was again divided into two portions. KCl (1.0 M) was added to one portion from the control culture and one from the drug-containing culture, and [^{14}C]betaine was added to each flask. Uptake of [^{14}C]betaine was determined as described in the text during the next 70 min.

betaine and quantitation by the periodide method showed that MRS medium contained 0.5 to 1.0 mM betaine.

The intracellular betaine decreased when the cells were washed with buffer of lower osmotic pressure. Thus, cells grown in 1 M KCl-supplemented MRS medium and washed with 1.0 M KCl still contained 92% of the betaine, and successive washings had no further effect. Cells washed one to three times with 50 mM citrate-phosphate buffer or with 50 mM MES buffer [2-(N-morpholino)ethane sulfonic acid], pH 6.2, however, lost 60 to 75% of the internal betaine. Adding 1.0 M KCl with or without 2 mM betaine to such washed cells did not restore the higher betaine levels, presumably because active transport of the osmolyte requires metabolic energy as well as increased external osmotic pressure.

The betaine transport system is not induced by high osmotic pressure. The immediate stimulation of betaine transport following osmotic upshock in nongrowing cells (Fig. 3) suggested that the system was activated, rather than induced, by osmotic stress. Activation, rather than induction, was also suggested by the finding that the rate of betaine transport (tested in buffer with 1.0 M KCl) was the same whether the cells were grown in MRS medium with or without 1.0 M KCl (4.7 and 4.5 nmol/min per mg of cells [dry weight], respectively). As a further test, we assayed the effect of chloramphenicol on betaine transport activity elicited by high osmotic pressure and found no effect of the protein synthesis inhibitor (Fig. 4). In this experiment chloramphenicol inhibited growth, as the cell density did not increase 1 h after addition of the antibiotic. However, these cells were able to take up betaine over the next 70 min in response to KCl addition, and the uptake was the same as

that of cells not exposed to chloramphenicol and whose growth had not been impaired. We concluded that the activity of the betaine transport carrier is stimulated by the rise in extracellular osmotic pressure.

DISCUSSION

Osmotolerance generally has not been considered a phenotypic trait of lactobacilli (25). Recent reports that some lactobacilli can tolerate high salt concentrations (11, 24) suggest that members of this genus may be more halotolerant than previously believed. *L. acidophilus* strains generally tolerate 2% (0.34 M) NaCl (51). One strain, *L. acidophilus* IAM 3532, possesses the cellular morphology, Gram reaction, and biochemical phenotype typical of this species (25) but was found to be relatively osmotolerant, growing in MRS medium containing up to 1.8 M (10.5%) NaCl. Other lactobacilli tested, including another strain of *L. acidophilus* and a strain of *L. bulgaricus*, behaved in a more usual way; that is, they tolerated no more than 0.3 M (1.75%) NaCl, while a strain of *L. delbrueckii* grew in 0.6 M (3.5%) NaCl added to the complex medium MRS.

The osmotolerance of strain 3532 of *L. acidophilus* can be attributed to its capacity to accumulate the osmoprotective compound glycine betaine. This conclusion is based on the following findings. (i) Betaine was found in high concentrations within cells growing in medium of high osmolarity, reaching approximately 430 mM in exponential-phase cells, while cells growing in low-osmolality medium contained <5 mM of the osmolyte. The internal betaine only partially compensated for the increase in medium osmotic pressure. Partial compensation has also been found for other bacteria (14, 39). (ii) Cells of strain 3532 were able to accumulate exogenous betaine both during growth and under nongrowing conditions. (iii) The rate of transport was proportional to the osmotic pressure of the incubation medium. (iv) Other, less osmoresistant lactobacilli transported betaine significantly more slowly. The other strains were not devoid of activity, however, the difference among strains being quantitative rather than absolute. Further experiments are needed to determine whether transport protein gene dosage explains the difference among these lactobacilli, as has been found in *Escherichia coli*, where increased *proU* gene dosage has been shown to enhance osmotolerance (17).

The source of the betaine accumulated by the lactobacilli during growth in high osmotic pressures was the medium. With a K_m of 50 μM, the medium contained sufficient betaine (0.5 to 1.0 mM) to saturate the transport carrier. It is not known whether these lactobacilli can synthesize betaine, as attempts to grow the cells in defined medium were not successful. In any case, the betaine in the medium most probably would have repressed betaine biosynthesis. Thus, induction of the transport system by betaine could not be determined; in enteric organisms the ProP and ProU betaine transport systems are not induced by betaine or by proline (5, 6).

The best-studied systems for accumulation of betaine by bacteria in response to osmotic stress are found in gram-negative enterics. *E. coli* (39, 49) and *Salmonella typhimurium* (5, 6) transport betaine, when it is available, by two transport systems, products of the *proP* and *proU* genes (5–7, 49). Synthesis of both the betaine transport systems (39) and the choline-glycine betaine pathway (30, 46) is inducible by increased external osmotic pressure, as is the K$^+$ uptake system in *E. coli* (13). In contrast, the *L. acidophilus* 3532 betaine carrier was not induced by increased medium osmolality.

In lactobacilli the H^+ gradient is established and maintained by the membrane H^+-extruding ATPase, utilizing glycolytically generated ATP (20, 37, 38). Treatments that decrease ATP synthesis would be expected to inhibit betaine uptake whether its transport system is proton gradient driven or ATP energized. Similarly, agents that dissipate the proton gradient could inhibit both ion gradient-driven and ATP-energized transport systems; in the latter case, ATP would be depleted by increased H^+-ATPase activity compensating for ion gradient dissipation. We found that betaine transport was inhibited by a variety of metabolic poisons, including those that inhibit ATP synthesis and those that dissipate the proton and other ion gradients across the cell membrane. Thus, in contrast to facultative anaerobes (42), in intact lactobacilli it is not possible to distinguish between the proton motive force and ATP as the energy-coupling mechanism for transport systems.

Once the betaine was accumulated within *L. acidophilus* 3532 cells it effluxed little, even in the absence of a fermentable energy source, unless the external osmotic pressure was lowered. Transport thus is regulated by the osmolality of the external medium. Activation by increased external osmolality is a possible mechanism for regulation of betaine carrier activity. Alternatively, the turgor pressure may be the regulatory factor, in analogy to the model proposed by Epstein and co-workers for the *E. coli* K^+ transport systems (12, 13, 29). A mechanism could involve deformation of the cell membrane due to a change in the turgor pressure, leading to a change in conformation of the membrane-embedded transport carrier; this, in turn, would stimulate the inward transport of betaine.

ACKNOWLEDGMENTS

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