The Physiological Response of *Lactobacillus plantarum* towards Salt and Non-electrolyte Stress

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ABSTRACT

In this paper we address the differential effects on the growth of *Lactobacillus plantarum* of raising the medium molarity by high concentrations of KCl or NaCl and iso-osmotic concentrations of non-ionic compounds. Analysis of cellular extracts for organic constituents by nuclear magnetic resonance spectroscopy showed that ‘salt-stressed’ cells do not contain detectable amounts of organic osmolytes, whereas ‘sugar-stressed’ cells contain sugar (and some sugar-derived) compounds. The cytoplasmic concentrations of lactose and sucrose in growing cells are always similar to the medium concentrations. By using the activity of the glycine betaine transport system as a measure of hyperosmotic conditions, we show that, in contrast to KCl and NaCl, high concentrations of sugars (lactose or sucrose) only impose a transient osmotic stress because external and internal sugars equilibrate after some time. Analysis of lactose (and sucrose) uptake also indicates that the corresponding transport systems are neither significantly induced, nor activated directly by hyper-osmotic conditions. The systems operate by facilitated diffusion and have very high apparent affinity constants for transport (>50 mM for lactose), which explains why low sugar concentrations do not protect against hyperosmotic conditions. The more severe growth inhibition by salt-stress as compared to equi-osmolal concentrations of sugars reflects, to our opinion, the inability of the cells to accumulate K\(^+\) (or Na\(^+\)) to high enough levels to restore turgor as well as deleterious effects of the electrolytes intracellularly.

INTRODUCTION

The response of micro-organisms towards growth-inhibiting salt concentrations has been studied extensively (for reviews see 1 and 3). In a number of cases, the effects both salt- and sugar-stress on the accumulation of individual compatible solutes such as K\(^+\) and glycine betaine have been determined (1, 5, 6, 8). However, only in a few studies, the osmolytes accumulated in salt-stressed cells have been compared with those in sugar (non-ionic)-stressed cells (2, 12). In one study, it was reported that accumulation of the major compatible solutes N-acetylglutaminyl-glutamine amide, glucosylglycerol and glutamate was similar irrespective of the type of osmotic stress (2), whereas large differences between
sugar- and salt-stressed cells were observed in another study (12).

Growth stimulation of osmotically stressed cells by exogenous glycine betaine is frequently observed, because most micro-organisms cannot synthesize this compound and, therefore, have to take up glycine betaine from the medium (4, 5, 8, 9, 16). The effects of glycine betaine on the growth of salt- and sugar-stressed cells are not always the same. In the Enterobacteriaceae (Escherichia coli, Salmonella typhimurium and Klebsiella pneumoniae) glycine betaine stimulated growth at ionic and non-ionic (sucrose) stresses (6), whereas in the lactic acid bacteria Lactococcus lactis and Lactobacillus plantarum a stimulatory effect of glycine betaine was only observed when a salt (KCl or NaCl)-stress was applied (5, 8). It should be emphasized that KCl and NaCl inhibited the growth of the lactic acid bacteria much more than equi-osmolar concentrations of sucrose.

In this study, the mechanisms underlying the response of Lactobacillus plantarum towards salt and sugar-stress have been investigated. The osmolyte pools in cellular extracts from cells cultured at high KCl, NaCl, sucrose, lactose, and sorbitol concentrations were determined by nuclear magnetic resonance (NMR) spectroscopy and by HPLC. Our data provide a rationale for the different effects exerted on the organism by high concentrations of inorganic ions and sugars.

**MATERIALS AND METHODS**

**Bacterial strains, culture conditions and media.** Lactobacillus plantarum ATCC 14917 was grown at 30°C in a chemically defined medium (CDM) at pH 6.7, containing 0.5% (w/v) glucose as described previously (5). High osmolarity media were obtained by adding KCl [0.8 M], NaCl [0.8 M], sucrose [35% (w/v)], lactose [35% (w/v)], sorbitol [30% (w/v)], or xylitol [30% (w/v)] to the standard CDM at concentrations indicated between the brackets. The osmolality of the solutions was measured by freezing-point depression using an Osmomat 030 (Gonotec, Berlin, F.R.G.). The osmolality of 0.8 M KCl (or NaCl) equalled 1.35 osm/kg, whereas a similar osmolality of a sucrose (or lactose) solution extrapolated to a concentration of about 1 M (~35% w/v) (data not shown).

**Cellular K⁺ and Na⁺ concentrations.** Exponentially growing cells of L. plantarum (about 1 mg of total cell protein) were collected on cellulose acetate filters with a pore size of 0.45 µm (Schleicher and Schuell GmbH, Dassel, Germany) by a manifold filtration apparatus. The filters were washed twice with 50 mM ‘sodium-free’ potassium phosphate, pH 6.5 (Na⁺ determination) or sodium phosphate, pH 6.5 (K⁺ determination), without (low-osmolarity cultures) or with 0.8 M KCl or NaCl (high-osmolarity cultures). The filters were transferred to
vials containing 0.3 ml of 5% (vol/vol) perchloric acid. After 30 min of incubation, the cellular extracts were centrifuged and the supernatant was used to determine the Na\(^+\) or K\(^+\) concentration by atomic absorption using the Perkin-Elmer 3030 spectrophotometer.

**Nuclear Magnetic Resonance Spectroscopy.** Exponentially growing cells of *L. plantarum* were harvested, washed with 50 mM potassium phosphate, pH 6.5, without (low-osmolarity cultures) or with 0.8 M KCl, (high-osmolarity cultures). Cellular extracts were obtained by incubating the cells (around 200 mg of protein per 10 ml) for 30 min with 5% perchloric acid containing 1 mM citric acid as internal standard. The mixture was neutralized by adding 5 N KOH, and the precipitate was centrifuged for 15 min at 48,000 x g. Subsequently, the supernatant was lyophilized, resuspended in 3-5 ml D\(_2\)O, centrifuged, lyophilized, and resuspended in circa 0.6 ml D\(_2\)O containing 0.5 - 3.0 mg EDTA-d\(_{12}\) (fully deuterated EDTA; to complex para-magnetic metal-ions). The recordings were performed on a Varian 400 Unity Plus spectrometer using either a 5 mm probe for four nuclei or a pulse-field gradient inverse broadband probe.

**Mass Spectrometry.** Electrospray mass spectra were recorded on a R 3010 quadrupole mass spectrometer (NERMAG, Argenteuil, France) equipped with a custom-built pneumatically assisted electrospray (IonSpray) ion source.

**Transport assays.** Uptake assays and enzymatic synthesis of \(^{14}\)C-glycine betaine from [N-methyl-\(^{14}\)C] choline (40-60 mCi/mmol) and uptake of \(^{14}\)C-lactose (55.8 mCi/mmol) and \(^{14}\)C-sucrose (540 mCi/mmol) were performed as described previously (5). To prevent carry over of osmolytes from the growth media, the cells were washed two times with a large excess (~100x the volume of the cell pellet) of buffer as specified in the figure legends.

**Miscellaneous.** Protein was determined by the method of Lowry et al. (7) 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 sterile low protein-binding microplates. Plate wells containing 200 \(\mu\)l of culture were sealed by adding 75 \(\mu\)l of sterile silicone oil (1.03 g/ml) and growth rates were determined from A\(_{620}\) increases using a Multiscan MCC/340 MKII (Flow Laboratories, Lugano, Switzerland). For the calculation of intracellular concentrations we used a value for the specific internal volume of 3 \(\mu\)l/mg of protein; the cytoplasmic volume was found to be constant under different culture and assay conditions (low and high osmolarities) as inferred from flow cytometry measurements (5).

**RESULTS**

**Main osmolytes under salt or sugar stress.** Previous experiments have demonstrated that addition of 2.5 mM glycine betaine to the medium was sufficient to alleviate growth inhibition caused by KCl or NaCl stress up to 1.2 M, whereas it did not affect the growth of sucrose-stressed *Lactobacillus plantarum* (5). Glycine betaine also did not stimulate the specific growth rates of *L. plantarum* under lactose, sorbitol, and xylitol stress (data not shown). To study the adaptation to high osmolality media further, the main osmolytes in cellular extracts of *L. plantarum* grown at low-osmolarity and under salt- or
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Sugar-stress were measured by nuclear magnetic resonance (NMR) spectroscopy.

*(i) Salt-stressed cultures.* Organic osmolytes could not be detected by proton NMR-spectroscopy in cells cultured in the presence of 0.8 M KCl or 0.8 M NaCl, however, increases in the accumulation of glutamate, alanine, and proline (additional accumulation of 223, 40, and 243 nmol/mg of protein, respectively) were measured by HPLC previously (5; Table I). When glycine betaine was added to the culture media, this compound was accumulated to concentrations of 850 nmol per mg of protein (Fig. 1A), whereas the concentrations of the amino acids were reduced significantly (see ref. 5). It was previously shown by atomic absorption spectrometry that large amounts (circa 5000 nmol/mg of protein; ~1.7 M) of potassium were associated with 0.8 M KCl-stressed cells, irrespective of whether glycine betaine was present in the medium (5). We have now established that the Na⁺ concentration increased to circa 2000 nmol/mg of protein in 0.8 M NaCl-stressed cells, but not in 0.8 M KCl-stressed cells. Increased concentrations of K⁺ and Na⁺ ions (about 2000 nmol/mg of protein) were only detected when the cells were stressed with the corresponding salts. Overall, the data suggest that *L. plantarum* is unable to respond adequately to osmotic stress by accumulating K⁺ as the increased potassium concentration was not observed in NaCl-stressed cells, even though the growth medium contained 35 mM of K⁺. As glycine betaine diminishes the growth inhibition by KCl- and NaCl-stress, whereas the amounts of K⁺ and Na⁺ associated with the cells are similar with or without this compatible solute (5), it may well be that the protective effect of glycine betaine is not only osmotic but also due to the stabilization of macromolecular structures at high cellular concentrations of ions.

*(ii) Sugar-stressed cultures.* Glycine betaine was detected at concentrations of 300 nmol per mg of protein in extracts from sucrose-stressed cells growing on 25 mM glucose, irrespective of whether glycine betaine was added to the culture medium (Fig. 1B; Table I). This was surprising since *L. plantarum* is unable to synthesise glycine betaine (5). Mass spectrometry analysis of standard sucrose solutions (Acros Organics, Biochemical grade), chromatographed on a Hypersil ammonium column, indicated that low amounts of glycine betaine were contaminating the sucrose. The concentration of glycine betaine present in a 35% (w/v) sucrose solution (the concentration used to stress the cells) could not be estimated accurately but fell in the range of several hundreds of micromolar, which is sufficient to restore the turgor partially through the uptake of glycine betaine. In addition to the relatively small amounts of glycine betaine, glutamate and alanine, the major osmolyte sucrose was present at circa 3600 nmol per mg
of protein (~1.2 M) in sucrose-stressed cells. In the case of cells grown in the presence of lactose (35% w/v ~ 1.0 M) or sorbitol (35% w/v ~ 1.7 M) plus 25 mM of glucose, about 3800 and 6100 nmol/mg of protein of the respective sugars were detected by NMR spectroscopy in cellular extracts. At a specific internal volume of 3 µl/mg of protein, this corresponds to internal lactose and sorbitol concentrations of ~1.3 and ~2.0 M, respectively, which is of the same order as the externally applied concentrations. Glycine betaine was only detected in lactose- or sorbitol-stressed cells when the compound was present in the medium (Table I). In cellular extracts from \textit{L. plantarum} cultured on CDM with 35% lactose, some phosphorylated sugar-like compounds were found in addition to the unmodified disaccharide (Fig. 1C). The proton undecoupled phosphorous spectrum, showed that the phosphate of one of the phosphorylated compounds forms a triplet and is therefore linked to a CH$_2$-group (= 6-phosphate), whereas the other one is most likely a sugar-bisphosphate (data not shown). A comparison with reference spectra identified one compound as the glycolytic intermediate glucose-6-phosphate. The phosphorylated compounds were quantified by using the inorganic phosphate from the buffer as an internal standard. They comprised less than 15% of the total amount of sugar(-derivatives) in the cellular extracts and are therefore thought to be of limited importance for maintaining the osmotic balance. Since \textit{L. plantarum} ATCC 14917 does not grow on lactose, whereas it does on the respective monosaccharides, the phosphorylated sugars cannot be derived from lactose, but must originate from glucose (carbon and energy source). Similar to sucrose-stressed cells, cells grown in the presence of 1 M lactose contained relatively large amounts of lactic acid (at least one order of magnitude higher than in control or KCl-stressed cells; Table I).

\textbf{TABLE I. Intracellular osmolyte concentrations in \textit{Lactobacillus plantarum}$^1$}

\begin{center}
\begin{tabular}{lccccccc}
\hline
Osmolyte$^2$ & No additions & 0.8 M KCL & 1 M Lactose & 1 M Sucrose$^3$\\
 & -GB & +GB$^3$ & -GB & +GB$^3$ & -GB & +GB$^3$ & -GB$^4$ \\
\hline
Glycine betaine & 0$^3$ & nd & 0$^3$ & 850 & 0$^3$ & ~200 & ~300 \\
Glutamate & 57 & 36 & 280 & 110 & ~550 & ~500 & ~550 \\
Alanine & 23 & 18 & 63 & 39 & ~700 & ~500 & ~700 \\
Proline & 7 & 4 & 250 & 10 & ~100 & ~100 & ~100 \\
Lactose(-derivative) & nd & nd & nd & nd & ~3800 & ~3200 & nd \\
Sucrose(-derivative) & nd & nd & nd & nd & nd & nd & ~3600 \\
Lactic acid & ~70 & ~65 & ~40 & ~65 & ~1200 & ~1200 & ~800 \\
\hline
\end{tabular}
\end{center}

Supplement to Chemically Defined Medium

1. In \textit{Lactobacillus plantarum}.
2. Inositol (GABA) auxotrophic strain used.
3. nd = not detected.
Part of the data were taken from ref. 5.

Concentrations are in nmol/mg of protein.

GB, Glycine betaine was added to a final concentration of 2.5 mM.

Sucrose solutions were contaminated with >100 µM glycine betaine.

'0' by definition because *L. plantarum* cannot synthesize glycine betaine.

nd, not detectable; ~, based on NMR data (less precise than HPLC analysis).

To substantiate the notion that *L. plantarum* cannot metabolize lactose or lactose-6-phosphate (after transport via a phosphoenolpyruvate-dependent sugar phosphotransferase system), the initial step in the breakdown, β-galactosidase or phospho-β-galactosidase activity, was measured using the chromogenic substrates o-nitrophenyl-β-D-galactopyranoside (ONPG) or o-nitrophenyl-β-D-galactopyranoside-6-phosphate (ONPG-P), respectively. Neither activity was present in *L. plantarum* grown on CDM containing 0.5% glucose plus 0.5% or 30% lactose, whereas β-galactosidase (ONPG hydrolysis) and phospho-β-galactosidase (ONPG-P hydrolysis) activities were measured in *Streptococcus thermophilus* ST 11 or *Lactococcus lactis* ML3, respectively. These results confirm that lactose is not metabolized by *L. plantarum* ATCC 14917. As also shown by the transport studies presented below, the intracellular concentration of lactose equilibrates with the extracellular one, which implies that lactose contributes significantly to the internal osmolality only when its medium concentration is high. Although *L. plantarum* can grow in the presence of sucrose or sorbitol as sole energy source, the intracellular concentrations of these sugars were also close to the extracellular ones, which indicates that decreases in turgor pressure upon addition of these sugars to the medium will only be transient.

The addition of glycine betaine to CDM supplemented with 35% lactose resulted in the accumulation of glycine betaine up to concentrations of about 200 nmol/mg of protein. Moreover, cells cultured on CDM containing 35% lactose or sucrose contained relatively large amounts of glutamate (500-550 nmol/mg of protein), alanine (500-700 nmol/mg of protein), and lactate (800-1200 nmol/mg of protein), irrespective of whether glycine betaine was present in the growth medium. These compounds were identified by means of gradient selected heteronuclear single quantum coherence (ghsqc) and gradient selected hetero-nuclear single quantum coherence total correlated spectroscopy (ghsqtocsy) (18).

**Restoration of osmotic imbalance.** Since sugar-stress resulted in much lower intracellular concentrations of glycine betaine than salt-stress, the uptake of glycine betaine under the two conditions was compared. The rationale for the experiment is that the ‘activated’ state of the glycine betaine uptake system can be used as a measure of osmotic imbalance following an osmotic upshift. Cells
were grown on CDM supplemented with 0.8 M KCl plus 0.5 % glucose, washed twice and resuspended in potassium phosphate, pH 6.5, and uptake was assayed in CDM. The final accumulation levels of glycine betaine were significantly lower in the presence of 30% (w/v) lactose (or sucrose) than when iso-osmotic concentrations of KCl were present (Fig. 2A). The final level of glycine betaine was even lower when cells were cultured on CDM supplemented with 30% (w/v) lactose (iso-osmotic with 0.8 M KCl) plus 0.5% (w/v) glucose and assayed in the presence of 30% (w/v) lactose (Fig. 2B). The cytoplasmic glycine betaine concentrations determined in these assays correlate well with those estimated from the NMR-spectra. Since the uptake rates under KCl and lactose (or sucrose) stress are initially the same, while the final accumulation levels differ, the data suggest that the osmotic imbalance is restored more rapidly in the presence of lactose (or sucrose) through uptake of these sugars. Hence, glycine betaine uptake is inhibited at an earlier time point by high concentrations of sugar (after circa 12 min) than by high concentrations of KCl.

**Regulation of sugar uptake by osmolarity.** Since *L. plantarum* has no detectable β-galactosidase and/or phospho-β-galactosidase activities, the uptake of lactose can be studied without interference of cellular metabolism. The lactose (and sucrose) uptake rates, assayed in 50 mM potassium phosphate, pH 6.5, were similar for cells cultured on CDM containing 0.5% glucose, 0.5% glucose plus 0.8 M KCl, or 0.5% glucose plus 0.5% lactose (or sucrose). However, when the cells were cultured in CDM containing 0.5% glucose plus 30% lactose (or sucrose), the rates of lactose (or sucrose) uptake increased 2 to 3-fold (Fig. 3 and 4; data not shown). This is consistent with induction of putative uptake systems for lactose and sucrose by high concentrations of the respective sugars.

As described previously for glycine betaine (4), the uptake of the sugars may be subject to osmotic regulation and the transport system(s) may be more active under hyper-osmotic conditions. To study osmotic regulation at the protein level (activation of the transporter), lactose uptake was performed in cells cultured on CDM supplemented with 30% (w/v) lactose plus 0.5% (w/v) glucose (fully induced cells). The cells were washed and resuspended in potassium phosphate, pH 6.5, and lactose uptake was initiated by dilution into potassium phosphate, pH 6.5, plus 10 mM glucose and 1.3 mM [14C]-lactose without further additions (low-osmolarity), or with 30% (w/v) lactose or 0.8 M KCl (high osmolarity). Activation of lactose uptake by hyper-osmotic conditions was not observed, i.e., 0.8 M KCl had no stimulating effect on the activity. However, the uptake rate of 14C-lactose in the presence of an additional 30% (w/v) of lactose increased approximately 50-fold (to 60 nmol/min x mg of protein), which was more than expected if one assumes an affinity constant for lactose in the submillimolar
range, i.e., as observed for lactose transport in other (lactic acid) bacteria (15). In fact, the data for L. plantarum point to an uptake system with a very high affinity constant for lactose (see next).

**Kinetic characterization of lactose uptake.** The kinetic constants for lactose uptake by L. plantarum were determined for cells cultured on CDM supplemented with 0.5% glucose plus 0.5% lactose and CDM supplemented with 0.5% glucose plus 30% lactose. In both cases the initial lactose uptake rates increased linearly up to a lactose concentration of at least 50 mM; the initial lactose uptake rates were highest in cells cultured in CDM containing 30% lactose (Fig. 3). Note that the uptake of lactose in Fig. 3 is downhill and involves no lactose metabolism. Measurements of uptake versus time showed that lactose was never taken up beyond equilibration level (data not shown). A lactose counterflow assay (14) was used to test whether the observed low-affinity uptake of lactose is facilitated by an uptake system or due to passive diffusion. If facilitated diffusion occurs one should observe an transient accumulation of [14C-\text{-}]lactose as a result of isotopic exchange with intracellular unlabeled lactose, whereas in case of passive influx the intra- and extracellular pool should only equilibrate. Indeed, counterflow was observed in cells that were cultured on CDM supplemented with 30% lactose plus 0.5% glucose as well as in cells cultured on CDM supplemented with 0.5% lactose plus 0.5% glucose. For the cells grown in the presence of 30% lactose, [14C-\text{-}]lactose was taken up by counterflow to about 3 nmol/mg of protein (~1 mM; after 4 to 5 min in Fig. 4), which is almost 15-fold higher than the extracellular concentration. As in the lactose uptake assays, the counterflow rates were increased approximately 2 to 3-fold in cells cultured on 30% lactose when compared to cells cultured on medium containing 0.5% lactose (Fig. 4). Surprisingly, the counterflow activity was subject to inhibition by glucose (Fig. 4, inset). These results together with the NMR-data suggest that lactose is entering the cells by facilitated diffusion rather than by passive diffusion across the cytoplasmic membrane. Since lactose uptake in resting and glucose-metabolizing cells is only observed to equilibration level, it seems that transport is not coupled to any form of metabolic energy (e.g., proton motive force or ATP).

**DISCUSSION**

In this paper we provide a rationale for the observation that raising the medium osmolality through the addition of electrolytes (KCl or NaCl) or non-electrolytes (various sugars) has different consequences for L. plantarum. The transport data
indicate that an osmotic upshock elicited by the addition of KCl or equi-osmolar amounts of lactose enhances the rate of glycine betaine uptake to the same extent (Fig. 2), which reflects the activation of the uptake system as a result of loss of turgor pressure (see also our previous study, 4). As lactose diffuses into cells, the osmotic conditions are restored and the net rate of glycine betaine uptake ceases. On the basis of the regulation of glycine betaine fluxes during osmostasis, hyper- and hypoosmotic shock (4), we infer that this decreased rate of uptake is due the lowering of the unidirectional uptake rate from ‘activated’ to ‘basal’ whereas the unidirectional efflux may increase somewhat. The uptake of sugar (to equilibration levels) together with the accumulation of glycine betaine may eventually result in hyper-osmolarity of the cytoplasm, which will then be compensated by net exit of glycine betaine. The decrease in glycine betaine levels after 15 min of uptake in the presence of lactose (Fig. 2B) is a strong indication that the cytoplasm becomes hypertonic relative to the external medium and that the glycine betaine exit systems become activated. The properties of the osmotically regulated efflux systems in *L. plantarum* have recently been described (4).

Although electrolytes and non-electrolytes impose an osmotic upshift initially, the facilitated influx of sugars diminishes the osmotic gradient in time. If one assumes an apparent affinity constant for lactose transport of 100 mM (the system is clearly not saturated at 50 mM; Fig. 3), the rate of lactose uptake at a medium concentration of 30% (0.83 M) lactose will approximately be 120 nmol/min x mg of protein. Taking this rate, the cytoplasmic concentration of lactose will increase with 40 mM/min and equilibrate with the external medium concentration after about 20 min. This value is in accordance with the transport data presented in Fig. 2B, given the uncertainty about the affinity constant for lactose transport.

The amount of K\(^+\) associated with the cells increases when the osmolarity of the medium is increased by the addition of 0.8 M KCl, but not with 0.8 M NaCl as stress factor. The cellular levels of K\(^+\) (either bound and/or free) are already high (~1M) in ‘unstressed’ cells and it seems that *L. plantarum* is unable to increase the K\(^+\) levels much further upon hyperosmotic (e.g., NaCl)-stress. This implies that under conditions of salt-stress, and in the absence of an alternative compatible solute, the cell turgor remains far from optimal. In fact, even in the presence of glycine betaine the increase in organic compatible solutes is insufficient to restore the turgor pressure in KCl-stressed cells completely. Therefore, the growth inhibition by KCl and NaCl may not only have an osmotic
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origin, but high salt in the cytoplasm may become inhibitory by binding to intracellular macromolecules. Because glycine betaine is able to restore the growth of salt-stressed *L. plantarum*, whereas the amounts of K$^+$ ions are associated with the cells are similar in the presence and absence of glycine betaine in the medium (5, this study), it seems likely that glycine betaine offers (additional) protection through stabilization of enzymes or other macromolecules.

The facilitated influx of lactose and sucrose uptake at high sugar concentrations also occurred when glycine betaine was present (Fig. 1B/C), indicating that the lower levels of glycine betaine in sugar-stressed cells are most likely due to the equilibration of sugar. During salt (KCl or NaCl)-stress, glycine betaine is taken up to higher levels than in sugar-stressed cells. Our interpretation of the data is that the cells are unable to increase the osmolality of the cytoplasm sufficiently by accumulating K$^+$ or Na$^+$. Support for this notion also comes from the experiments on the ‘activated state’ of the glycine betaine uptake system upon salt- and sugar stress (Fig. 2), as discussed above. Thus, the addition of glycine betaine to media with high concentrations of salts leads to the accumulation of this osmolyte, partial restoration of the osmotic imbalance, possible stabilization of macromolecules, and, consequently, to an increased specific growth rate. The addition of lactose, sucrose, or sorbitol (at a concentration of 14 mM) to KCl stressed-cells does not increase the specific growth rate of *L. plantarum* (unpublished results), because the organism fails to accumulate these sugars against their concentration gradients.

At this point we cannot (and do not) discriminate between growth inhibition by an osmotic upshift as a result of a decrease in turgor pressure or perhaps plasmolysis, i.e., retraction of the cytoplasmic membrane from the cell wall. In contrast to Gram-negative bacteria, Gram-positive bacteria, in general, do not plasmolyse (10, 13, 19). The reason for the failure of Gram-positive bacteria to plasmolyse might be the strong adhesion between the cytoplasmic membrane and peptidoglycan. Alternatively, these bacteria may not plasmolyse because of their very high internal osmotic pressure (turgor pressures of 15 to 25 atm), which means that much higher external osmolalities are needed before the turgor drops to zero than in Gram-negative bacteria (turgor pressures of 1 to 5 atm).

A few other points relevant for the discussion of the present paper require explanation. In a previous study we observed accumulation of glycine betaine to levels as high as 1500 nmol/mg of protein for cells cultured in the presence of 0.8 M KCl or NaCl (5), whereas levels of 850 nmol/mg of protein are reported here.
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The present measurements, however, were performed in CDM from which other osmolytes were accumulated, most notably glutamate (Table I), rather than in phosphate buffer. The co-accumulation of these osmolytes will have its impact on the restoration of turgor pressure, and, consequently, reduce the accumulation of glycine betaine.

To obtain equi-osmolar conditions in case of salt or sugar addition to CDM or phosphate buffer, the osmolality was measured by freezing point depression rather than calculated from the number of particles in the solution. We chose to measure the osmolality because the amount of solute added to a solvent does not usually produce a proportional increase in osmotic pressure due to interaction of the solute with the solvent, which in case of CDM is likely to be very complex because several other solutes are present as well. Although measurements of osmolality by freezing point depression (or any other method) has its limitations (see ref.15), deviations in the actual osmolality as experienced by the cells will not significantly affect the interpretation of the present data.

In conclusion, the response of *L. plantarum* to hyperosmotic conditions is different from that of other microorganisms. Mainly on the basis of studies in enteric bacteria it was found that K\(^+\) uptake is activated and K\(^+\) ions accumulate to high levels upon an osmotic upshock (1). To maintain electroneutrality, the accumulation of K\(^+\) is accompanied by the uptake anions (e.g., glutamate) and/or exit of other cations (e.g., protons). At later times during hyperosmotic stress, (part of) the potassium is replaced by neutral osmolytes that can either be synthesized or taken up from the medium (1). Our findings indicate that *L. plantarum* cells are unable to compensate for a decrease in turgor by increased accumulation of K\(^+\), and an exogenous organic osmolyte that can be accumulated to high levels (e.g., glycine betaine) is needed to restore growth by reversing at least partly the decrease in turgor pressure upon ‘salt-stress’. Hyperosmotic conditions imposed by ‘sugar-stress’ are much less detrimental and only transient, because the cells are able to equilibrate the extra- and intracellular concentrations of lactose (and sucrose). The uptake of these sugars most likely occurs by facilitated diffusion via system(s) with a very low affinity for the substrates, which is consistent with the inability of the sugars to serve as compatible solutes (at low substrate concentration) in *L. plantarum*.

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