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Regulation of the Glutamate-Glutamine Transport System by Intracellular pH in *Streptococcus lactis*

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Various methods of manipulation of the intracellular pH in *Streptococcus lactis* result in a unique relationship between the rate of glutamate and glutamine transport and the cytoplasmic pH. The initial rate of glutamate uptake by *S. lactis* cells increases more than 30-fold when the intracellular pH is raised from 6.0 to 7.4. A further increase of the cytoplasmic pH to 8.0 was without effect on transport. The different levels of inhibition of glutamate and glutamine transport at various external pH values by uncouplers and ionophores, which dissipate the proton motive force, can be explained by the effects exerted on the intracellular pH. The dependence of glutamate transport on the accumulation of potassium ions in potassium-filled and -depleted cells is caused by the regulation of intracellular pH by potassium movement.

In the last few years the regulation of cytoplasmic pH in bacteria has been studied intensively (for recent reviews, see references 3 and 16). In neutrophilic bacteria, the intracellular pH appears to be regulated by proton extrusion via the respiratory chain or the F_0F_1-ATPase and cation influx at low pH, whereas cation-proton antiporters regulate the pH at high values (3). Recently, it has been proposed that the cytoplasmic pH of *Streptococcus faecalis* is regulated by the F_0F_1-ATPase only (9). This regulation would be exerted by the pH dependence of the F_0F_1-ATPase and changes in the amount of enzyme present in the cytoplasmic membrane (9, 11). Besides the F_0F_1-ATPase in *S. faecalis*, many cation transport systems which are thought to be involved in pH control are regulated by intracellular pH (1, 2, 11). For example, sodium efflux via the sodium-proton antiporter of *Escherichia coli* is stimulated by an imposed pH gradient only when the cytoplasmic pH exceeds 6.5 (2).

The proton motive force or one of its components provides the driving force for the translocation of a large number of solutes (secondary transport systems) in a variety of organisms (12). Studies of the effects of intracellular pH on these transport activities are often complicated by the difficulty in modulating the internal pH without disturbing the proton motive force. Some transport systems in lactic acid bacteria require ATP or another form of phosphate bond energy (for a review, see reference 5 and W. N. Konings, W. de Vrij, A. J. M. Driessen, and B. Poolman, *in Sugar Transport and Metabolism in Gram-Positive Bacteria*, in press). For instance, in *S. faecalis* several cation transport systems belong to this category (5). Phosphate bond energy is also involved in the accumulation of glutamate and glutamine in *Streptococcus lactis* and *Streptococcus cremoris* (B. Poolman, E. J. Smid, and W. N. Konings, J. Bacteriol., in press). Although the mechanism of energy coupling differs from that of the secondary transport systems, inhibition of glutamate and glutamine transport is observed after the addition of uncouplers and ionophores which dissipate the transmembrane pH gradient ($Z\Delta pH$). In this report, we present evidence that the activity of the glutamate-glutamine transport system in *S. lactis* ML3 is strictly controlled by intracellular pH.

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MATERIALS AND METHODS

Culture conditions. *S. lactis* ML3 was grown overnight at 30°C in a complex medium (MRS) (4) at pH 6.4 containing 1.0% (wt/vol) galactose and 10 mM arginine, as described previously (Poolman et al., in press).

Transport assays. Cells were harvested, deenergized, and suspended in buffer as described previously by Poolman et al. (in press). Uptake of [^{14}C]glutamate and [^{14}C]glutamine (280 mCi/mmol) was assayed by the filtration method (Poolman et al., in press). Initial rates of uptake were measured in duplicate between 5 and 30 s of incubation. Conditions are further specified in the legends to the figures or in the text.

Determination of $\Delta\psi$ and $Z\Delta pH$. The membrane potential ($\Delta\psi$) and the $Z\Delta pH$ (inside alkaline) were measured simultaneously with the uptake of amino acids by using ion-selective electrodes for tetraphenylphosphonium ion (TPP^+) and salicylate, respectively (Poolman et al., in press). Reaction mixtures (2.0 ml) contained a cell suspension of 1 to 2 mg of protein per ml; TPP^+ and potassium or sodium salicylate were added to 4 and 100 μM, respectively. After 5 min of energization with lactose (10 mM) or arginine (5 mM), ^{14}C-labeled amino acids were added to the concentrations given in the figure legends. Samples (100 μl) were taken at various time intervals to measure the amino acid uptake and the intracellular ATP concentrations. These samples were further handled as described in another paper (Poolman et al., in press).

To determine pH gradients, the inside alkaline or acidic with respect to the extracellular medium, the distribution of [U-^{14}C]benzoic acid (50 mCi/mmol) and [U-^{14}C]methylamine (56 mCi/mol), respectively, was measured by the silicon oil centrifugation method (16a). The $\Delta\psi$ was calculated with the Nernst equation from the distribution of TPP^+ between the bulk phase of the medium and the cytoplasm after correction for concentration-dependent binding of TPP^+ to the cytoplasmic membrane (13).

Other analytical procedures. Intracellular potassium concentrations were determined by flame photometry after separation of the cells from the external medium by silicon oil centrifugation. Intracellular concentrations were calculated after correction for the amount of extracellular water in the perchloric acid extract.

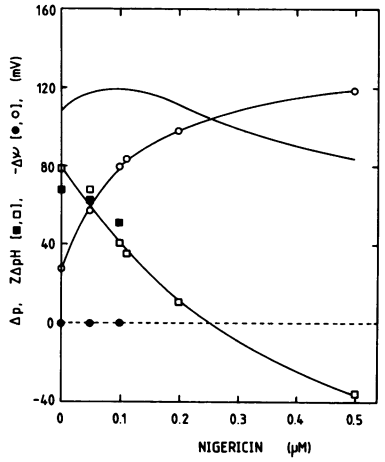

FIG. 1. The effect of increasing concentrations of nigericin on the magnitude of the ZΔpH and the Δψ in *S. lactis* ML3. Cells were suspended to a final protein concentration of 2.3 mg/ml in 50 mM potassium MES (morpholineethanesulfonic acid)–50 mM potassium PIPES (piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)–5 mM $MgSO_4$ buffer (pH 6.0) containing 100 μM potassium salicylate and 4 μM TPP^+. The cells were incubated with various concentrations of nigericin after which 10 mM lactose was added. After 5 min of energization, the ZΔpH (inside alkaline), the Δψ, and the intracellular ATP concentrations (see the text) were determined simultaneously. The measurement of the ZΔpH (inside alkaline) and the Δψ was performed with ion-selective electrodes. For the determination of the ZΔpH (inside acidic) the cells were incubated with 17.8 μM [^{14}C]methylamine and separated from the external medium by silicon oil centrifugation. ZΔpH in the absence (□) and presence (■) of 1.6 μM valinomycin, Δψ in the absence (○) and presence (●) of 1.6 μM valinomycin, and the sum of the ZΔpH and Δψ (Δp) in the absence of valinomycin (——) are shown.

Extracellular water adhering to the cells was determined from the difference in partition of ^3H_2O (1 mCi/ml) and D-[U-^{14}C]sorbitol (3.6 μM; 333 mCi/mmol) as markers of the total and extracellular volumes, respectively (16a). Intracellular ATP concentrations were determined with the firefly luciferase assay as described previously (15). Protein was measured by the method of Lowry et al. (14) with bovine serum albumin as the standard.

RESULTS

Effect of intracellular pH on the initial rate of glutamate uptake. In *S. lactis*, transport of glutamate proceeds independently of the magnitude and composition of the proton motive force (Poolman et al., in press). During this study, it was observed that the addition of nigericin to glycolyzing-cell suspensions caused, under certain conditions, a partial or even a full inhibition of glutamate and glutamine transport. The reason for this inhibition was further investigated. *S. lactis* cells, metabolizing lactose at pH 6.0, maintained a ZΔpH of approximately 80 mV, corresponding to an intracellular pH of 7.3 (Fig. 1). The steady-state value of Δψ was low under these conditions, i.e., between −30 and −40 mV. Increased concentrations of nigericin dissipated the ZΔpH. At nigericin concentrations between 200 and 500 nM, the polarity of the ZΔpH even reversed, making the cytoplasm acidic relative to the external medium. The decrease in ZΔpH was fully compensated by an increase in Δψ as long as the intracellular pH was above 6 (Fig. 1). The initial rate of glutamate uptake dropped with increasing concentrations of nigericin (Fig. 2, inset). A similar effect of nigericin on transport and on ZΔpH was found when the cells were preincubated with a saturating amount of valinomycin to collapse the membrane potential (Fig. 1; Fig. 2, inset). The addition of nigericin also caused a decrease in the intracellular ATP concentrations. At 200 nM nigericin, the ATP concentration was lowered from 2.1 to 1.5 mM (data not shown). The strong influence of the cytoplasmic pH on transport activity was revealed when the initial rate of glutamate uptake was plotted as a function of the intracellular pH (Fig. 2). A similar pH dependence of glutamate transport was found when the external pH was kept at 5.0. Transport activities are expressed as the percentage of the maximum rates observed at an intracellular pH of 7.4 in order to distinguish the influence of the internal pH from the effects of the extracellular pH on the initial rate of uptake by changes in the apparent affinity constant. The affinity constant for glutamate transport in *S. lactis* has been shown to be 11.2 and 77 μM at pH 5.1 and 6.0, respectively (Poolman et al., in press). The glutamate concentration used at pH 6.0 (41.6 μM) was therefore not saturating, which explains the differences in absolute transport rates at pH 5.0 and 6.0 (see the legend to Fig. 2).

Effect of ionophores and uncouplers on glutamine transport and intracellular pH. At pH 7.5, the ZΔpH is close to zero and the addition of uncouplers and ionophores should not affect the rate of glutamate and glutamine transport. However, nigericin caused a 50% inhibition of glutamate transport, whereas valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) had only minor effects (Table 1). In this experiment, glutamine was used instead of glutamate because the concentration of the transported species, (undissociated) glutamic acid, is extremely low at pH 7.5 (Poolman et al., in press). The inhibition of glutamine transport by nigericin at pH 7.5 appears also to be due to the decrease of

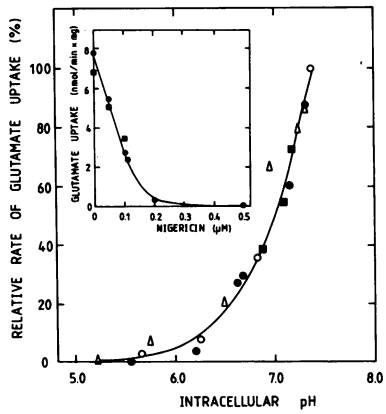

FIG. 2. The dependence of the initial rate of glutamate uptake on intracellular pH. The relative uptake rates were normalized to the rate at an intracellular pH of 7.4. *S. lactis* ML3 cells were suspended in buffer at pH 5.0 (○) and pH 6.0 (● and ■), as described in the legend to Fig. 1. The effect of nigericin on the rate of glutamate uptake at pH 6.0 is shown in the inset of the figure. An additional titration experiment was carried out at pH 5.0. The final [^{14}C]glutamate concentration was 41.6 μM. Measurements in the presence (■) and absence (●) of 1.6 μM valinomycin are shown. The maximal rates of glutamate uptake (100% values) were 14.4 and 9.0 nmol/min per mg of protein at pH 5.0 and 6.0, respectively. The intracellular pH dependence of glutamate transport in potassium-depleted cells (see the legend to Fig. 3) was also plotted (△). The 100% value of the glutamate transport rate in these cells corresponded to 8.5 nmol of glutamate per min per mg of protein.

the intracellular pH. At pH 7.5, the addition of a glycolytic substrate resulted in the generation of a $\Delta\psi$ of -30 to -50 mV and a $Z\Delta pH$ of 0 mV under steady-state conditions. The addition of nigericin, which catalyzes the electroneutral exchange of potassium ions for protons, increased the $\Delta\psi$ to -91 mV and decreased the $Z\Delta pH$ from 0 to -36 mV (inside acidic). Consequently, the intracellular pH fell from 7.5 to 6.9 (Table 1). The addition of valinomycin instead of nigericin at pH 7.5 led to an increase of the intracellular pH to 7.97 (Table 1), whereas the addition of CCCP caused a decrease to pH 7.35. The effects of valinomycin and CCCP on the initial rate of glutamine uptake indicate that glutamine transport reaches its maximal rate at internal pH values around 7.5.

With arginine as the source of energy for ATP synthesis instead of lactose, the initial rates of glutamine uptake were lower since arginine acts as a weak competitive inhibitor of the glutamate-glutamine transport system in *S. lactis* and *S. cremoris* (Poolman et al., in press). The effects of ionophores and CCCP on glutamine uptake under these conditions supported the conclusions reached above. A stimulation of glutamine transport was elicited when the intracellular pH was increased to 7.5, whereas a decrease of the internal pH caused inhibition of transport (Table 1). No net uptake or significant exchange of glutamine (or glutamate) was observed in the absence of an energy source.

Effects of potassium and sodium ions. Although the glutamate-glutamine transport system of *S. lactis* translocates only the neutral species, glutamic acid and glutamine (Poolman et al., in press), the accumulation of glutamate may require the compensatory movement of either cations or anions to preserve the cytoplasmic pH. The translocation of potassium ions in particular could perform this function. To study the effect of potassium ions on glutamate transport, *S. lactis* cells were washed and suspended in a potassium-free medium. The intracellular potassium concentration remained approximately 600 mM in these (potassium-filled) cells despite the large gradient of potassium ions. The rate of glutamate transport in these washed cells was about 50% of that in the presence of potassium (Fig. 3). The stimulation of glutamate transport was maximal at 2 mM KCl externally.

TABLE 1. Effect of ionophores and uncouplers on the initial rate of glutamine uptake and the intracellular pH of *S. lactis* ML3[a]

Energy source	Addition (μM)	Initial rate of glutamine uptake (nmol/min per mg of protein)	Intracellular pH
None	None	0.0	7.05
Lactose	None	33.1	7.49
	Nigericin (0.5)	15.5	6.90
	Valinomycin (1.0)	30.5	7.97
	CCCP (1.0)	27.6	7.35
Arginine	None	3.7	7.22
	Nigericin (0.5)	2.5	6.87
	Valinomycin (1.0)	4.2	7.50
	CCCP (1.0)	3.7	7.21

[a] Cells were suspended to a final protein concentration of 1.54 mg/ml in 50 mM potassium PIPES (pH 7.5)–5 mM $MgSO_4$ buffer containing either 17.9 μM [^{14}C]methylamine or 20 μM [^{14}C]benzoate for determination of the intracellular pH. No energy source, lactose (10 mM), or arginine (5 mM) was added, and after 5 min of incubation the cells were separated from the external medium by silicon oil centrifugation. Transport of [^{14}C]glutamine (final concentration, 40.9 μM) was measured in parallel samples as described in Materials and Methods.

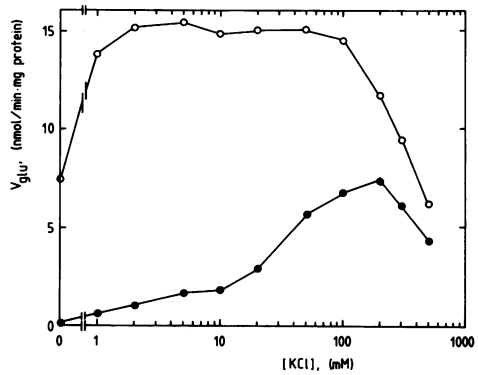

FIG. 3. Effect of extracellular KCl on the initial rate of glutamate uptake in *S. lactis* ML3 cells. Cells were washed twice and suspended to a final protein concentration of approximately 80 mg/ml in 50 mM Tris-PIPES (pH 5.8)–1 mM $MgCl_2$ buffer (potassium-filled cells) (○). The same procedure was performed after incubation of the cells with valinomycin (2 nmol/mg of protein) to deplete the intracellular potassium pool (potassium-depleted cells (●). Subsequently, the cells were diluted 50-fold into 50 mM Tris-PIPES (pH 5.8)–1 mM $MgCl_2$–10 mM lactose containing various concentrations of KCl. Transport of glutamate (final concentration, 100 μM) was measured after 5 min of preenergization.

Above 200 mM KCl, the rate of glutamate transport decreased. Parallel measurements of the components of the proton motive force indicated that the stimulation of the rate of glutamate transport was caused primarily by an increase in the intracellular pH. The addition of potassium to a glycolyzing-cell suspension at pH 5.8 resulted in an increase of the $Z\Delta pH$ from 55 to 90 mV and in a decrease of the membrane potential from -69 to -25 mV. The $Z\Delta pH$ values correspond to internal pH values of 6.7 and 7.3 in the absence and presence of potassium, respectively. Figure 4 shows that the net uptake of potassium in these cells proceeded up to an intracellular concentration of approximately 850 mM and an intracellular pH of 7.4. The depolarization of the membrane potential with the addition of KCl to potassium-filled cells in potassium-free medium indicates that the accumulation of potassium is electrogenic. In the presence of sodium ions (10 mM), both the cytoplasmic pH (Fig. 4) and the rate of glutamate uptake increased slightly. Sodium became inhibitory at external concentrations above 50 mM owing to a lowering of the internal pH (data not shown).

The intracellular potassium concentration could be decreased to 150 mM after the cells were washed with potassium-free medium in the presence of valinomycin (2 nmol/mg of protein). The initial rate of glutamate uptake was 0.15 nmol/min per mg of protein in the potassium-depleted cells in the absence of external potassium (Fig. 3). The rate of glutamate transport in potassium-depleted cells increased with increasing concentrations of external potassium. The control rate, i.e., the rate in potassium-filled cells, was approached only at concentrations above 200 mM. The extent of glutamate uptake was essentially independent of the extracellular potassium concentration (data not shown).

Potassium-depleted cells were unable to regulate their intracellular pH in the absence of potassium or at low external potassium concentrations (Fig. 4). Net uptake of potassium was observed in glycolyzing potassium-depleted cells which matched the increase of the intracellular pH with increasing external potassium concentrations. The relative rate of glutamate uptake in these cells at various external

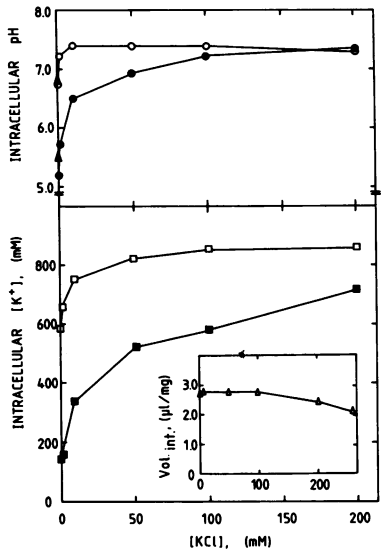

FIG. 4. Effect of external concentration of potassium ions on cytoplasmic pH and intracellular potassium concentration in potassium-filled and -depleted glycolyzing cells of *S. lactis* ML3. Potassium-filled (open symbols) and potassium-depleted (closed symbols) cells were prepared as described in the legend to Fig. 3. The intracellular pH was determined from the distribution of [^{14}C]benzoic acid (20 μM) after separation of the cells from the external medium by silicon oil centrifugation. Parallel samples were prepared for the analysis of the intracellular potassium concentration. The measurements were performed after 5 min of energization with lactose. The final protein concentrations were 1.58 and 1.65 mg/ml for the potassium-filled and -depleted cells, respectively. The values for the internal volumes ($Vol._{int.}$; inset) were used to calculate the intracellular potassium concentrations and the intracellular pH at the corresponding external potassium concentrations. Samples to which 10 mM NaCl instead of KCl was added are indicated by triangles.

potassium concentrations is shown as a function of the intracellular pH in Fig. 2. The dependence of the rate of glutamate transport on potassium ions in potassium-depleted cells (Fig. 3) now seems to be linked through the dependence of the cytoplasmic pH on the (electrogenic) uptake of potassium ions mediated (predominantly) by valinomycin.

DISCUSSION

The results presented in this study provided evidence for the regulation of the rate of glutamate-glutamine transport by intracellular pH. The rate of glutamate and glutamine transport is not dependent on the magnitude of the proton motive force or one of its components (Poolman et al., in press). It is easily observed, however, that uncouplers and ionophores which dissipate the ZΔpH inhibit transport of these amino acids. These results can now satisfactorily be explained by the effects on the internal pH. The picture that emerges from these experiments is that the rate of glutamate transport increases more than 30-fold from an intracellular pH of 6.0 to 7.4 (Fig. 2). A further increase of the intracellular pH to 8.0 does not lead to a further increase of the rates of glutamate and glutamine transport (Table 1). It is unlikely that the intracellular pH dependency of glutamate and glutamine transport is affected by the energy status of the cell, since the cellular ATP levels remain above 1.5 mM when the internal pH is between 6 and 8 (Poolman et al., in press). For manipulation of the intracellular pH by the addition of the ionophore nigericin to a glycolyzing-cell suspension of *S. lactis* at various external pH values, one has to take into account that the internal pH may become lower than that of the outside (Table 1). It has been found that an excess of nigericin results in an intracellular pH which is 0.7 to 0.8 units lower than that of the external medium between pH 5.5 and 8.7 (B. Poolman, unpublished results).

Regulation of solute transport by the intracellular H^+ concentration has thus far been described for cation transport systems which themselves are involved in pH regulation (1, 2). Interestingly, the rates of transport of serine and alanine in membrane vesicles of *S. cremoris* also rely on the absolute value of the cytoplasmic pH (A. J. M. Driessen and W. N. Konings, submitted for publication). The pH profiles for the rates of uptake of serine and alanine are the opposite of those for glutamate and glutamine uptake. A 5- to 10-fold inhibition of serine and alanine transport was observed when the intracellular pH was increased from 6.0 to 7.5. The relationship between the rate of arsenate transport and the cytoplasmic pH in *S. faecalis* has also been studied (6). The rate of arsenate transport increases with the intracellular pH in a similar manner as glutamate and glutamine transport in *S. lactis* except that the rate of arsenate transport starts to increase at pH 7.0, whereas it is already maximal at an internal pH of 7.5. The uptake of aspartate by *S. faecalis* cells was found to be less sensitive to changes in the cytoplasmic pH (6).

The stimulation of glutamate transport by potassium ions both in potassium-depleted and -filled cells of *S. lactis* appears to be caused by the effects of potassium on the intracellular pH. The components involved in pH regulation in *S. lactis* are not known but may be similar to those of *S. faecalis* (3, 10), in which the cytoplasmic pH appears to be raised by the extrusion of protons by the F_0F_1-ATPase and the electrogenic influx of cations. When cations move into the cell, the magnitude of the membrane potential decreases, which allows more protons to be pumped out, and consequently, the magnitude of the pH gradient increases. The fact that the pH gradient is similar in the presence and absence of valinomycin suggests that the route of potassium uptake is not important for pH regulation, provided the flow is electrogenic and sufficiently fast. That indeed an electrogenic flow of cations is important for pH regulation is indicated by the observation that, in a mutant of *S. faecalis* which is defective in the extrusion of sodium ions, the cytoplasmic pH can be regulated in the presence of sodium, whereas in the wild-type strain it cannot (10). Our data suggest that a leak pathway or a specific transport system for sodium uptake does not contribute to the alkalinization of the cytoplasm in *S. lactis* at pH 5.8.

The mode of alkalinization of the cytoplasm in *S. lactis* appears to be similar to that of *S. faecalis* (3, 10) and other neutrophilic bacteria, such as *E. coli* (3). The absolute value of the internal pH in *S. lactis* ML3 is about 0.5 pH unit lower than that in *S. faecalis* (9, 10), but the intracellular pH is approximately 0.5 pH unit higher than that in the atypical *S. lactis* ATCC 7962 (8). Furthermore, *S. lactis* ML3 is able to maintain its cytoplasmic pH above 7 at external pH values as low as 5, whereas in *S. faecalis* the intracellular pH drops below an external pH of 6.5 (9, 10).

Glutamate transport is inhibited at high KCl concentrations. This inhibition, which is also observed with isotonic concentrations of sucrose or potassium HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), coincides with a fall in the internal pH and a drop in the intracellular ATP pool (unpublished results).

Growth of *S. cremoris* at low pH depends on the composition of the medium (7, 17). The presence of weak acids and the sodium concentrations appear to be important parameters for growth by determining the maintenance of the internal pH (17). The sharp decline in the glutamate transport rate when the internal pH falls below 7.5 (Fig. 2), which has also been observed for the uptake of aspartate, asparagine, phosphate, and leucyl-leucine (B. Poolman, H. M. J. Nijssen, and A. van Boven, unpublished results), might be the primary target of growth inhibition at low pH values.

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