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Relation of Growth of *Streptococcus lactis* and *Streptococcus cremoris* to Amino Acid Transport

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The maximum specific growth rate of *Streptococcus lactis* and *Streptococcus cremoris* on synthetic medium containing glutamate but no glutamine decreases rapidly above pH 7. Growth of these organisms is extended to pH values in excess of 8 in the presence of glutamine. These results can be explained by the kinetic properties of glutamate and glutamine transport (B. Poolman, E. J. Smid, and W. N. Konings, J. Bacteriol. 169:2755–2761, 1987). At alkaline pH the rate of growth in the absence of glutamine is limited by the capacity to accumulate glutamate due to the decreased availability of glutamic acid, the transported species of the glutamate-glutamine transport system. Kinetic analysis of leucine and valine transport shows that the maximal rate of uptake of these amino acids by the branched-chain amino acid transport system is 10 times higher in *S. lactis* cells grown on synthetic medium containing amino acids than in cells grown in complex broth. For cells grown on synthetic medium, the maximal rate of transport exceeds by about 5 times the requirements at maximum specific growth rates for leucine, isoleucine, and valine (on the basis of the amino acid composition of the cell). The maximal rate of phenylalanine uptake by the aromatic amino acid transport system is in small excess of the requirement for this amino acid at maximum specific growth rates. Analysis of the internal amino acid pools of chemostat-grown cells indicates that passive influx of (some) aromatic amino acids may contribute to the net uptake at high dilution rates.

The question of why bacteria cannot grow infinitely fast has recently been addressed by Koch (7). For a fast-growing organism like *Escherichia coli*, it has been observed that chloramphenicol inhibits the growth rate progressively from 0 μM and higher, i.e., without a threshold concentration of the drug (3). Since chloramphenicol only inhibits protein synthesis in *E. coli* through blockage of peptide bond formation by ribosomal peptidyl transferases, these results have been interpreted to indicate that growth of *E. coli* is rate-limited by some step in protein synthesis or rRNA maturation (3, 6). The fastest specific growth rates reported for *E. coli* are about 2.6 h^{-1} (corresponding with a doubling time of 16 min). This growth rate approaches the theoretical rate (i.e., 4.2 h^{-1}) which has been calculated for a cell that would be pure ribosomes and associated factors (autocatalytic ribosome) (7).

In contrast to *E. coli*, which can grow on a medium containing minimal salts plus glucose, lactic acid streptococci require, in addition to a minimal medium, various amino acids, vitamins, nucleic acid bases, and other substrates for growth (8; R. Otto, Ph.D. thesis, University of Groningen, Haren, The Netherlands, 1981). Although large differences have been observed among strains, lactic acid streptococci grow relatively fast, and maximum specific growth rates up to 1.1 h^{-1} have been reported (18; this study). The maximum specific growth rates in milk are lower than those in other media (3a). Growth in milk, however, can often be stimulated by the addition of amino acids, indicating that casein hydrolysis, transport of amino acids (or peptides), or both are rate-limiting (3a, 12).

The optimum pH for the maximum specific growth rate of lactic acid streptococci usually varies between 6.0 and 6.5 and decreases rapidly at higher and lower pH values (4; Otto, Ph.D. thesis). Since *Streptococcus cremoris* and *Streptococcus lactis* can regulate their internal pHs between 7.0 and 7.5 when external pHs range from 5.5 to 7.5 (15, 19), the failure to grow at alkaline (and to a lesser extent, at acid) pH values is unlikely caused by a limitation of cytoplasmic processes. This suggests that the external pH affects growth by influencing one or more membrane-associated processes, for instance, transport of essential nutrients. In this report we demonstrate that growth of *S. lactis* and *S. cremoris* at alkaline pH is rate-limited by the capacity to accumulate glutamate. At high growth rates the demands for other essential amino acids like leucine, isoleucine, valine, and phenylalanine can (to varying extents) be satisfied by the maximum activity of the corresponding transport systems. Under some conditions passive diffusion, in addition to carrier-mediated transport, may contribute to the net uptake of (hydrophobic) amino acids.

MATERIALS AND METHODS

Organisms and culture conditions. *S. lactis* ML3 and *S. cremoris* Wg2 were grown anaerobically at 30°C on a synthetic medium or MRS broth (1). The composition of the synthetic medium has been described previously (11), except that individual amino acids were added at the following concentrations, unless indicated otherwise, (in mg/liter) sodium aspartate, 420, or asparagine, 350; sodium glutamate-H_2O, 500, or glutamine, 390; serine, 340; threonine, 225; glycine, 175; arginine, 125; alanine, 240; proline, 675; valine, 325; methionine, 125; leucine, 475; isoleucine, 210; tryptophan, 50; phenylalanine, 275; lysine, 440; histidine, 150; and tyrosine, 200. Further modifications of the synthetic medium are given for the different modes of cultivation.

(i) Batch cultivation. Cells were grown overnight in synthetic medium containing 30 mM lactose. For the determination of the maximum specific growth rates, the organisms

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were cultivated in screw-cap tubes which fit in a spectrophotometer (UC200; Vitatron Scientific Instruments, Dieren, The Netherlands). To increase the buffer capacity, the phosphate concentration was raised to 7.5 g of K_2HPO_4 per liter and 9.0 g of KH_2PO_4 per liter. The pH was adjusted to 6.4, unless indicated otherwise. Growth of *S. lactis* and *S. cremoris* was monitored at 660 nm to a final optical density of 0.3.

(ii) Continuous cultivation. Chemostats were used as described previously (17). Organisms were grown in the synthetic medium as indicated above, with glucose (30 mM) instead of lactose as the growth-limiting substrate. In addition, the arginine concentration was raised to 4 mM. The pH of the medium was kept at 6.4 by titrating it with 1 N NaOH.

(iii) Cultivation on solid media. Culture suspensions were diluted stepwise with 50 mM potassium phosphate (pH 6.4)–5 mM $MgSO_4$. Subsequently, 100 μl of cell suspension was spread onto agar plates containing synthetic medium or complex broth (1), 1.5% (wt/vol) agar (Becton Dickinson and Co., Paramus, N.J.), and 15 mM lactose or glucose. The plates were incubated for about 2 days at 30°C.

Transport assays. Cells were harvested by centrifugation, washed twice, and suspended in 50 mM K-PIPES [potassium piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.0] and 5 mM $MgSO_4$ at 1 to 2 mg of protein per ml. Deenergization of the cells was performed as described previously (16). Carrier-mediated transport was assayed at 30°C in 200 μl of incubation mixture. Initial uptake rates were determined by adding the appropriate concentration of ^{14}C-labeled amino acid to the cell suspension after 5 min of preenergization with 10 mM lactose. Initial rates of uptake were measured in duplicate after 10 s of incubation by the filtration method (16). To measure the passive influx of amino acids, deenergized cells (1 to 2 mg of protein per ml) were incubated for 60 min at 30°C with 10 mM diethylpyrocarbonate (DEPC) and 0.5 mM *N*-naphthylmaleimide (NNM). Subsequently, the cells were washed and suspended to 60 to 90 mg of protein per ml. Amino acid uptake was measured for 5 s at 30°C, after the cells were diluted to a final protein concentration of 5 to 6 mg/ml, into 50 mM K-PIPES (pH 6.0) and 5 mM $MgSO_4$ (final volume, 200 μl) containing the appropriate concentration of ^{14}C-labeled amino acid. The cells were separated from the medium after the addition of 3.0 ml of ice-cold 0.1 M LiCl by rapid filtration over glass microfiber filters (diameter, 2.5 cm; GF/F; Whatman, Inc., Clifton, N.J.). The amount of amino acid taken up after 5 s of incubation was always less than 25% of the equilibration level.

Intracellular amino acid concentrations. Procedures to separate the cells from the medium by silicon oil centrifugation have been described previously (14, 17). Amino acids were analyzed after derivation with dansyl chloride and separated by reversed-phase high-performance liquid chromatography on a C_{18} column (3.9 mm by 30 cm; μBondapak C18; Waters Associates, Inc., Milford, Mass.), as described previously (17). Intracellular amino acid concentrations were calculated from the amount that was present in the cell extracts after correction for the medium that adhered to the cellular surface during silicon oil centrifugation (17). The ratio of extracellular water (that adhered after silicon oil centrifugation) over intracellular water was about 1 for *S. lactis* cells.

Amino acid composition. The amino acid composition of *S. lactis* cells was determined after acid hydrolysis (in 6 N HCl) of the samples for 40 h. Amino acid analysis was performed by P. Jekel with an amino acid analyzer (Kontron Liquimat III) at the Department of Biochemistry, University of Groningen.

Protein determination. Protein was measured by the method described by Lowry et al. (9) by using bovine serum albumin as a standard.

Calculations. Rates (theoretical) of amino acid uptake (V_t) by growing cells were calculated from the amino acids in total cell hydrolysates (N) (in nanomoles per milligram of protein) and the specific growth rate (μ [in hours^{-1}]). Upon integration of the amino acid uptake in time (i.e., during exponential growth), V_t is given by $N \times \mu/60$ (nanomoles per minute × [milligram of protein]).

Materials. [U-^{14}C]glutamate (280 mCi/mmol), [U-^{14}C]leucine (348 mCi/mmol), [U-^{14}C]valine (285 mCi/mmol), and [U-^{14}C]phenylalanine (522 mCi/mmol) were obtained from Amersham Corp. (Buckinghamshire, England). All other chemicals were reagent grade and were obtained from commercial sources.

RESULTS

Relation between glutamate uptake and growth inhibition at alkaline pH. Glutamate uptake by *S. lactis* ML3 and *S. cremoris* Wg2 cells is catalyzed by a transport system which translocates glutamic acid instead of glutamate anion (16). At various external pH values, the initial rate of glutamate transport (when plotted versus the glutamic acid concentration) followed the same Michaelis-Menten kinetics (Fig. 1A). The apparent affinity constant (K_t) of the transport system for glutamine acid was 1.5 μM, and the maximal rate of uptake (V_{max}) was about 20 nmol/min × (mg of protein). Despite the high affinity for glutamic acid, at alkaline pH values the transport system is not saturated for this substrate, even when glutamate (i.e., the sum of glutamate anion plus glutamic acid) is present at millimolar concentrations. The physiological consequences of the operation of a glutamic acid instead of a glutamate anion uptake system were investigated.

Glutamate is an essential amino acid for growth of lactic acid streptococci (8). The optimal glutamate concentration for growth of *S. cremoris* in synthetic medium at pH 6.5 has been estimated to be 2 to 3 mM (Otto, Ph.D. thesis). Our standard growth medium usually contains 2.67 mM (0.5 g/liter) sodium glutamate (i.e., glutamate anion plus glutamic acid). The fraction of glutamic acid in this medium decreased sodium logarithmically with increasing pH, resulting in glutamic acid concentrations below 1.5 μM (i.e., the K_t of transport) above pH 7.5 (Fig. 1B). Consequently, the rate at which glutamate can be taken up by *S. lactis* and (*S. cremoris*) cells decreases with increasing pH (Fig. 1B).

To estimate the amount of glutamate that needs to be taken up to meet the maximum growth rate of *S. lactis*, the amino acid composition of total cell hydrolysates was determined (Table 1). The molar percentage of glutamate plus glutamine was 10.6, corresponding to 853 nmol/mg of protein. It was not necessary to quantitate the fraction of glutamate and glutamine separately since streptococci can only synthesize glutamine from glutamate in the absence of glutamine (and vice versa). The rates of glutamate uptake minimally required for growth (V_t^{glu}) can be calculated from the amino acid composition at any growth rate (see above). The actual rates of glutamate uptake are determined by the concentrations of glutamic acid and, thus, at a constant glutamate concentration by the pH of the medium (Fig. 1B). The maximum specific growth rates in the standard growth medium ([Glu] = 2.67 mM) could then be calculated as a

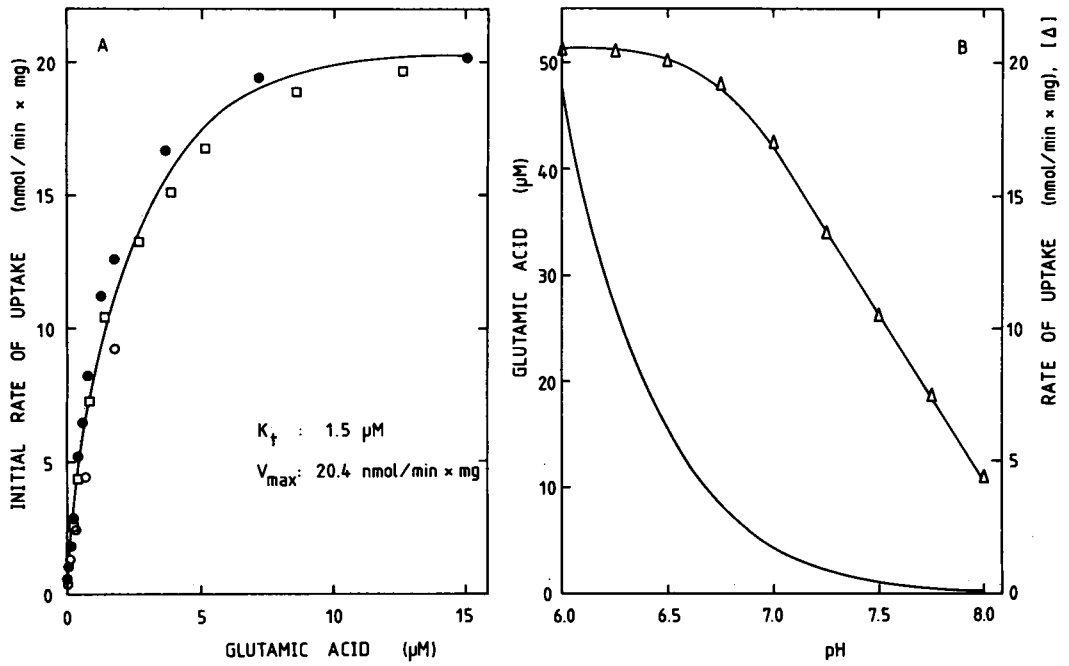

FIG. 1. Kinetic parameters and maximal activity of glutamate transport by *S. lactis* ML3 cells in a synthetic growth medium. (A) Dependence of the initial rate of glutamate transport at pH 5.1 (□), 6.0 (●), and 7.0 (○) on the concentration of glutamic acid. Data were obtained (after modification) from a previously published report (16). The glutamic acid concentration was calculated from the total glutamate concentration and pH of the medium, according to the Henderson-Hasselbach equation. (B) pH dependence of the rate of glutamate transport and the glutamic acid concentration in a synthetic growth medium containing 2.67 mM glutamate. The rates of glutamate uptake were calculated on the basis of the kinetic parameters of glutamate transport (see panel A) and the glutamic acid concentration in the synthetic growth medium.

function of pH. If growth of *S. lactis* is restricted only by the capacity to accumulate glutamate, the growth rate decreases linearly with increasing pH above pH 7 (i.e., on the assumption that glutamic acid uptake is not affected [competitively] by other solutes) (Fig. 2, solid line). On the basis of the V_{max} of glutamate transport, a maximum specific growth rate of 1.4 h^{-1} would be possible at a saturating glutamic acid concentration.

The glutamate transport system appeared to be highly specific for glutamic acid and glutamine (16). Aspartic acid and asparagine competitively inhibit glutamate transport, with K_i values of approximately 1 mM (B. Poolman and L. Truong, unpublished data). Competitive inhibition of glutamate (or glutamine) uptake is most pronounced for arginine (K_i = 257 μM) (16). In the presence of a competitive inhibitor of glutamate transport (with K_i = 250 μM and at an inhibitor concentration of 1 mM), the maximum attainable specific growth rates decrease to lower pH values (Fig. 2, dotted line).

To test whether growth of *S. lactis* and *S. cremoris* at alkaline pHs is indeed limited by the ability to accumulate glutamate, both organisms were grown in a synthetic medium in the presence of glutamate or glutamine at various pH values (Fig. 3). For both organisms the maximum specific growth rate declined rapidly above pH 6.5 to 7.0, when only glutamate was present as source of glutamate and glutamine for biosynthesis. Further support for the contention that growth at alkaline pH is inhibited by the uptake of glutamate was obtained by studying the pH dependence of the growth rate with glutamine instead of glutamate as a source of glutamate and glutamine. It has been shown previously that glutamine is taken up by the same transport system with a K_t of about 2 μM, independent of the external pH (17). With

TABLE 1. Amino acid composition and rates of amino acid transport necessary to meet the growth requirements of *S. lactis* ML3

Amino acid	Amino acid composition (mol%)[a]	V_t (for μ = 1.0 h^{-1}) (nmol/min × [mg of protein])[b]
Asp + Asn	10.9	14.6
Thr	5.7	7.6
Ser	3.3	4.4
Glu + Gln	10.6	14.3
Gly	12.9	17.4
Ala	12.9	17.4
Val	7.1	9.5
Met	2.2	3.0
Ile	5.6	7.5
Leu	8.3	11.0
Tyr	2.0	2.6
Phe	3.3	4.4
Lys	4.0	5.4
His	2.2	3.0
Arg	3.6	4.9
Pro	5.1	6.9
Cys	0.1	<0.1
Trp	ND[c]	

[a] Amino acid composition of total cell hydrolysates, including proteins and peptidoglycan amino acids. *S. lactis* ML3 cells were grown to an A_{660} of 0.8 on MRS broth containing 30 mM galactose and 25 mM arginine, as described in the text. Cells were harvested, washed twice, and suspended in 100 mM potassium phosphate (pH 7.0) prior to the amino acid analysis.

[b] Transport rates were calculated for a specific growth rate of 1.0 h^{-1} on the basis of the amino acid composition of total cell hydrolysates (see text).

[c] ND, Not determined.

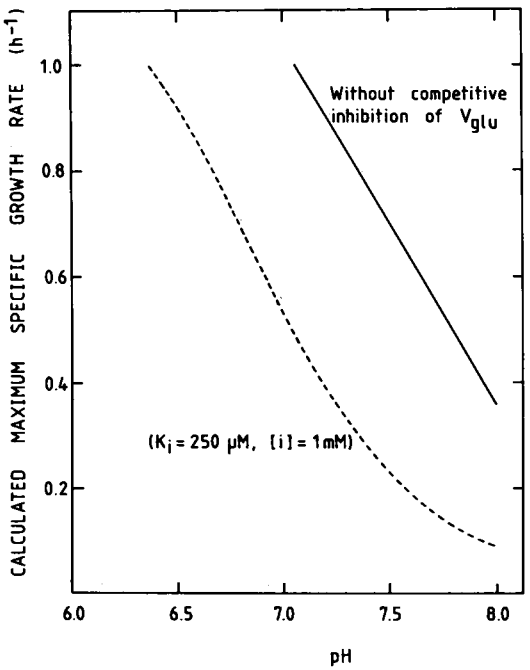

FIG. 2. pH dependencies of calculated maximum specific growth rates based on the kinetic parameters of glutamate transport of *S. lactis* ML3. The solid line represents the pH dependence of the growth rate on the basis of the rates of glutamate uptake in the synthetic medium ([Glu] = 2.67 mM) (Fig. 1) and the glutamate plus glutamine requirement (calculated from the amino acid composition [Table 1]) for growth. The dotted line represents the pH dependence of the growth rate by taking into account competitive inhibition of glutamate transport. Competitive inhibition was assumed to occur with a K_i of 250 μM and at an inhibitor concentration (*i*) of 1 mM.

glutamine present in the (synthetic) growth medium, high growth rates were observed up to pH 8. When *S. lactis* was grown in the presence of glutamate-asparagine (2.65 mM) instead of glutamate-aspartate (2.65 mM), growth at alkaline pH was even further decreased, as would be expected as a result of the higher concentration of effective (competitive) inhibitor, i.e., 2.65 mM asparagine versus micromolar concentrations of aspartic acid (pK_a = 3.86) (Fig. 2; data not shown).

The observation that glutamine is a better source of glutamate than glutamate itself at high pH values suggests that the media used for growth of *S. lactis* and *S. cremoris* can be improved. Growth of *S. cremoris* (and, to a lesser extent, *S. lactis*) strains on synthetic (solid) media is usually poor and non-reproducible in comparison with growth on complex media (unpublished data). By replacing glutamate with glutamine in our defined medium, growth of *S. cremoris* (expressed as CFU) became as good as that on agar plates containing complex broth. Furthermore, the lag phase preceeding exponential growth on synthetic liquid media was highly reduced in the presence of glutamine (data not shown).

Are carrier-mediated amino acid uptake rates sufficient to supply the amino acid requirements for growth? (i) Branched-chain amino acids. The minimally required uptake rate of any amino acid at a distinct growth rate can be calculated from the amino acid composition of the cell (Table 1). To sustain a maximum specific growth rate of 1.0 h^{-1}, *S. lactis* needs to take up leucine, isoleucine, and valine at rates of 11.0, 7.5, and 9.5 nmol/min × (mg of protein), respectively. Results of studies of the membrane vesicles of *S. cremoris* (2) have indicated that these (essential) amino acids are taken up by a single kinetically distinguishable transport system. The activity of the branched-chain amino acid carrier in intact cells, therefore, must be at least 28.0 nmol/min × (mg of protein) to fulfil the leucine, isoleucine, and valine requirements. The

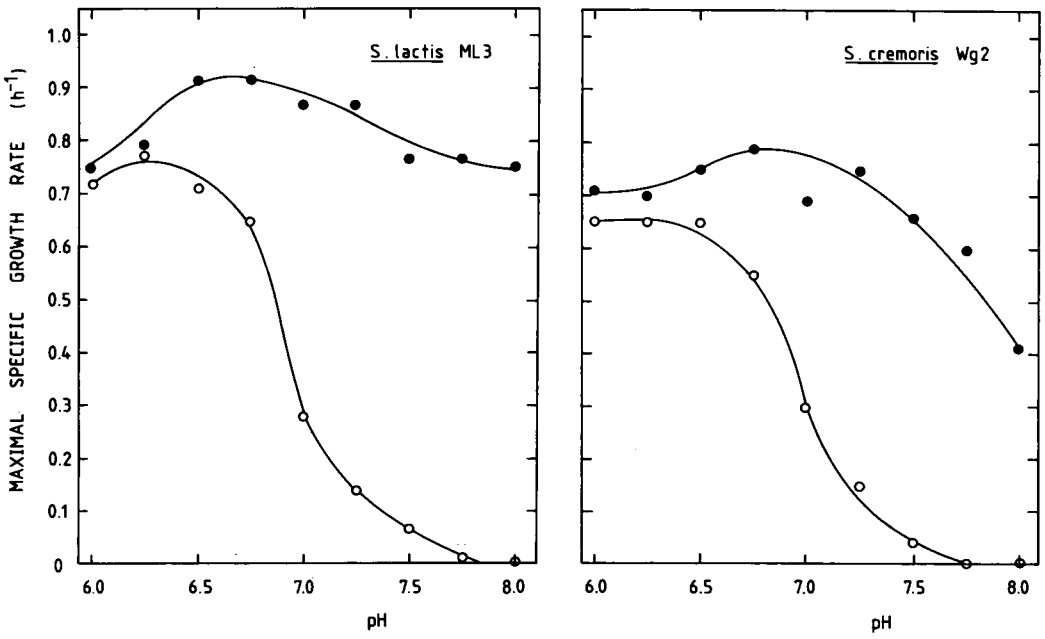

FIG. 3. pH dependence of growth of *S. lactis* ML3 (A) and *S. cremoris* Wg2 (B). *S. lactis* and *S. cremoris* were grown in a synthetic medium containing 2.67 mM glutamate (○) or glutamine (●) as a source of glutamate and glutamine for biosynthesis. The growth medium always contained aspartate together with glutamate and asparagine together with glutamine.

actual rates (and pathways) of leucine (and valine) uptake by cells grown on different media were determined.

The kinetic parameters of proton motive force (Δp)-driven leucine and valine uptake were estimated in the presence of a glycolytic substrate. In cells of *S. lactis* grown on synthetic medium containing amino acids, the V_{max} for leucine (and valine; data not shown) uptake was about 130 nmol/min × (mg of protein) (Fig. 4). Since Δp values in glycolyzing and growing cells are very similar (15, 19, 20), these rates can be compared with those obtained under growing conditions. In contrast to glutamate transport (data not shown), the V_{max} of leucine (and valine) uptake was about 10 times lower in cells grown on complex broth (Fig. 4). The K_t values for leucine and valine transport in these cells were 5.9 and 12.0 μM, respectively. The affinities did not differ significantly between cells grown on synthetic and complex media. These results indicate that the rates of Δp-driven uptake of branched-chain amino acids (for cells grown on the synthetic medium) are sufficient to account for the leucine, isoleucine, and valine requirements at high growth rates, whereas the rates are insufficient for cells grown on complex broth. It is possible that the needs of lactic acid streptococci for branched-chain amino acids are (partially) fulfilled by the presence of peptides in these media for which separate transport systems are present. Under conditions in which the internal concentrations of leucine, isoleucine, and valine are lower than the external concentrations, the cells should also be able to take up these hydrophobic amino acids by passive influx (2a).

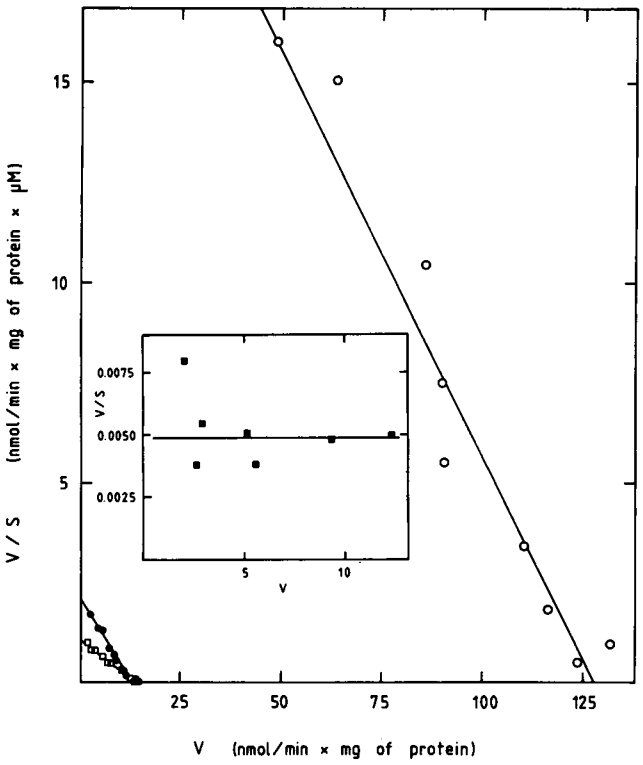

FIG. 4. Kinetic properties of leucine and valine transport in *S. lactis* ML3 cells. Cells were grown on synthetic medium containing glutamine plus asparagine (○) or complex broth (●, □) in the presence of 30 mM lactose. Cells were suspended to a final protein concentration of 0.12 (○) or 0.95 (●, □) mg/ml in 50 mM K-PIPES (pH 6.0) and 5 mM $MgSO_4$ containing 10 mM lactose. After 5 min of preenergization, various concentrations (1.5 to 225 μM) of [^{14}C] leucine (○, ●) or [^{14}C]valine (□) were added. Uptake was measured in duplicate for 10 s, after which transport was terminated as described in the text. (Inset) Kinetic properties of leucine influx in DEPC-NNM-treated cells of *S. lactis*. A concentrated cell suspension was diluted to 5.6 mg of protein per ml in 50 mM K-PIPES (pH 6.0)–5 mM $MgSO_4$ containing various concentrations of [^{14}C]leucine (0.25 to 2.5 mM). Uptake was measured in duplicate for 5 s. The data are plotted in the form of Eadie-Hofstee plots.

To determine whether passive diffusion of branched-chain amino acids could (in principle) occur, the influx of leucine in DEPC-NNM-treated (amino acid depleted) cells of *S. lactis* was estimated. DEPC-NNM treatment resulted in complete inhibition of carrier-mediated leucine transport (data not shown). The initial rates of leucine uptake in these cells did not exhibit saturation kinetics up to leucine concentration of 2.5 mM (Fig. 4, inset), suggesting that this transport component is due to passive diffusion. The first-order rate constant for leucine influx in DEPC-NNM-treated cells was about 1.3 min^{-1}.

(ii) Aromatic amino acids. In contrast to tyrosine and tryptophan, phenylalanine is essential for growth of *S. lactis*. The molar percentage of phenylalanine in the amino acid composition of *S. lactis* was 3.3 (Table 1). For a specific growth rate of 1.0 h^{-1}, *S. lactis* needs to transport phenylalanine at a rate of 4.4 nmol/min × (mg of protein). Since phenylalanine transport in *S. lactis* is (competitively) inhibited by tyrosine and tryptophan (unpublished data), the capacity of this transport system must be at least 8 to 9 nmol/min × (mg of protein) (on the basis of the molar percentages for phenylalanine and tyrosine presented in Table 1, and assuming a molar percentage of 1 for tryptophan [7]). This rate does not take into account the possible differences in the K_t values for uptake of aromatic acids or differences in the concentrations of these amino acids in the synthetic medium. Kinetic analysis of phenylalanine transport in glycolyzing cells indicated a V_{max} of about 8 nmol/min × (mg of protein), in addition to a nonsaturable transport component which may represent passive influx of phenylalanine. The first-order rate constant of this process was estimated to be 2.1 min^{-1}. Based on the V_{max} of phenylalanine uptake, carrier-mediated transport of aromatic amino acids seems hardly sufficient to support the observed maximum specific growth rates.

Amino acid concentration gradients in growing cells. The intra- and extracellular amino acid concentrations were determined in chemostat-grown cells of *S. lactis* at various imposed growth rates. Limitation of growth by amino acid transport was reflected in a decrease of the intracellular amino acid pool with increasing growth rate.

The branched-chain amino acids were accumulated up to twofold (almost) independent of the dilution rate (Fig. 5A). The intracellular concentrations of the aromatic amino acids decreased with increasing dilution rate (Fig. 5B). At high growth rates the intra- and extracellular concentrations of phenylalanine and tyrosine became similar, whereas the internal concentration of tryptophan became significantly lower than the external concentration (Fig. 5B). Under these conditions passive influx of tryptophan could play a role in the net uptake process. The intracellular concentrations of alanine and glycine (Fig. 5C), which were found with the highest molar fractions in *S. lactis* cells (Table 1), also decreased with increasing dilution rate. Although these amino acids are not essential for growth (8), the decrease in the external concentration indicated that these amino acids were preferentially taken up at any dilution rate instead of being synthesized. The internal proline and histidine concen-

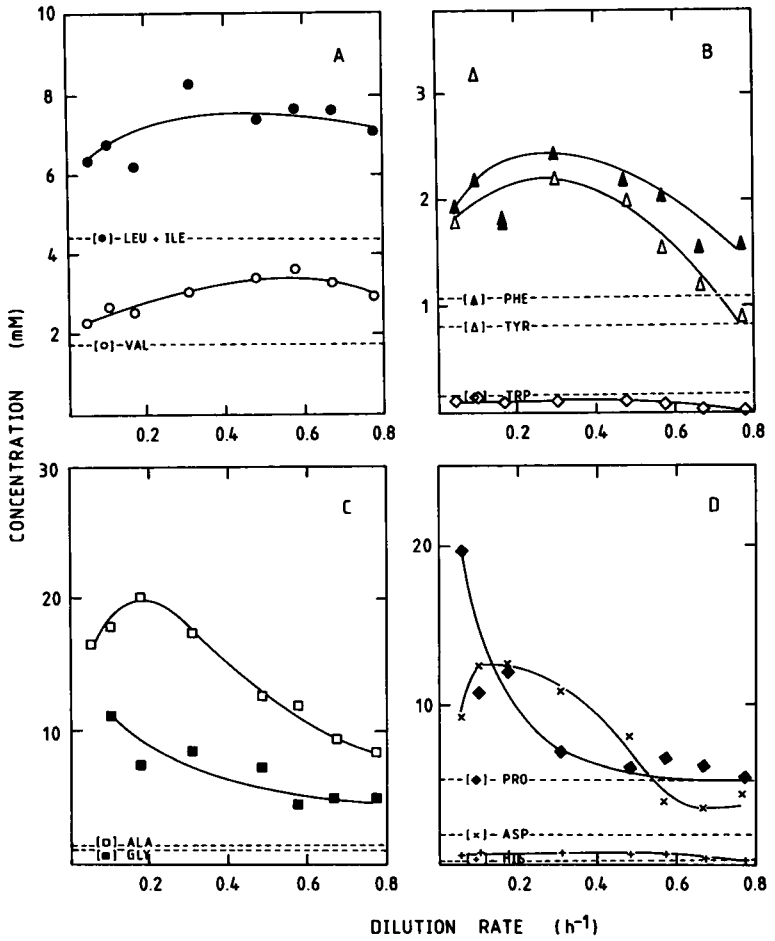

FIG. 5. Effect of imposed growth rate on the intracellular concentrations of amino acids in chemostat-grown cells of *S. lactis* ML3. The (steady-state) intra- and extracellular concentrations are indicated by solid and dotted lines, respectively. The concentrations represent the average of two independent determinations.

trations fell to equilibration levels with an increase in the dilution rate (Fig. 5D). The concentration gradients of other essential amino acids like glutamate and methionine remained essentially constant (data not shown). The failure to maintain concentration gradients for a number of amino acids (i.e., phenylalanine, tyrosine, tryptophan, proline, and histidine) at high dilution rates indicates that the net uptake rates are close to the rates of amino acid consumption under these conditions.

Effect of the concentrations of branched-chain amino acids on the growth rate. Although the K_t values of amino acid transport in streptococci are in the micromolar range, maximum specific growth rates have frequently been shown to be affected by changes in amino acid concentration in the millimolar range (8; Otto, Ph.D. thesis). These effects could be due to changes in the relative fluxes of amino acids, which share the same transport system(s), as a result of changes in the relative amounts of amino acids. Despite a large overcapacity of the branched-chain amino acid carrier to accumulate leucine, isoleucine, and valine (i.e., when cells are grown on the synthetic medium), uptake of a single amino acid can become insufficient when its concentration is decreased with respect to the other branched-chain amino acids. It can be calculated on the basis of the K_t, V_{max}, and the absolute amino acid concentrations that a 20-fold decrease in the concentration of one branched-chain amino acid results in inhibition of growth. A 20-fold decreased concentration of leucine, isoleucine, and valine still exceeds (at least 10-fold) the K_t for transport of each amino acid (Fig. 4). Since the V_{max} of the branched-chain amino acid carrier is similar for leucine, isoleucine, and valine, the flux of each amino acid is determined by the relative ratios of the amino acid concentration in the medium divided by the K_t. Consequently, a reduction in the concentration of valine (K_t = 12 μM; medium concentration, 2.8 mM) has more pronounced effects on the maximum specific growth rate than does a similar decrease in the concentration of leucine (K_t = 6 μM; medium concentration, 3.6 mM). From these data and the V_{max} of the branched-chain amino acid carrier, leucine and valine uptake rates of 8.9 and 2.0 nmol/min × (mg of protein), respectively, can be calculated when the concentration of the corresponding amino acid in the growth medium is reduced 20-fold. The effect of these (decreased) transport rates on growth can be deduced by comparing these rates with those given in Table 1. The maximum specific growth rates of *S. lactis* and *S. cremoris* were decreased by only 10% with a 20-fold decrease of the concentration of all branched-chain amino acids (Table 2). Growth of these organisms was more severely affected when the concentration of a single amino acid was reduced to the same extent. As expected, the effects were most pronounced when the valine concentration was reduced.

TABLE 2. Effect of the concentration of branched-chain amino acids on the maximum specific growth rates of *S. lactis* ML3 and *S. cremoris* Wg2

Amino acid (concn [%])[a]	Maximum specific growth rate (h^{-1}) of[b]:	
	S. lactis ML3	*S. cremoris* Wg2
Leu, Ile, Val (100)	1.11	0.83
Leu, Ile, Val (20)	1.10	0.79
Leu, Ile, Val (5)	0.99	0.77
Leu (20)	1.07	0.84
Leu (5)	0.84	0.55
Val (20)	0.96	0.49
Val (5)	0.33	0.16

[a] Cells were grown in the synthetic growth medium (pH 6.4) containing glutamine and asparagine, as described in the text, except that the concentrations of branched-chain amino acids were varied, as indicated.

[b] Maximum specific growth rates represent the average of three separate measurements.

DISCUSSION

In this report we have provided quantitative information on the requirements for essential amino acids during growth of *S. lactis* (and *S. cremoris*) and the capacity (and pathways) to accumulate these amino acids. Growth of *S. lactis* (and *S. cremoris*) at alkaline pH values is shown to be limited by the capacity to accumulate glutamate. Furthermore, the kinetic properties of the glutamate-glutamine transport system predict relatively well the pH dependence of the maximum specific growth rate above pH 7 (Fig. 2 and 3). At pH 6 or below, the maximum specific growth rate of *S. lactis* was the same with glutamate or glutamine as the source of glutamate and glutamine for biosynthesis. Complex broth is routinely autoclaved instead of filter sterilized, and this results in the cyclization of glutamine to ammonium pyrrolidone carboxylate (10). Consequently, glutamate (residues) could also be the only source of glutamate and glutamine in complex broth, which explains the growth inhibition at alkaline pH in these media (4, 13). Growth of *S. lactis* and *S. cremoris* at alkaline pH values in the presence of glutamate may (in principle) be stimulated by raising the glutamate concentration, but to keep the glutamic acid concentration constant, the glutamate concentration must be raised by 1 order of magnitude per pH unit. However, glutamate concentrations above 2 to 3 mM have been shown to be inhibitory for growth of *S. cremoris* (Otto, Ph.D. thesis), possibly by interference (competitive inhibition) with other transport systems that catalyze the uptake of essential nutrients (see below).

In *S. lactis* aspartate has also been shown to be taken up by a transport system that translocates the acid instead of the anion (unpublished data). Since aspartate is not essential for the growth of *S. lactis* and *S. cremoris* (8; unpublished data), inhibition of the maximum specific growth rate at alkaline pH values is not likely due to a limited capacity to accumulate aspartate. Actually, growth at alkaline pH values in the presence of glutamate is even more severely inhibited in the presence of asparagine than in the presence of aspartate, most likely as a result of the stronger competitive inhibition by asparagine than by aspartate (at the same concentration).

The kinetic properties of glutamate uptake of *Streptococcus faecalis* are distinct from those of *S. lactis* (and *S. cremoris*), which explains why *S. faecalis* can grow at alkaline pH values in the presence of glutamate (6). The *S. faecalis* transport system has no preference for either the acidic or the anionic form of glutamate; or even when the organism only transports the glutamate anion, the dominant species at alkaline pHs, uptake of glutamate does not become a limiting factor for growth between pH 6 and 8 (13).

The amino acid composition of *S. lactis* (Table 1) indicates a relatively high requirement for leucine, isoleucine, and valine which can be satisfied by the V_{max} of the branched-chain amino acid carrier for cells grown on synthetic medium containing amino acids. On the other hand, the V_{max} of the branched-chain amino acid carrier of cells grown on complex broth is insufficient to account for the observed maximum specific growth rates in these media (4; unpublished data). Under these conditions, it is most likely that other sources of branched-chain amino acids (i.e., peptides) are present. The contribution of passive influx to the net uptake of branched-chain amino acids may become important for growth of lactic acid streptococci when the level of expression of the branched-chain amino acid carrier is reduced (for instance, by genetic means) and the internal concentrations are decreased below the external concentrations. The kinetic data of phenylalanine uptake (a V_{max} which can hardly explain the observed growth rates when competitive inhibition by tyrosine and tryptophan is taken into account) indicate that passive influx of (some) aromatic amino acids could contribute to net uptake at high growth rates (in media containing amino acids and no peptides). This suggestion is supported by the observation of the negative concentration gradients (i.e., the internal concentration is less than the external concentration) for tryptophan in chemostat-grown cells (Fig. 5B). Although tryptophan is not essential for growth of *S. lactis*, the low intracellular concentrations at high dilution rates (Fig. 5B) suggest that the rate of cellular synthesis of tryptophan is limited. Limitation of growth by nonessential amino acids like tryptophan has been observed for thermophilic streptococci (8).

The effect of reducing the concentration of a single (branched-chain) amino acid in the growth medium shows that the relative rather than the absolute concentrations of amino acids can determine the maximum specific growth rate. These effects can only be predicted when the kinetic properties of the transport systems (pathways) concerned are known.

The cause for the large differences in V_{max} values of leucine transport between cells grown on synthetic and complex media has not yet been investigated further. The increase in the V_{max} of leucine (and valine) transport is most likely due to an increased expression of the carrier since the same difference in carrier activity was also observed for artificial ion diffusion potential-driven leucine uptake in membrane vesicles derived from these cells (R. Kieviet, unpublished data). It is possible that the synthesis of the branched-chain amino acid carrier in cells grown on complex broth is (partially) repressed by the presence of peptides.

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