

## Aspartate Biosynthesis Is Essential for the Growth of *Streptococcus thermophilus* in Milk, and Aspartate Availability Modulates the Level of Urease Activity<sup>∇</sup>

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**We investigated the carbon dioxide metabolism of *Streptococcus thermophilus*, evaluating the phenotype of a phosphoenolpyruvate carboxylase-negative mutant obtained by replacement of a functional *ppc* gene with a deleted and inactive version,  $\Delta pp c$ . The growth of the mutant was compared to that of the parent strain in a chemically defined medium and in milk, supplemented or not with L-aspartic acid, the final product of the metabolic pathway governed by phosphoenolpyruvate carboxylase. It was concluded that aspartate present in milk is not sufficient for the growth of *S. thermophilus*. As a consequence, phosphoenolpyruvate carboxylase activity was considered fundamental for the biosynthesis of L-aspartic acid in *S. thermophilus* metabolism. This enzymatic activity is therefore essential for growth of *S. thermophilus* in milk even if *S. thermophilus* was cultured in association with proteinase-positive *Lactobacillus delbrueckii* subsp. *bulgaricus*. It was furthermore observed that the supplementation of milk with aspartate significantly affected the level of urease activity. Further experiments, carried out with a *p<sub>ureT</sub>-gusA* recombinant strain, revealed that expression of the urease operon was sensitive to the aspartate concentration in milk and to the cell availability of glutamate, glutamine, and ammonium ions.**

*Streptococcus thermophilus* is a major component of dairy starter used for the manufacture of yogurt and cheeses. One of the main roles of *S. thermophilus* growing in milk is to provide rapid acidification as a consequence of the production of lactic acid. Previous studies have shown that the availability of peptides and amino acids is critical for the optimal growth of this bacterial species in milk (8, 11). While for other species, such as *Lactococcus lactis* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, growth in milk depends largely on the activity of cell-wall-anchored proteinases, for *S. thermophilus* the amino acid requirement is satisfied by the efficiency of its biosynthetic capacities and by the cooperation with other bacterial species growing in association in the dairy environment, such as *Lactobacillus delbrueckii* subsp. *bulgaricus*. Cell-wall-anchored proteinases are reported to be present only in a minority of *S. thermophilus* strains studied until now (9), and their physiological and technological roles have been investigated (8, 11). The capacity of *S. thermophilus* to satisfy its amino acid requirement during growth in milk is different from that of other lactic acid bacterial species. Some *S. thermophilus* strains appear auxotrophic for at least four amino acids, Glu, Cys, His, and Met, while other lactic acid bacteria are known to be more exigent (16, 19). The relevance of the amino acid biosynthetic

pathways for the metabolism of *S. thermophilus* have been investigated for branched-chain amino acids, proline, and glutamine (13, 20, 24). *S. thermophilus* genome analysis has revealed a relatively high conservation of functional amino acid biosynthetic genes, which might reflect the importance of amino acid synthesis for the growth of this species in milk (2, 16). In this work, we investigated the aspartic acid biosynthesis of *S. thermophilus*, focusing our attention on phosphoenolpyruvate (PEP) carboxylase activity and its relationship with urease, an ammonia and carbon dioxide-generating enzyme. Ammonia produced from urea hydrolysis was recently reported to be involved in glutamine biosynthesis (24). An overview of the potential metabolic connections among the aspartate biosynthetic pathway, glutamine synthesis, and urease activity is shown in Fig. 1.

In a previous study on carbon dioxide fixation by *S. thermophilus*, PEP carboxylase was found to be mainly responsible for bicarbonate fixation in this species (21). This enzymatic activity (EC 4.1.1.31) catalyzes the reaction between carbon dioxide and PEP to obtain oxalacetic acid. Oxalacetic acid is then converted to aspartic acid by an aspartate aminotransferase (EC 2.6.1.1). In the present work, the aspartic acid biosynthesis was studied by analyzing the phenotype of a PEP carboxylase-negative mutant obtained by replacement of a functional *ppc* gene with a deleted and inactive version,  $\Delta pp c$ , demonstrating the following: (i) this enzymatic activity is fundamental for the growth of *S. thermophilus* in milk; (ii) the aspartic acid concentration in milk is not sufficient for *S. thermophilus* growth; (iii) aspartate, glutamate, glutamine, and ammonia availability mod-

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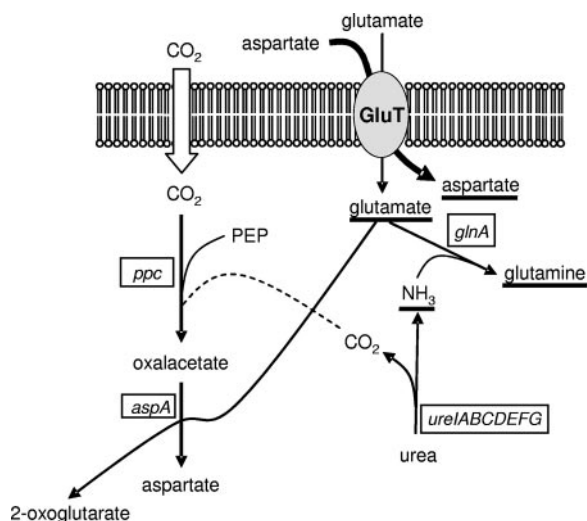


FIG. 1. Schematic representation of the enzymatic activities involved in aspartate biosynthesis and urea metabolism in *Streptococcus thermophilus*. The molecules underlined act on transcription of ure operon. *ppc*, gene coding for phosphoenolpyruvate carboxylase; *aspA*, gene coding for aspartate aminotransferase; *glnA*, gene coding for glutamine synthetase, *ureIABCDEFGF*, operon coding for urease; GluT, hypothetical glutamate membrane transporter. The wide arrow crossing the GluT represents the competitive effect of aspartic acid on the uptake of glutamate.

ulate the level of urease activity through a transcriptional regulation mechanism sensitive to a nitrogen-limited condition. Furthermore, culture association between the  $\Delta ppc$  mutant and the proteinase-positive/proteinase-negative *Lactobacillus delbrueckii* subsp. *bulgaricus* strains was also analyzed with the aim of contributing to a more complete understanding of the protooperation between these two lactic acid bacterial species.

#### MATERIALS AND METHODS

**Bacterial strains, growth conditions, and reagents.** Wild-type PpC<sup>+</sup> *S. thermophilus* DSM 20617<sup>T</sup> and its PpC<sup>-</sup> derivative, A18( $\Delta ppc$ ), were maintained in M17 broth (Difco Laboratories, Detroit, MI) at 37°C. *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842, CNRZ 397, and 1159 (CNRZ 397 PrtB negative) were maintained in MRS broth at 37°C. Plasmid-containing *S. thermophilus* strains were maintained in M17 broth (10 g liter<sup>-1</sup> lactose) supplemented with 5  $\mu$ g of erythromycin per ml at 28°C, while strains containing the pG<sup>+</sup>host9-derived vector integrated into the chromosome were maintained in the same medium supplemented with 2  $\mu$ g of erythromycin per ml at 42°C. *Escherichia coli* strains were routinely maintained in Luria broth at 37°C with aeration supplemented with 10  $\mu$ g of kanamycin per ml and when necessary with 200  $\mu$ g of erythromycin per ml. The auxotrophy for aspartic acid and the growth behavior of *S. thermophilus* wild type and A18( $\Delta ppc$ ) in the presence of urea, L-aspartate, or NaHCO<sub>3</sub> was evaluated in a chemically defined medium (CDM) derived from the medium described by Reiter and Oram (30). It contained lactose (10 g liter<sup>-1</sup>), sodium acetate (1 g liter<sup>-1</sup>), ascorbic acid (0.5 g liter<sup>-1</sup>), NH<sub>4</sub>Cl (0.5 g liter<sup>-1</sup>), adenine (5 mg liter<sup>-1</sup>), guanine (5 mg liter<sup>-1</sup>), xanthine (5 mg liter<sup>-1</sup>), uracil (5 mg liter<sup>-1</sup>), pyridoxal hydrochloride (2 mg liter<sup>-1</sup>), niacin (1 mg liter<sup>-1</sup>), thiamine hydrochloride (1 mg liter<sup>-1</sup>), riboflavin (1 mg liter<sup>-1</sup>), calcium pantothenate (1 mg liter<sup>-1</sup>), *para*-aminobenzoic acid (10  $\mu$ g liter<sup>-1</sup>), biotin (10  $\mu$ g liter<sup>-1</sup>), folic acid (1  $\mu$ g liter<sup>-1</sup>), vitamin B12 (1  $\mu$ g liter<sup>-1</sup>), MgCl<sub>2</sub> · 6H<sub>2</sub>O (200 mg liter<sup>-1</sup>), CaCl<sub>2</sub> (50 mg liter<sup>-1</sup>), FeCl<sub>3</sub> · 6H<sub>2</sub>O (5 mg liter<sup>-1</sup>), ZnSO<sub>4</sub> · 7H<sub>2</sub>O (5 mg liter<sup>-1</sup>), CoCl<sub>2</sub> · 6H<sub>2</sub>O (2.5 mg liter<sup>-1</sup>), CuSO<sub>4</sub> · 5H<sub>2</sub>O (2.5 mg liter<sup>-1</sup>), NiSO<sub>4</sub> · 7H<sub>2</sub>O (10 mg liter<sup>-1</sup>), MnSO<sub>4</sub> · 1H<sub>2</sub>O (10 mg liter<sup>-1</sup>), L-cysteine hydrochloride (200 mg liter<sup>-1</sup>), L-leucine (200 mg liter<sup>-1</sup>), L-alanine (150 mg liter<sup>-1</sup>), L-lysine hydrochloride (800 mg liter<sup>-1</sup>), L-arginine (450 mg liter<sup>-1</sup>), L-proline (540 mg liter<sup>-1</sup>), L-isoleucine (100 mg liter<sup>-1</sup>), L-methionine

(70 mg liter<sup>-1</sup>), L-phenylalanine (100 mg liter<sup>-1</sup>), L-serine (200 mg liter<sup>-1</sup>), L-threonine (100 mg liter<sup>-1</sup>), L-tryptophan (100 mg liter<sup>-1</sup>), L-valine (150 mg liter<sup>-1</sup>), glycine (200 mg liter<sup>-1</sup>), L-histidine hydrochloride · 1H<sub>2</sub>O (550 mg liter<sup>-1</sup>), L-glutamate (3.2 g liter<sup>-1</sup>), L-glutamine (3.2 g liter<sup>-1</sup>), and L-tyrosine (100 mg liter<sup>-1</sup>). The medium was reconstituted using concentrated stock solutions containing the nutrients. A mixture containing lactose, salts, and vitamins was prepared at a concentration double that in the CDM. After its pH was adjusted to 7 using NaOH, the mixture was autoclaved for 15 min at 110°C. The amino acids were prepared as a five-times-concentrated solution filter sterilized (0.22  $\mu$ m) after adjustment of its pH to 7. When appropriate, this medium was supplemented with L-Asp, urea, and NaHCO<sub>3</sub> using filter-sterilized concentrated solution. For growth measurements, *S. thermophilus* strains were grown in reconstituted (10% [wt/vol]) skimmed milk (SM) (Difco Laboratories, Detroit, MI) sterilized for 10 min at 110°C.

**PCR protocols and DNA sequencing.** As previously described, total bacterial DNA was extracted (25) starting from 100  $\mu$ l of M17 broth culture. A PCR approach for the amplification of the *ppc* gene was developed on the basis of the genome sequence of *S. thermophilus* LMG13811 and CNRZ1066 (accession numbers CP000023 and CP000024) (2). The amplification of a DNA region of about 3,200 bp encompassing all the *ppc* gene was performed as recommended by the supplier using 0.5  $\mu$ mol liter<sup>-1</sup> of primers PpCF (5'-GGAAATCGGT GAGAAAGCT-3') and PpCR (5'-CTGCTCAGCTTTTCAATCGT-3') and 2 U of ExTaq DNA polymerase (Takara Bio, Inc., Shida, Japan). The PCR conditions were as follows: 35 cycles at 94°C for 1 min, 60°C for 35 s, and 72°C for 2 min and a single final extension at 72°C for 7 min. All amplification reactions were performed in a Mastercycler (Eppendorf Italia s.r.l., Milan, Italy). The PCR product was purified (NucleoSpin extract; Machery-Nagel GmbH and Co, Düren, Germany) and sequenced using the PpCF and PpCR primers followed by primer walking. The sequence reactions were analyzed in a 310 automatic DNA sequencer (Applera, Monza, Italy) with fluorescent dideoxy chain terminators (Big Dye Terminator cycle sequencing kit version 2.0; Applera, Monza, Italy). The sequence obtained was analyzed with ORF Finder and BLAST services at the National Center for Biotechnology Information and subsequently manually aligned with the homologous *ppc* gene of *S. thermophilus* LMG13811 and CNRZ1066.

**Phylogenetic analysis.** Sequences of *ppc* genes of *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus mutans*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidophilus*, and *Bifidobacterium longum* were obtained from genome sequence data (GenBank/EMBL accession numbers AE009948, AE007317, AF004092, AE014133, CR954253, CP000033, and AE014295), while the *ppc* sequence of *Lactobacillus gasseri* was obtained from genome sequence data of strain ATCC 33323 produced by the Joint Genome Institute ([http://genome.jgi-psf.org/mic\\_home.html](http://genome.jgi-psf.org/mic_home.html)). Sequences were phylogenetically aligned using ClustalX v.1.83 (32). Alignments were checked manually, and poorly aligned or divergent regions were eliminated using Gblocks software v.0.91b (5) with a minimum block of five and allowed gap positions equal to half. Tree construction was done with Treecon v.1.3b, using neighbor-joining methods and a bootstrap analysis with 1,000 replicates.

**Replacement of *ppc* gene with a deleted version,  $\Delta ppc$ .** DNA manipulation of the pG<sup>+</sup>host9 vector and derivatives was carried out using *Escherichia coli* VE7108. Plasmid isolation was performed using the Nucleospin plasmid kit according to the manufacturer's instructions (Machery-Nagel GmbH and Co, Düren, Germany).

Strain A18 contains a deletion of 1,106 bp in the *ppc* gene, referred to as  $\Delta ppc$ . The  $\Delta ppc$  gene was obtained by PCR as previously described (26; also data not shown). Briefly, DNA fragments located upstream and downstream of the 1,106-bp deletion were independently amplified using the PpC1-PpC2 and PpC3-PpC4 primer pairs (PpC1, CAGGAAATCATCTCTGAAGA; PpC2, ACCAATCCACATCCCATTG; PpC3, ATAATCCAACACCTATTACAATGGGAATGTGGATTGGTTCAGAACGAGTTGACCAAGA; PpC4, CAAGACGTTGAGATATGGCA). Primer PpC3 has a 38-bp 5' region complementary to the 5' region of amplified product obtained using the PpC1-PpC2 primer set. These two PCR fragments were diluted to a final concentration of 100 fmol and mixed with 5  $\mu$ l of 10 $\times$  PCR buffer, 200  $\mu$ M of each deoxynucleoside triphosphate, and 1.5 U of *Taq* DNA polymerase in 50  $\mu$ l (Amersham-Pharmacia Biotech, Milan, Italy) with the aim of generating a new template DNA containing a deleted version of the *ppc* gene using the following thermal protocol: denaturation at 94°C for 2 min, reassociation at 40°C for 5 min, and extension at 72°C for 10 min. Following this step, primers PpC5 and PpC6 (PpC5, TTATTACTGCAGCTGCAGATGATGCAACACGAGGCATA; PpC6, TTATTACTGCAGCTGCAGAGCACCATATCCACGTTAGA) carrying a PstI site at their 5' end were added to the reaction mixture at a final concentration of 0.5  $\mu$ M and subjected to the following amplification protocol: 40 cycles consisting of 94°C for 45 s, 58°C

for 35 s, and 72°C for 50 s, followed by a final extension at 72°C for 10 min. The resulting PCR fragment,  $\Delta ppc$ , was ligated into the dephosphorylated PstI site of pG<sup>+</sup>host9, generating pMI49. pMI49 was introduced into *S. thermophilus* DSM20617<sup>T</sup> using a previously described protocol (26). The procedure of gene replacement described by Biswas and colleagues (1) was then applied to the *ppc* gene. The resulting PEP carboxylase-negative mutant was named A18( $\Delta ppc$ ).

**Evaluation of PEP carboxylase activity.** At the end of the exponential growth phase, *S. thermophilus* cells were harvested by centrifugation of 250 ml of culture for 15 min at 14,000 × g and 4°C. They were then washed with 250 ml of water, resuspended in 1.5 ml of Tris-HCl buffer (100 mM, pH 7.8), and placed in a 2-ml tube containing 600 mg of 0.1-mm-diameter glass beads (PolyLabo, Strasbourg, France). They were then disrupted in a cell disruptor (FP120 FastPrep; Savant Instruments, Inc.) for 30 s at 4°C and at speed 6.5. The cell crude extract was recovered after centrifugation for 5 min at 21,000 × g and 4°C. The protein content of the total cell crude extract was evaluated using the Bradford method (4). PEP carboxylase activity was evaluated (17) by coupling the malate dehydrogenase reaction to the spectrophotometric determination of NADH at 340 nm. Briefly, the enzymatic reaction was assayed at 37°C with Tris-HCl buffer (pH 7.2; 100 mM), MnSO<sub>4</sub> (5 mM), PEP (5 mM), NADH (0.6 mM), malate dehydrogenase (5 U ml<sup>-1</sup>), and total cell crude extract (about 0.2 mg protein). The reaction was started with the addition of KHCO<sub>3</sub> (10 mM). Specific activity was expressed as nanomoles of reacted PEP per minute per milligram of protein. The values were averages of three independent determinations.

**Evaluation of casein hydrolysis by cell-wall-anchored proteinases.** *Streptococcus thermophilus* and *Lactobacillus delbrueckii* strains were subjected to evaluation of casein hydrolysis by PrtS and PrtB cell-wall-anchored proteinases. Briefly, 10 ml of an overnight culture was harvested by centrifugation, washed with 0.1 M Tris-HCl, pH 8, and resuspended in 500 µl of the same buffer with or without the addition of 2 to 10 mM of CaCl<sub>2</sub>. One hundred microliters of cell suspension containing 100 µg of caseins (VWR, Milan, Italy) per ml was incubated at 37°C for 3 h. After incubation, the cell suspension was centrifuged at maximum speed and the supernatant recovered and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 12% acrylamide gel in a Mini-Protean III apparatus (Bio-Rad, Milan, Italy). A solution containing 100 µg of caseins per ml in Tris-HCl buffer, incubated at 37°C for 3 h, was used as a negative control (undigested caseins).

**Growth curves and measurement of acidifying activity.** Growth curves of DSM 20167<sup>T</sup> and the A18 derivative mutant were evaluated in triplicate in CDM and in SM at 37°C. Briefly, cells from an overnight M17 culture were harvested by centrifugation, washed twice, resuspended in sterile NaCl (9 g liter<sup>-1</sup>) and inoculated at a concentration equivalent to 0.7 absorbance unit (575 nm) in CDM and reconstituted SM. Sterile 8-ml tubes were filled with 7.8 ml of inoculated CDM or milk and hermetically sealed. Growth in CDM was measured spectrophotometrically. Growth in SM was measured with the M680 microplate reader (Bio-Rad Laboratories, Hercules, CA). A clarification procedure was necessary for the evaluation of growth in milk (10). Some cultures were done in the presence of different combination of the following chemicals: aspartic acid, urea, NaHCO<sub>3</sub>, and fluorofamide, a potent inhibitor of urease. For the evaluation of milk acidifying activity, the pH was continuously measured during 24 h using a CINAC apparatus (Ysebaert, Frépillon, France).

**Mixed cultures of *S. thermophilus* and *L. delbrueckii*.** Mixed cultures of *S. thermophilus* and *L. delbrueckii* and their derivative mutants were performed at 37°C by inoculating SM with approximately 5 × 10<sup>6</sup> CFU per ml of stock cultures of each strain. For each strain, cells from stock cultures were washed three times in sterile NaCl (9 g liter<sup>-1</sup>) before inoculation to avoid peptides or amino acids being supplied by the mixed cultures. The bacterial population was estimated by the plating method on specific medium, M17 for *S. thermophilus* and MRS (pH 5.5) for *Lactobacillus delbrueckii*. The L-Asp content of uncultured milk and L-Asp changes during growth of mixed cultures were determined by ion exchange chromatography using liquid chromatography equipment (Biochrom 30; Biochrom, Cambridge, United Kingdom) operating with ninhydrin (Flucka, Buchs, Switzerland) postcolumn derivatization and detection at 530 nm. The chromatographic conditions described by Resmini et al. (31) and a multipoint calibration were adopted.

**GusA assay.** A recombinant *S. thermophilus* strain harboring the pMI200 vector was grown in SM or in M17 broth supplemented with 0.75 to 2 mM L-Asp and/or 0.75 mM NH<sub>4</sub>Cl, 1 mM L-Glu, or 0.75 mM L-Glu. Vector pMI200 (26) is a pNZ273 (29) derivative containing a transcriptional fusion between a 219-bp region located upstream of the ureI gene and containing p<sub>ureI</sub> and the promoterless *gusA* reporter gene coding for the β-glucuronidase (GusA) enzyme. Cells from milk cultures were harvested at the end of the exponential growth phase after a clarification procedure (10), washed once with GusA buffer (10 mM β-mercaptoethanol, 1 mM EDTA, 0.1% Triton X-100 in 50 mM sodium phos-

phate buffer at pH 7), and then resuspended in 200 µl of the same buffer. The cell density was measured spectrophotometrically. Cell suspensions were subjected to mechanical disruption in the presence of 100 µl of glass beads (0.1 mm diameter) by homogenization for 5 min at 4°C. The amount of total proteins of each lysate was measured using the Bradford method (4) with bovine serum albumin as the standard. For the determination of GusA activity, 50 µl of protein extract was added to 950 µl of GusA buffer containing 1.25 mM *para*-nitro-β-D-glucuronic acid (VWR International, Milano, Italy). Measurement of GusA activity was performed at 37°C by monitoring the optical density at 420 nm (OD<sub>420</sub>) with the microplate reader M680 (Bio-Rad Laboratories, Hercules, CA) programmed for a reading set of 60 repetitions with intervals of 30 s. The GusA activity was expressed in milli-OD<sub>420</sub> units per mg of protein for four independent determinations.

**Urease activity.** Urease activity was determined on cell biomass obtained from 40 ml of SM culture after a clarification procedure as previously described. Cells were suspended in 1 ml of distilled water, and the cell density (OD<sub>600</sub>) was evaluated. Cells were recovered by centrifugation and suspended in 1 ml of urease assay solution containing 0.3 M urea, 85 mM NaCl, 0.002% phenol red, 6% (vol/vol) ethanol, 13 mM potassium buffer, pH 6.5, for the evaluation of urease activity. Measurement of the urease activity was performed at 37°C. The generation of ammonium ions due to urea hydrolysis led to a pH change and a development of a red-violet color detected at 580 nm. Urease activity was measured as the increment of OD<sub>580</sub> units after 15 min of incubation at 37°C, as the mean of four independent determinations. The OD<sub>580</sub> measurement was performed using the microplate reader M680.

## RESULTS AND DISCUSSION

Optimal growth of *S. thermophilus* in milk requires hydrolysis of caseins, internalization and degradation of the resulting peptides, and de novo amino acid biosynthesis. Genomic analysis of *S. thermophilus* has revealed that it has maintained the capacity to synthesize several amino acids throughout evolution (16). Despite the relevance of amino acid biosynthetic pathways in milk adaptation of *S. thermophilus*, only the functionality of genes involved in branched-chain-amino-acid, proline, and glutamine biosynthesis was experimentally investigated in this species (13, 20, 24).

In this study the aspartate biosynthesis of *S. thermophilus* was studied, focusing attention on the first step of the pathway, the fixation of CO<sub>2</sub> by a phosphoenolpyruvate carboxylase. The *ppc* gene coding for PEP carboxylase of strain DSM 20617<sup>T</sup> was sequenced, and the mutant A18( $\Delta ppc$ ) was obtained by using a gene replacement approach.

**Sequence and phylogenetic analysis of *ppc* gene.** *Streptococcus thermophilus* DSM 20617<sup>T</sup> *ppc* gene sequence analysis (AM167938) revealed a high sequence similarity (99%) with *ppc* gene sequences of the genomes of strains LMG13811 and CNRZ1066. The *ppc* gene consisted of 2,833 bp and encoded a 940-amino-acid PpC protein with a predicted molecular mass of 107 kDa. The average percent G+C of the *ppc* gene was 39.0%, as expected for the *S. thermophilus* species (2, 16). In the available genome of streptococci and lactic acid bacterial species, *ppc* genes were identified in pathogenic *S. pneumoniae*, *S. mutans*, *S. pyogenes*, and *S. agalactiae* and in *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, and *L. gasseri*. *Lactococcus lactis*, *Lactobacillus plantarum*, and *Lactobacillus salivarius* were characterized by the presence of pyruvate carboxylase (E.C. 6.4.1.1) and/or PEP carboxykinase (E.C. 4.1.1.49). Other lactic acid bacterial species did not show genes coding for these enzymatic activities, which are involved in the anaplerotic assimilation of carbon dioxide. Phylogenetic analysis revealed that the *ppc* gene of *S. thermophilus* clustered with the orthologs of pathogenic streptococci but represented an

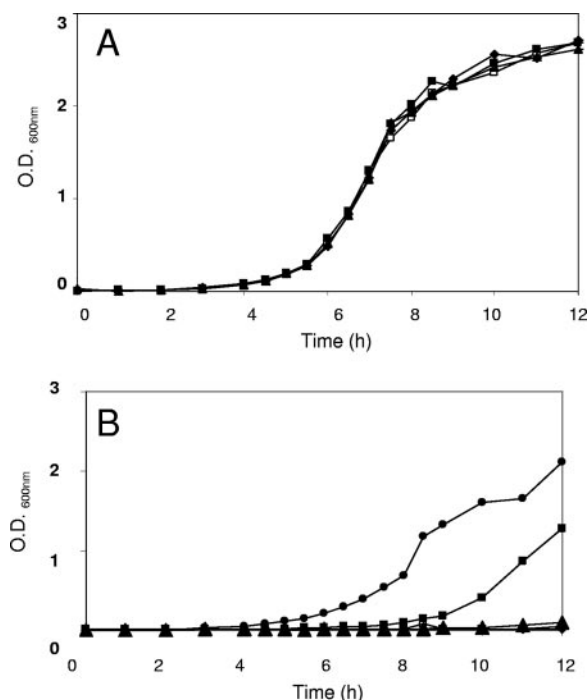


FIG. 2. Growth of *S. thermophilus* wild type (A) and PEP carboxylase-negative mutant A18( $\Delta ppc$ ) (B) in CDM ( $\square$ ) or CDM supplemented with L-Asp at a 0.075 mM ( $\blacklozenge$ ), 0.23 mM ( $\blacktriangle$ ), 0.75 mM ( $\blacksquare$ ), or 2.25 mM ( $\bullet$ ) concentration. All CDM cultures were repeated three times, and all standard deviations were  $<0.05$  OD<sub>600</sub> units.

independent lineage. The *ppc* gene of streptococci appeared well separated from those of *Lactobacillus* and *Bifidobacterium* species, with the *L. delbrueckii* subsp. *bulgaricus* *ppc* gene clustering in a phylogenetic branch with *Bifidobacterium longum*, according to the high G+C content of the *L. delbrueckii* genome and the evolutionary scenario recently depicted by van de Guchte et al. (33).

#### PEP carboxylase-negative mutant is auxotroph for L-Asp.

The wild type and the A18( $\Delta ppc$ ) mutant were subjected to evaluation of PEP carboxylase activity. The wild type displayed similar enzymatic activities whether the cells were grown in M17 ( $97 \pm 25$  U), in CDM ( $137 \pm 20$  U), or in CDM supplemented with L-Asp (0.75 mM) ( $126 \pm 20$  U). As expected, no PEP carboxylase activity could be detected in strain A18( $\Delta ppc$ ).

Evaluation of the growth of the wild type and the PEP carboxylase-negative mutant in CDM revealed that strain A18( $\Delta ppc$ ) is auxotrophic for L-Asp (Fig. 2). The addition of aspartate to the CDM medium was found to meet only partially the nutritional requirement of A18( $\Delta ppc$ ). The cell density of mutant A18( $\Delta ppc$ ) did not reach a level similar to that of the parental strain even when aspartate was added to the medium at a final concentration of 2.25 mM (Fig. 2). The supplementation of CDM with aspartate in the range of concentration between 0.075 and 2.25 mM did not show any effect on the growth of the wild type. Addition of urea to CDM (urea hydrolyzed by urease activity yields ammonia and carbon dioxide, a substrate for PEP carboxylase activity) did not have any effect on the growth of either the parental or the mutant

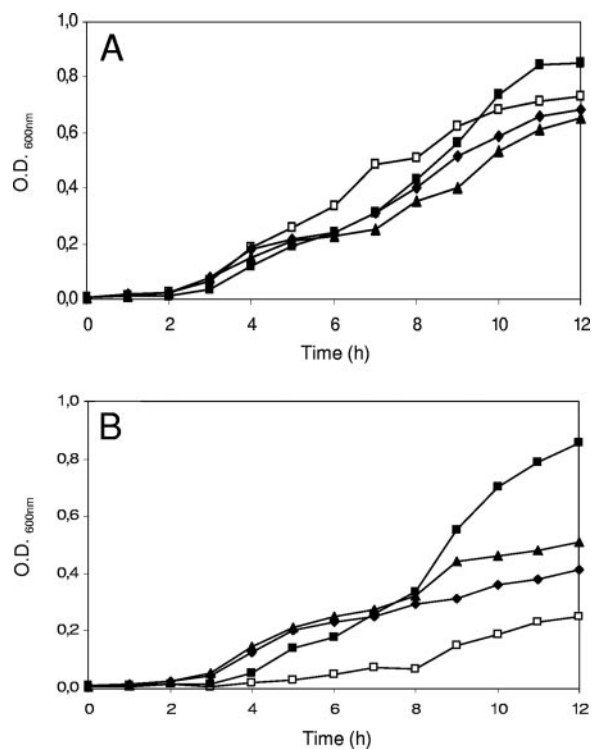


FIG. 3. Growth of *S. thermophilus* wild type (A) and PEP carboxylase negative mutant A18( $\Delta ppc$ ) (B) in reconstituted SM ( $\square$ ) or SM supplemented with L-Asp at 0.075 mM ( $\blacklozenge$ ), 0.23 mM ( $\blacktriangle$ ), or 0.75 mM ( $\blacksquare$ ). All milk cultures were repeated three times, and standard deviations were always  $<0.08$  OD<sub>600</sub> units.

strain, indicating that carbon dioxide dissolved in the medium was not present in a limiting concentration. Similar data were obtained when bicarbonate was added to CDM (data not shown).

#### PEP carboxylase activity is essential for growth of *S. thermophilus* in milk.

The A18( $\Delta ppc$ ) mutant showed only limited growth in milk and was unable to coagulate milk within 24 h of incubation (Fig. 3). Supplementation of SM with 0.75 mM aspartic acid resulted in a growth rate similar to that obtained with the parental strain. The use of a higher aspartate concentration, 2 mM, was found to inhibit the growth of the parental strain. The growth-inhibiting effect due to a high aspartate concentration in milk was not observed when aspartate supplementation was counterbalanced by the addition of glutamic acid (data not shown), thus confirming the existing competition in the uptake system of these two amino acids, as reported by Bracquart et al. (3). The inhibitory effect of a high aspartate concentration on *S. thermophilus* growth was not observed in CDM, probably because in this medium glutamate was present in a higher concentration (20 mM) than in milk (0.30 mM). The extremely low growth rate of mutant A18( $\Delta ppc$ ) was also reflected in limited milk acidification, less than 0.6 pH units in 12 h of incubation (Fig. 4). An acidification rate similar to that of the parental strain was not observed for the mutant A18( $\Delta ppc$ ), even when aspartate was supplemented at the highest concentration. After 12 h of incubation, the parental strain acidified the medium to pH 5.1 while strain A18( $\Delta ppc$ )

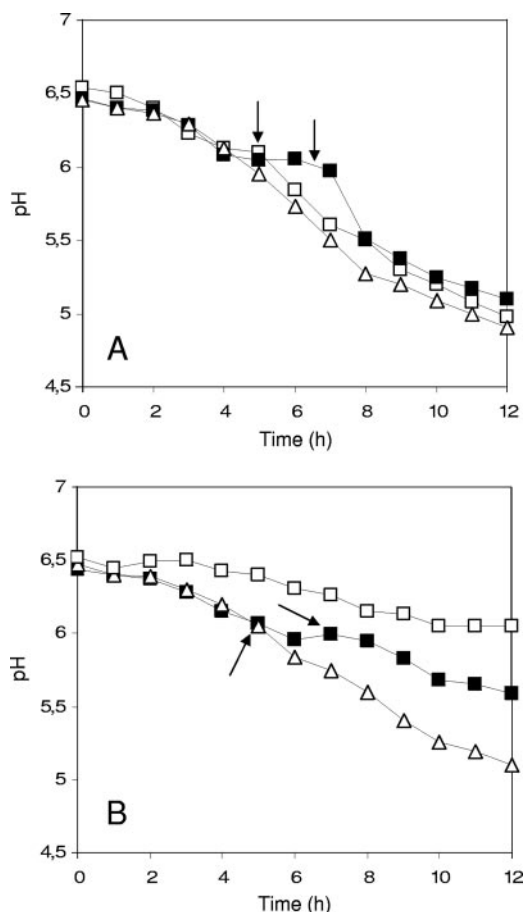


FIG. 4. Acidification rate of *S. thermophilus* wild type (A) and PEP carboxylase-negative mutant A18( $\Delta ppc$ ) (B) in reconstituted SM (□), 0.75 mM L-Asp (■), or 0.75 mM L-Asp and fluorofamide (10  $\mu$ M) ( $\Delta$ ). The decrease in the acidification rate due to urease activity is indicated by arrows. All milk cultures were repeated three times, and the standard deviations were always  $<0.08$  pH units.

grown in aspartate-supplemented milk reached the pH value of 5.6.

Inhibition of growth by a high aspartate concentration was reported by Bracquart et al. (3) and was explained by the competitive effect of aspartic acid on the uptake of glutamic acid. The authors reported that *S. thermophilus* harbors a glutamate transport system characterized by a marked specificity in the uptake of glutamic acid with aspartic acid being a strong competitive inhibitor. During growth in milk, glutamate is required in large amounts for the optimal growth of *S. thermophilus*, auxotrophic for this amino acid, and the presence of an excess of aspartic acid saturates the entry sites inhibiting glutamate uptake and therefore bacterial growth. Unfortunately, experimental data on *S. thermophilus* amino acid transport systems are very limited, and in silico analysis performed on the available genomes has revealed the presence of several symporters, a branched-chain amino acid ABC transporter, and several glutamine ABC transporters, but a specific system for the uptake of glutamate has not been identified (16).

**The availability of aspartic acid affects the rate of milk acidification of *S. thermophilus*.** It is interesting to note that in

aspartate-supplemented milk, the typical reduction of the acidification rate due to the urease activity detectable when the culture pH is near 6 (Fig. 4A) is remarkably different from that observed without the addition of aspartate. The same phenomenon was observed with mutant A18( $\Delta ppc$ ) (Fig. 4B). The addition of fluorofamide, a potent urease inhibitor, to the aspartate-supplemented milk confirmed that the reduction of the acidification rate detected near pH 6 was related to urea hydrolysis as previously demonstrated (22, 26, 28). Moreover, the addition of fluorofamide in aspartate-supplemented milk allowed mutant A18( $\Delta ppc$ ) to reach a pH value similar to that of the parental strain.

**Aspartate-dependent glutamate uptake deficiency revealed that urease is transcriptionally regulated by a mechanism sensitive to a nitrogen-limited condition.** The data obtained in this work and in a previous study (3, 24) and the information deduced by analysis of the *S. thermophilus* genome highlight close relationships among the aspartic acid biosynthetic pathway, glutamate/glutamine cellular equilibrium, and urease activity, as shown in Fig. 1. Carbon dioxide, generated by urea hydrolysis, might be used as a substrate in several anaplerotic reactions mainly involved in amino acid and nucleic acid biosynthesis, while ammonia has been previously reported to be involved in glutamine biosynthesis (24). In light of these considerations, the effect of aspartate availability on the levels of urease activity was investigated.

With the aim of verifying our hypothesis, the recombinant strain MIM200 was grown in milk in the presence of different combinations of aspartate, glutamate, and glutamine or aspartate and ammonium chloride for the evaluation of GusA as reporter activity. In the recombinant strain MIM200, GusA reporter activity was under the control of the urease operon promoter  $p_{ureI}$  (27). With the aim of highlighting the influence of aspartate, glutamate, glutamine, and ammonia concentrations on the expression levels of urease and to avoid the induction of urease biogenesis due to the culture acidification (27), *S. thermophilus* MIM200 was cultured in forced glutamate starvation obtained by adding to milk a growth-inhibiting concentration of aspartate (2 mM). The experiments were performed using an early induction approach in which recombinant *S. thermophilus* MIM200 was cultured for 2 h in milk in the presence of aspartate in a growth-inhibiting concentration (2 mM), followed by an additional 10 min of incubation without or with the addition to the culture of glutamate, glutamine, or ammonium chloride. The results obtained and shown in Table 1 underlined that urease expression was sensitive to aspartate, glutamate, glutamine, and ammonia availability. Here the addition of glutamate, glutamine, and ammonia to *S. thermophilus* cells cultured in the presence of a growth-inhibiting concentration of aspartate resulted in a significant reduction (25 to 33%) of urease expression. The decrease in the expression level was confirmed by the reduction of urease activity (26 to 53%) in the same experimental condition (Table 1). In conclusion, the data obtained suggest that apart from the primary transcriptional regulation by environmental pH, reported in a previous study (27), urease biogenesis also appears to be regulated through a transcriptional regulation system sensitive to a nitrogen-limited condition and specifically sensitive to glutamate, glutamine, and ammonia concentrations. The induction of *ure* genes under a nitrogen-limited

TABLE 1. Influence of aspartate, glutamate, and glutamine availability on urease operon transcriptional level of *S. thermophilus* DSM 20617, measured as GusA reporter activity

Culture conditions	GusA activity <sup>a</sup>	Urease activity (%) <sup>b</sup>
Milk, aspartate (2 mM)	862 ± 28	100
Milk, aspartate (2 mM), glutamate (0.75 mM)	579 ± 18	53
Milk, aspartate (2 mM), glutamine (0.75 mM)	649 ± 22	33
Milk, aspartate (2 mM), NH <sub>4</sub> Cl (0.75 mM)	634 ± 24	26

<sup>a</sup> GusA activity was expressed as milli-OD<sub>420</sub> units per mg of protein as the mean of four independent determinations ± the standard deviation. Experiments were performed by incubating recombinant *S. thermophilus* MIM200 for 2 h in milk in the presence of aspartate at a growth-limiting concentration (2 mM), followed by 10 min in the presence of glutamate, glutamine, or ammonium chloride or without any other addition.

<sup>b</sup> Urease activity was evaluated with the same conditions used for GusA activity experiments and was expressed as a percentage of urease activity measured in aspartate-supplemented milk as a mean of three independent determinations; standard deviations were always less than 12%. The pH of the cultures at the end of the incubation time was 6.6.

condition has been previously demonstrated for *Bacillus subtilis* (12) but was not investigated with *Streptococcus salivarius*, the closest urease-positive neighbor of *S. thermophilus* (6, 7). Glutamic acid and glutamine have a central role in the nitrogen metabolism of many bacteria (23). Previous studies carried out on the glutamine synthetase activity of *S. thermophilus* (24) supported the idea that one of the physiological functions of urease was to supply ammonia for the synthesis of glutamine. In this context, the transcriptional regulation of the *ure* operon by glutamate availability corroborates this hypothesis, highlighting a key role of urease activity in the nitrogen metabolism of *S. thermophilus* (Fig. 1). Despite the relevance of nitrogen sources for the growth of lactic acid bacteria in milk, relatively little is known about central nitrogen regulation of this group of bacteria (18). Only recently it was demonstrated that glutamine synthetase, the enzyme that converts glutamate and ammonium into glutamine, is a key aspect of the metabolic adaptation of *Lactococcus lactis* to milk (15) and is essential for the growth in milk of *Streptococcus thermophilus* (24). In this context, the modulation of urease activity in aspartate-supplemented milk might be interpreted as a cellular response to a nitrogen-limited condition due to glutamate and glutamine starvation.

**Behavior of wild type and mutant A18( $\Delta$ *ppc*) in mixed culture with proteinase-positive *Lactobacillus delbrueckii* subsp. *bulgaricus*.** With the aim of testing whether the aspartate requested by mutant A18( $\Delta$ *ppc*) may be provided by the proteolytic activity of other lactic acid bacterial species traditionally used in association with *S. thermophilus* in dairy preparations, mixed cultures with *L. delbrueckii* subsp. *bulgaricus* were performed. All the strains used in mixed cultures were preventively checked for the presence and activity of cell-wall-anchored proteinases. *S. thermophilus* DSM 20617<sup>T</sup> (wild type) did not show any activity on casein solution and gave a negative response to a *prtS*-specific PCR assay and was therefore designated proteinase negative/*PrtS* negative. On the contrary, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 and CNRZ 397

showed a CaCl<sub>2</sub>-dependent proteolytic activity against casein, as expected for a *PrtB* cell wall proteinase. Mutant CNRZ 1159 (CNRZ 397 *PrtB* negative) (14) did not show any activity against casein solution (data not shown). *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 and CNRZ 397 were characterized by a different growth rate in milk, and despite both being proteinase positive, the behavior of strain ATCC 11842 in milk was similar to that observed for mutant CNRZ 1159 (CNRZ 397 *PrtB* negative) (data not shown).

Mutant A18( $\Delta$ *ppc*) was grown in association with strains *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 and CNRZ 397. As shown in Fig. 5, the aspartate requirement of A18( $\Delta$ *ppc*) was not satisfied by the proteolytic activity of *L. delbrueckii* strains. Despite the weakly increased growth rate of A18( $\Delta$ *ppc*) during association with CNRZ 397, cell density reached at the end of growth was low. The growth of parental strain DSM 20617<sup>T</sup> was not incremented during association with CNRZ 397 but was significantly reduced in association with mutant CNRZ 1159 (CNRZ 397 *PrtB* negative) (Fig. 5).

Monitoring of the L-Asp concentration during growth of mixed cultures (Table 2) revealed that the concentration of this amino acid decrease rapidly in the first hours of growth up to

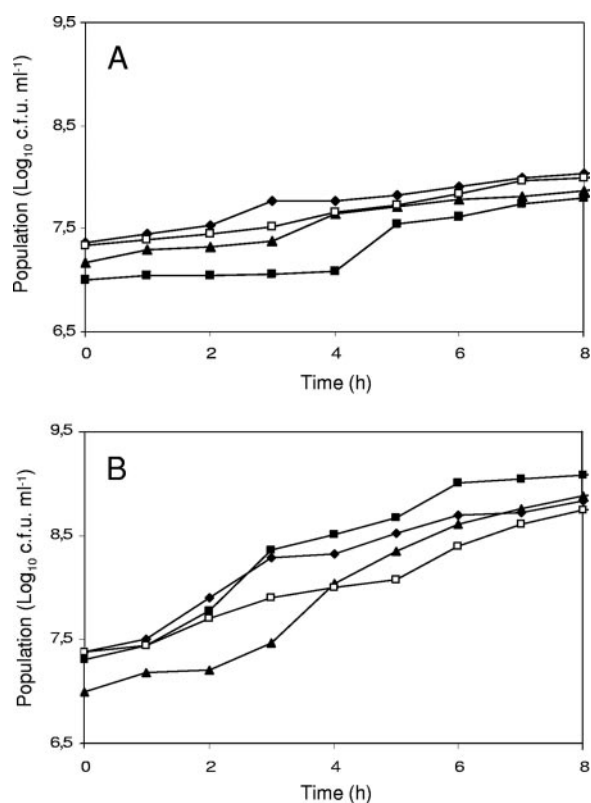


FIG. 5. Growth of mixed cultures of *S. thermophilus* mutant A18( $\Delta$ *ppc*) and DSM 20617 wild type with *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842, CNRZ 397, or CNRZ 1159 (CNRZ 397 *PrtB* negative) in reconstituted SM. (A) Population of A18( $\Delta$ *ppc*) cultured alone (◆) or A18( $\Delta$ *ppc*) in mixed culture of A18( $\Delta$ *ppc*)/ATCC 11842 (▲), A18( $\Delta$ *ppc*)/CNRZ 397 (■), or A18( $\Delta$ *ppc*)/CNRZ 1159 (□). (B) Population of DSM 20617 cultured alone (◆) or DSM 20617 in mixed culture of DSM 20617/ATCC 11842 (▲), DSM 20617/CNRZ 397 (■), or DSM 20617/CNRZ 1159 (□). All milk cultures were repeated three times, and standard deviations were always  $<0.2 \log_{10} \text{CFU ml}^{-1}$ .

TABLE 2. Changes in level of aspartic acid during growth of *S. thermophilus* wild type and mutant A18( $\Delta$ *ppc*) in association with *Lactobacillus delbrueckii* subsp. *bulgaricus* strains

Mixed culture	L-Asp concn ( $\mu$ M) <sup>a</sup>		
	3 h	5 h	8 h
DSM 20617/ATCC 11842	12.3 $\pm$ 0.6	12.8 $\pm$ 0.7	20.7 $\pm$ 1.0
DSM 20617/CNRZ 397	16.0 $\pm$ 0.9	24.5 $\pm$ 1.4	67.1 $\pm$ 3.4
DSM 20617/CNRZ 1159	20.5 $\pm$ 1.4	24.4 $\pm$ 1.0	17.7 $\pm$ 1.1
A18( $\Delta$ <i>ppc</i> )/ATCC 11842	14.6 $\pm$ 0.8	10.6 $\pm$ 0.6	11.2 $\pm$ 0.5
A18( $\Delta$ <i>ppc</i> )/CNRZ 397	17.0 $\pm$ 0.9	16.8 $\pm$ 0.9	37.9 $\pm$ 2.2
A18( $\Delta$ <i>ppc</i> )/CNRZ 1159	27.6 $\pm$ 1.4	21.2 $\pm$ 1.2	18.0 $\pm$ 0.8
DSM 20617	15.0 $\pm$ 1.2	14.3 $\pm$ 1.0	12.2 $\pm$ 0.8
A18( $\Delta$ <i>ppc</i> )	14.1 $\pm$ 1.1	11.5 $\pm$ 0.7	9.0 $\pm$ 0.5
ATCC 11842	25.0 $\pm$ 1.4	17.9 $\pm$ 1.0	24.4 $\pm$ 1.3
CNRZ 397	21.7 $\pm$ 1.2	31.5 $\pm$ 1.8	103.1 $\pm$ 5.0
CNRZ 1159	23.6 $\pm$ 1.3	25.4 $\pm$ 1.2	31.4 $\pm$ 1.6

<sup>a</sup> The L-Asp concentration in uncultured SM is 28.2  $\pm$  1.5  $\mu$ M.

the beginning of the exponential phase. After 8 h of incubation, the aspartate concentration was most likely increased by the activity of the *L. delbrueckii* proteinase. The increase of the aspartate concentration was not observed in the A18( $\Delta$ *ppc*)/ATCC 11842 culture or in the mixed cultures performed with mutant CNRZ 1159. In a comparison of the overall changes in the aspartate concentration, it was evident that the highest consumption of aspartate was detected, as expected, in mixed cultures containing mutant A18( $\Delta$ *ppc*) and/or CNRZ 1159. Nevertheless, the free aspartate was not totally consumed and remained detectable at values ranging between 11 and 18  $\mu$ M.

The overall data obtained showed that the amounts of aspartic acid present in milk or generated as a consequence of the proteolytic activity of *L. delbrueckii* strains were always lower than those required for the optimal growth of the A18( $\Delta$ *ppc*) mutant. Moreover, additional experiments showed that the growth rate of mutant A18( $\Delta$ *ppc*) was significantly increased in amino acid-enriched milk and not in casein peptone-enriched milk, underlining the presence in *S. thermophilus* of an oligopeptide uptake system unable to compete with *L. delbrueckii* to satisfy its amino acid requirements (data not shown).

It was therefore concluded that peptides and amino acids generated by *L. delbrueckii* proteolysis were not sufficient to restore the growth of an *S. thermophilus* phosphoenolpyruvate carboxylase-negative mutant. If we consider that *S. thermophilus* during its adaptation to the milk environment retained its ability to synthesize almost of all the amino acids (2), it could be hypothesized that the supplementation of oligopeptides and amino acids by *L. delbrueckii* should not be considered the main aspect of the protooperation with *S. thermophilus*. Nevertheless, a previous study (8) has demonstrated that the activity of *L. delbrueckii* PrtB was necessary for the optimal growth and acidification of *S. thermophilus*. In light of these considerations, it could be deduced that the number of factors that play a relevant role in the protooperation between these two species is strain dependent. It is possible to say that perhaps some strains of *S. thermophilus* adapted to the milk environment independently, while others coevolved with protease-producing *L. bulgaricus* as already hypothesized by van de Guchte et al. (33).

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