

Branched-Chain Amino Acid Biosynthesis Is Essential for Optimal Growth of *Streptococcus thermophilus* in Milk

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Lactic acid bacteria are nutritionally demanding bacteria which need, among other things, amino acids for optimal growth. We identified the branched-chain amino acid (BCAA) biosynthesis pathway as an essential pathway for optimal growth of *Streptococcus thermophilus* in milk. Through random insertional mutagenesis, we isolated and characterized two mutants for which growth in milk is affected as a consequence of *ilvB* and *ilvC* gene interruptions. This situation demonstrates that the BCAA biosynthesis pathway is active in *S. thermophilus*. BCAA biosynthesis is necessary but not sufficient for optimal growth of *S. thermophilus* and is subject to retro-inhibition processes. The specificity of the BCAA biosynthesis pathway in *S. thermophilus* lies in the independent transcription of the *ilvC* gene encoding a keto acid reductoisomerase acting on acetolactate at the junction of the BCAA and acetoin biosynthesis pathways. The possible advantages for *S. thermophilus* of keeping this biosynthesis pathway active could be linked either to adaptation of the organism to milk, which is different than that of other dairy bacteria, or to the role of the pathway in maintaining the internal pH.

The search for nutriment and especially for amino acids constitutes a real challenge for lactic acid bacteria. These organisms are auxotrophic for several amino acids which they cannot synthesize from simpler nitrogen sources (6, 8, 26). Only proteinase-positive lactic acid bacteria, which are capable of hydrolyzing caseins, usually grow significantly in milk, which contains only small amounts of amino acids and short peptides (39).

Most of the functions identified as being essential for optimal growth of *Lactococcus lactis* in milk concern the amino acid supply. Quite some time ago, Thomas and Mills (39) underlined the importance of the cell wall-anchored lactococcal proteinase (PrtP) to growth in milk. If the proteinase is absent, caseins are not hydrolyzed and *L. lactis* cell density reaches only 10% of that of a proteinase-positive (Prt⁺) strain. The cell wall proteinase liberates oligopeptides from caseins, some of which are transported into the cytoplasm by the oligopeptide transport system. This transport system is essential for optimal growth of *L. lactis* in milk (44). When internalized, the oligopeptides are further hydrolyzed into amino acids by intracellular peptidases. The most important peptidases in this process have been identified by using negative mutants. Suppression of these enzymes significantly reduces the *L. lactis* growth rate in milk (24). *L. lactis* can also synthesize amino acids, at least to some extent. The aspartate biosynthesis pathway is active in *L. lactis*, and lack of this pathway reduces the growth rate in milk to half the normal rate (45).

In the present work we identified, for the first time, the branched-chain amino acid (BCAA) biosynthesis pathway as a key pathway for optimal growth of *Streptococcus thermophilus* in milk. Since the BCAA are a quantitatively important group of amino acids in bacterial proteins (they account for 20% of the total protein amino acids in *Escherichia coli* and *L. lactis* [25, 27]), the BCAA biosynthesis pathways have been extensively studied and are well-known in several bacterial genera.

They share the following features: first, they involve three enzymes common to the three amino acid synthesis pathways; and second, they are composed of steps which are specific to leucine and isoleucine-valine biosynthesis. In addition, some metabolic intermediates are linked to other metabolic pathways, such as acetoin, butanediol, and coenzyme A synthesis (Fig. 1). As a consequence, the BCAA biosynthesis pathway is under complex control, including retro-inhibition processes and regulation at the transcription level (5, 11, 14, 32, 42).

Investigation of the amino acid requirements of *L. lactis* by using a single-omission technique revealed that the dairy lactococcal strains are auxotrophic for at least six amino acids (Glu, Met, Leu, Ile, Val, and His) while lactococci from vegetal origins are prototrophic for all amino acids (5, 6). The amino acid auxotrophies, including those for BCAA, are due to minor genetic lesions that, in most cases, are repairable by single-step mutations (8, 13).

The thermophilic bacterium *S. thermophilus* requires fewer amino acids than lactococci and lactobacilli (9). Only glutamine and glutamic acid, along with the sulfur amino acids, are essential for all of the strains that have been tested (3, 28; data not shown). This situation could be attributed to active amino acid biosynthesis pathways not yet described for this species. In the present work, we demonstrated that the *S. thermophilus* BCAA synthesis pathway is functional, while the BCAA synthesis pathway is not functional in *L. lactis* of dairy origin. Moreover, this biosynthesis pathway is essential for optimal growth of *S. thermophilus* in milk.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. *E. coli* TG1 RepA⁺ (TG1, which contains a chromosomal copy of the *repA* gene, was kindly provided by P. Renault and referenced as TIL206) and *E. coli* TG1 RepA⁺ containing the pG⁺h9::ISS1 plasmid (29) (= strain TIL401) were used. Both strains were grown on Luria-Bertani medium (35) at 37°C with shaking and in the presence of erythromycin (150 µg/ml) when required. *S. thermophilus* Prt⁺, plasmid-free strain St18 was provided by Rhodia-Food (Dangé Saint-Romain, France). Four media were used for cultures of *S. thermophilus*. Two of them were milk based and were used specifically for screening mutants. The first medium was Fast Slow Difference Agar (FSDA) (19), which contained erythromycin (5 or 3 µg/ml) when it was needed. This medium is a milk-based agar medium, which made it possible to differentiate bacteria that exhibited slow or limited growth in milk

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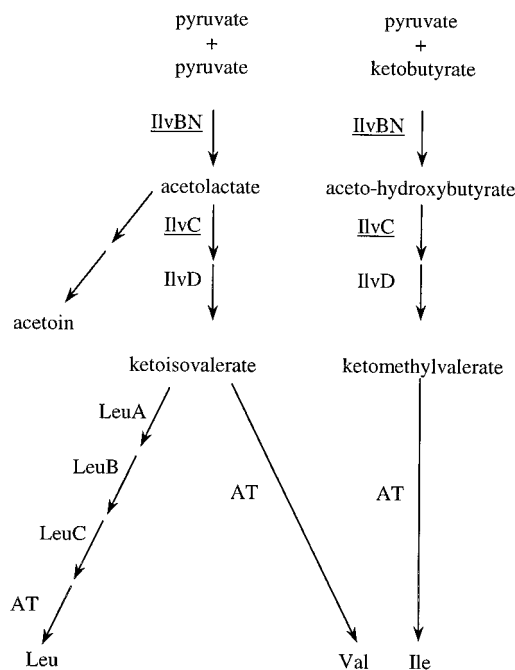


FIG. 1. Branched-chain biosynthesis of the three BCAA. *IlvBN*, acetolactate synthase; *IlvC*, keto acid reductoisomerase; *IlvD*, dihydroxy acid dehydratase; *LeuA*, isopropyl malate synthase; *LeuB*, isopropyl malate dehydrogenase; *LeuC*, isopropyl malate dehydratase; *AT*, aminotransferase. The proteins whose encoding genes have been disrupted are underlined.

from bacteria that exhibited rapid or optimal growth after 48 h of incubation at 37°C under anaerobic conditions. The second medium was reconstituted low-heat 10% (wt/vol) skim milk (Nilac; Nederlands Instituut von Zuivelonderzoek, Ede, The Netherlands) that was autoclaved at 110°C for 12 min, buffered with 2.5% 3 M sodium glycerophosphate, and in some cases contained 3 g of Bacto Tryptone (pancreatic digest of casein; Difco Laboratories, Detroit, Mich.) per liter. Bacterial growth was monitored by measuring the optical density at 480 nm (OD_{480}) after clarification of milk by 10-fold dilution in a solution containing 2 g of EDTA (pH 12) per liter (40). Two other media were used for general manipulation and growth rate experiments. The first of these was M17Lac medium (38), in which bacterial growth was monitored by measuring the OD_{600} . The second was a chemically defined medium (CDM) containing nucleotides, vitamins, amino acids, salts, potassium phosphate buffer (pH 6.7), and 1% (wt/vol) lactose (31), which was sterilized by filtration. When required, in some CDM growth experiments isoleucine, leucine, or valine was omitted or the corresponding precursor keto acids, β -ketoisovalerate and ketomethylvalerate, were added at concentrations of 0.8 and 0.2 g/liter, respectively (Fig. 1). Growth rate experiments were then performed at 37°C with a Microbiology Reader Bioscreen C (Labsystems, Helsinki, Finland) in 100-well sterile covered microplates. Each well contained 200 μ l of culture medium. Overnight M17Lac cultures of *S. thermophilus* were washed twice and resuspended in a volume of sterile phosphate buffer equal to the culture volume. Four microliters of the suspension was used to inoculate each well. The OD_{600} was measured every 20 min after gentle shaking. The apparent growth rate was defined as the maximum slope of the semi-logarithmic graph of growth determined by measuring optical density.

***S. thermophilus* mutagenesis.** The method used for insertional mutagenesis with $pG^+h9::ISS1$ in *S. thermophilus* St18 was adapted from the method previously described by Maguin et al. (22). Plasmid $pG^+h9::ISS1$ was first purified from *E. coli* TIL401. *S. thermophilus* St18 was transformed by electroporation (18) with 1 μ g of purified $pG^+h9::ISS1$, and plasmid-containing bacteria were selected on M17Lac medium containing erythromycin (5 μ g/ml) at 28°C under anaerobic conditions. Integration was performed as follows. A saturated overnight culture of an erythromycin-resistant (Em^r) colony containing $pG^+h9::ISS1$ was cultivated in M17Lac medium supplemented with erythromycin (5 μ g/ml) and then diluted 1:100 with fresh M17Lac medium without erythromycin and incubated at 28°C for 2.5 h. To reduce the plasmid copy number, the culture was incubated at 42°C for another 2.5 h. The culture was diluted and plated on FSDA in the presence or in the absence of erythromycin and incubated at 42°C (under anaerobic conditions) to induce chromosomal integration of the plasmid. At this step, the concentration of erythromycin was only 3 μ g/ml to limit tandem insertion of $pG^+h9::ISS1$. Em^r mutants were selected after 24 h. To excise transposed $pG^+h9::ISS1$ and obtain stable mutants, a method similar to that described previously (22) was used.

Selection of mutants whose growth in milk was affected. Mutants whose growth in milk was affected were selected in two steps. The first selection was made on FSDA. On this medium, colonies whose growth in milk was affected remained small and translucent while the colonies with normal growth were large and white. This first selection was confirmed by comparing the growth of mutants in milk to that of the wild-type strain.

DNA manipulation and sequencing. Plasmid DNA manipulation and transformation of *E. coli* TIL206 were performed as previously described (35). RNA was prepared by using *S. thermophilus* grown in M17Lac medium. The DNAs of mutants were digested by *EcoRI* or *HindIII* and ligated. TIL206 electrocompetent cells were transformed with ligation products, and Em^r colonies were screened by PCR after 24 h of incubation at 37°C. PCR amplifications were performed with a Gene Amp 2400 PCR system (Perkin-Elmer Corp., Norwalk, Conn.) by using *Taq* polymerase (Appligene Oncor, Illkirch, France) and oligonucleotides from the $pG^+h9::ISS1$ sequences (5' ACT ACT GAC AGC TTC CAA GGA 3' and 5' ATA GTT CAT TGA TAT ATC CTC 3' for *EcoRI* digestion and 5' GTA AAA CGA CGG CCA GTG 3' and 5' TAT CTA CTG AGA TTA AGG TCT 3' for *HindIII* digestion). A dye terminator kit and a 310 genetic analyzer (Applied Biosystems, Foster City, Calif.) were used for DNA sequencing; each strand was sequenced twice by using independent PCR products. DNA sequences were analyzed with Genetics Computer Group sequence analysis software from the University of Wisconsin (10) and Mail Fasta (National Center for Biotechnology Information). Southern and Northern hybridizations were performed by using a positively charged nylon membrane (Appligene Oncor) for transfer according to the instructions for the ECL detection system (Amersham, Buckinghamshire, England).

Nucleotide sequence accession number. The GenBank, EMBL, and DDBJ nucleotide sequence accession number for a 1,955-bp partial sequence of the *ilvBNC* operon of *S. thermophilus* St18 is AF220670.

RESULTS

Set of *S. thermophilus* mutants resulting from random mutagenesis. The transformation yield of *S. thermophilus* St18 (72 transformants/ μ g of $pG^+h9::ISS1$) was low but comparable to that which has been previously described for other *S. thermophilus* strains (23). We obtained 1.183×10^4 Em^r mutants on FSDA. The integration frequency (i.e., the ratio of the number of Em^r mutants to the total number of clones) was 7.7×10^{-3} , a value which is quite similar to that obtained for *L. lactis* (22). On the basis of their phenotypes on FSDA and their slow growth in milk, we isolated 72 integrants.

Characterization of two mutants that exhibited slower growth in milk. After Southern analysis of digested chromosomal DNAs of the mutants that grew slowly in milk, we selected 14 clones in which $pG^+h9::ISS1$ was integrated at only one locus, and the locus was distinct for each clone. In 12 of

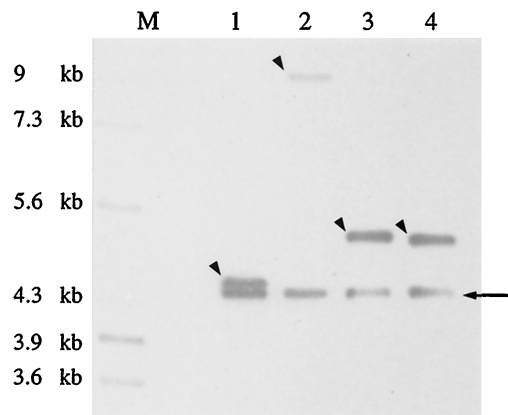


FIG. 2. Southern analysis of $pG^+h9::ISS1$ mutants 1 and 2. Chromosomal DNAs of mutants were digested by *EcoRI* (lanes 1 and 3) or *HindIII* (lanes 2 and 4) and probed with pG^+h9 . Two bands were observed for mutants 1 and 2 (lanes 1 and 2 and lanes 3 and 4, respectively), which corresponded to tandem transposition. One band corresponded to $pG^+h9::ISS1$ (arrow), and the other band corresponded to the integrated structure of the chromosome (arrowheads). No hybridization was observed with the wild-type strain DNA (data not shown). The RAOUL marker (Appligene) was used as a size reference (lane M).

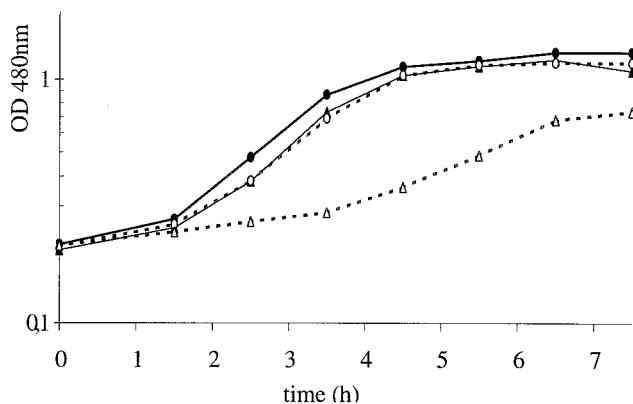


FIG. 3. Comparison of the growth curves of wild-type strain St18 in milk (○) and in milk containing 3 g of Bacto Tryptone per liter (●) with the growth curves of mutant 1 in milk (△) and in milk containing 3 g of Bacto Tryptone per liter (▲).

these clones, pG⁺h9::ISS1 was tandemly integrated, which gave two hybridization bands when pG⁺h9 was used as a probe. These two observations are illustrated in Fig. 2 for two of the mutants, mutants 1 and 2, which were characterized further in the present work.

Mutants 1 and 2 had similar growth curves in both milk and milk with Bacto Tryptone (Fig. 3). Their growth rates in milk (0.37 h⁻¹) were significantly lower than that of the wild-type strain (0.73 h⁻¹). Rapid growth was restored by addition of Bacto Tryptone (growth rate for the mutants 0.71 h⁻¹; growth rate for the wild-type strain, 0.85 h⁻¹), suggesting that the affected functions were related to nitrogen nutrition.

Identification of disrupted genes as BCAA biosynthesis genes. Sequences of the interrupted genes were determined by PCR with oligonucleotides from pG⁺h9::ISS1. We obtained 116- and 1,419-bp sequences for mutants 1 and 2, respectively. A search for homologues in databases revealed that the two mutants were affected in the same BCAA biosynthesis pathway. The *ilvB* and *ilvC* genes, coding for the large subunit of

acetolactate synthase and keto acid reductoisomerase, were interrupted in mutants 1 and 2, respectively (Fig. 1). Using oligonucleotides corresponding to the extremities of the two sequences obtained from mutants 1 and 2, we performed additional PCRs. We obtained a unique 1,955-bp DNA fragment that included the sequences from mutants 1 and 2 and contained three open reading frames (ORFs) (partial *ilvB* gene, whole *ilvN* gene encoding the small subunit of acetolactate synthase, and whole *ilvC* gene). Protein sequences deduced from the whole DNA sequence showed the highest homology with the sequences of similar proteins from *L. lactis* (46% identity for IlvN to 78% identity for IlvC) (14), *Bacillus subtilis* (44% identity for IlvN to 58% identity for IlvC) (30), and *Leuconostoc mesenteroides* subsp. *cremoris* (50% identity for IlvB to 52% identity for IlvC) (4).

Analysis of the sequence revealed the presence of a putative -10 extended promoter sequence and two short inverted repeat sequences that were 72 and 50 bp, respectively, upstream of the ATG start codon of *ilvC* and the presence of a putative terminator 19 bp downstream of the stop codon of the same sequence (Fig. 4). Downstream of the *ilvC* gene, we found a partial ORF encoding a potential protein with 51% identity to tyrosyl tRNA synthetase of *B. subtilis* (17). No trace of *leu* genes was found in a 417-bp sequence located 3.5 kb upstream of *ilvB* from *S. thermophilus*. No ORF encoding proteins with similarity to either the IlvY regulator from *E. coli* (46) or the α-acetolactate decarboxylase present in *L. lactis* (15) were found in the vicinity of *ilvC*. After RNA was prepared from strain St18 grown in M17Lac medium or CDM containing all of the amino acids, Northern blot analysis revealed the presence of a 1,070-bp transcript that hybridized with the 1,955-bp *ilvBNC* sequence and, more precisely, with the 1,070-bp *ilvC* sequence (Fig. 5A). As expected, the 1,070-bp transcript was not visible when the same experiment was done with the IlvC-negative mutant (Fig. 5A). In this case, only the two bands with unusual shapes, probably resulting from larger degraded *ilv* transcripts stacked with 23S and 16S rRNAs, were visible. Similar bands have been observed previously for other large transcripts (21). An *ilvBN* probe ending at the putative *ilvC*

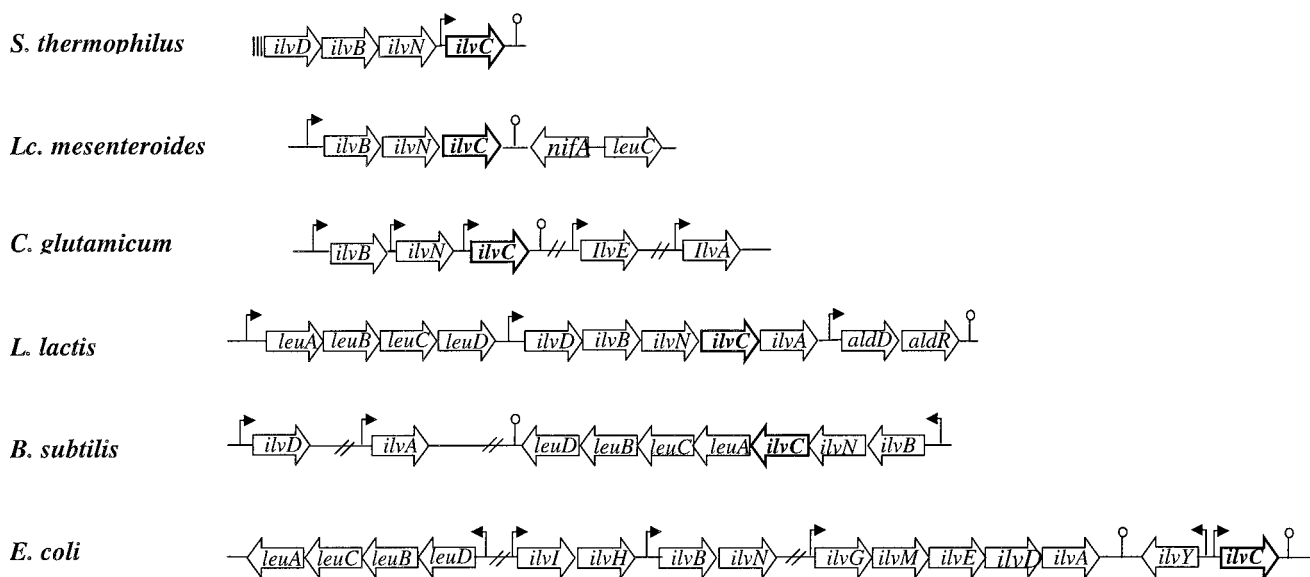


FIG. 4. Organization of the *ilv* and *leu* genes in different bacteria. ▸, putative promoter; ⊙, putative terminator; |||, partial ORF; //, interrupted sequence. Data for *S. thermophilus* were obtained from this study; data for *L. mesenteroides*, *C. glutamicum*, *L. lactis*, *B. subtilis*, and *E. coli* were obtained from references 4, 7, 14, 30, and 2, respectively.

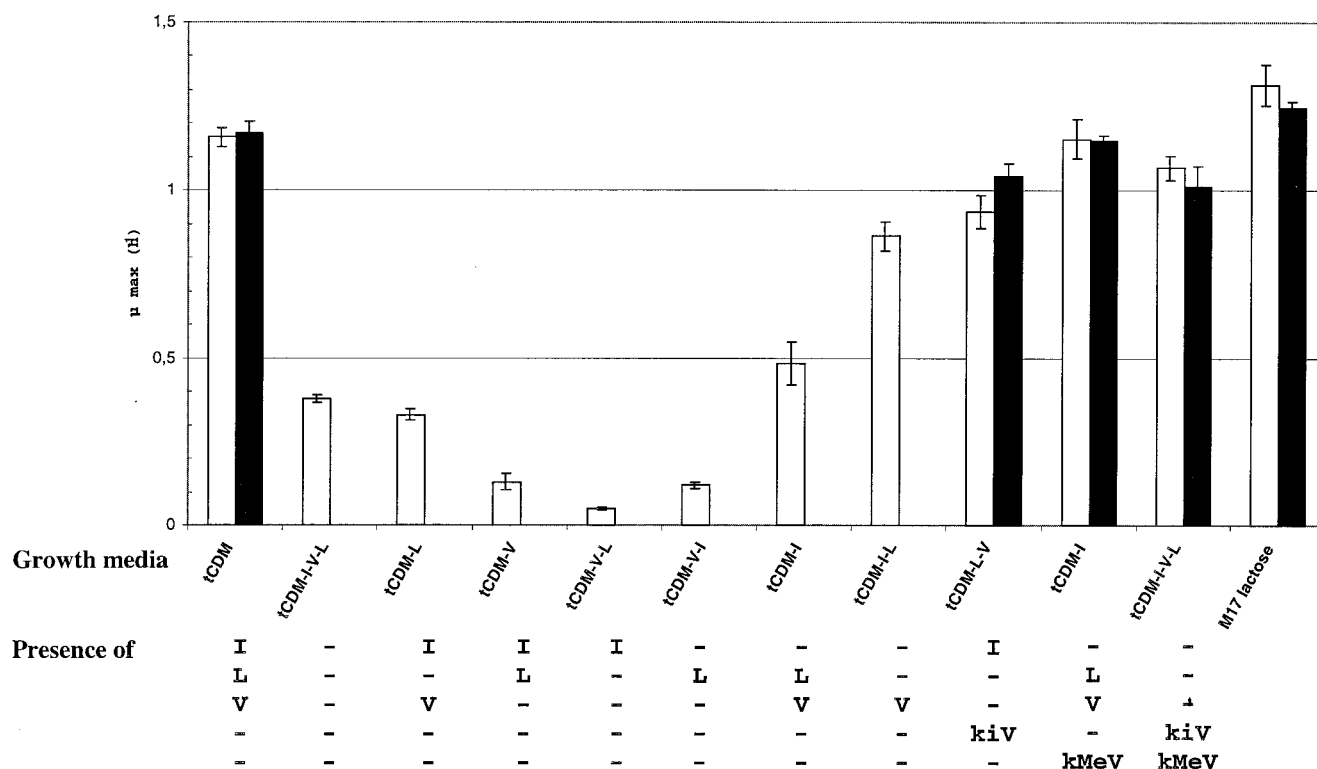


FIG. 7. Growth rate (μ_{max}) obtained with the wild-type strain (open bars) and mutant 1 or 2 (solid bars) in CDM in which the nitrogen source was variable. The data are averages obtained from four independent experiments. tCDM is CDM which contained all of the amino acids. I, isoleucine; L, leucine; V, valine; kiV, ketoisovalerate; kMeV, ketoisomethylvalerate.

Evolution and conservation of active amino acid biosynthesis pathways. The fact that dairy starter bacteria very frequently possess inactive amino acid biosynthesis pathways raises the question whether auxotrophies are beneficial to the bacteria (43, 47). The BCAA biosynthesis pathway, which is not functional in the dairy starter bacteria *L. lactis* and *L. mesenteroides* subsp. *cremoris* but is active in lactococci from vegetal origins (4, 14), reflects the different evolutionary pathways of these organisms. Godon et al. (13) have suggested that auxotrophy of dairy *L. lactis* strains could be a consequence of an adaptation to milk. In the present work, we demonstrated that the same BCAA biosynthesis pathway is functional in another dairy starter species, *S. thermophilus*. This finding contradicts what was previously suggested and can be explained in two ways. First, if we look at a cell wall proteinase capable of providing bacteria with peptides containing BCAA, we see that it appears more frequently in *L. lactis* than in *S. thermophilus* (36). *L. lactis* does not, therefore, really need to maintain a functional biosynthesis pathway. Mutants 1 and 2 grew rapidly again after addition of either Bacto Tryptone (Fig. 3) or an amino acid mixture (data not shown) to milk. This observation strongly suggests that the main role of the *S. thermophilus* BCAA biosynthesis pathway is to supply BCAA, a role complementary to the BCAA transport role already described for *S. thermophilus* (1). The difficulty in finding BCAA in milk encountered by *S. thermophilus* has been observed, to a much lesser extent, with *L. lactis* (12). In most cases, this difficulty is probably overcome by coculture of *S. thermophilus* with *Lactobacillus delbrueckii* subsp. *bulgaricus*, which produces amino acids and peptides and stimulates *S. thermophilus* growth (33). The second possible explanation is linked to the different genetic organizations of the BCAA biosynthesis pathways in

L. lactis (14) or *L. mesenteroides* (4) and *S. thermophilus* (this study). In this case, independent translation of *ilvC* probably allows finer regulation of the pathway, which is sufficiently beneficial to *S. thermophilus* to keep the BCAA biosynthesis pathway active.

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