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Gene Cloning, Sequencing, and Inactivation of the Branched-Chain Aminotransferase of *Lactococcus lactis* LM0230

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A branched-chain aminotransferase gene (*ilvE*) from *Lactococcus lactis* LM0230 was identified on a 9-kb chromosomal insert by complementation in *Escherichia coli* DL39. Sequencing of a 2.0-kbp fragment resulted in the identification of a 1,023-bp open reading frame that could encode a 340-amino-acid protein. Sequence analysis of the deduced amino acid sequence revealed 62% identity to IlvE of *Haemophilus influenzae* and high similarity to IlvEs from a variety of organisms found in GenBank classified as class IV aminotransferases. Under logarithmic growth in complex medium, *ilvE* is transcribed monocistronically as a 1.1-kb transcript. Hydrophobicity plot analysis of the deduced amino acid sequence and the lack of a signal peptide sequence suggest IlvE is a cytosolic protein. A derivative of LM0230 lacking IlvE activity was constructed by gene replacement. Comparison of the IlvE-deficient strain's ability to grow in defined media lacking an amino acid but containing its α-keto acid biosynthetic precursor to that of the wild-type strain indicated that IlvE is the only enzyme capable of synthesis of Ile and Val from their biosynthetic precursors. Comparison of the aminotransferase activity of the IlvE mutant to LM0230 revealed that the mutant retained <2, 4.5, 43, 40, and 76% of its aminotransferase activity with Ile, Val, Leu, Met, and Phe, respectively. No difference in growth or acidification rate between LM0230 and the IlvE-deficient strain was observed in milk.

Lactic acid bacteria (LAB) contribute to the manufacture of fermented dairy products by lowering the pH of milk via fermentation of lactose to lactic acid and providing enzymes and metabolites that contribute to texture and flavor (33). The physiological traits of LAB related to their ability to grow in milk and hence lower its pH have been studied in significant detail, while traits that impact product texture or flavor have received less attention. For example, significant advances have been made in understanding proteolytic systems of LAB and how these systems function to provide essential amino acids for growth in milk (22, 30). However, our understanding of how the products of casein hydrolysis, amino acids, are catabolized by these organisms and converted to cheese flavor compounds remains poorly understood.

A number of enzyme classes capable of initiating amino acid catabolism have been described. These include aminotransferases, deaminases, decarboxylases, and lyases (16). Aminotransferases catalyze the reversible transamination of α-amino groups and are essential for synthesis and catabolism of many amino acids. Three aminotransferases commonly found in eucaryotic and procaryotic organisms are the acidic, aromatic, and branched-chain aminotransferases (IlvE). All of these enzymes require pyridoxal phosphate as a coenzyme (16). It is known that strains of *Lactococcus lactis* isolated from milk are auxotrophic for many amino acids (19), most likely due to selective pressure resulting from adaptation to milk, a nitrogen-rich environment. Since numerous strains of *L. lactis* which are auxotrophic for Leu, Ile, and Val still encode IlvE activity, it seemed possible this enzyme is involved in the catabolism of the branched-chain amino acids (BCAAs). Aminotransferases active on the BCAAs and the products of BCAA transamination are used by bacteria for the generation of metabolic energy and carbon (24), regulation of the NADH/NAD^+ ratio (28), recycling of Glu from α-ketoglutarate (21), production of siderophores (20), biosynthesis of pantothenic acid (4), and the production of branched-chain fatty acids for cell membrane synthesis (8). Whether an IlvE homolog from *L. lactis* has similar roles is unknown.

There is evidence that the amino acids released from casein via the action of proteolytic enzymes are converted to aldehydes, alcohols, fatty acids, esters, and thiols (6, 25, 26, 33). Moreover, it is believed that many amino acids also serve as precursors for the production of volatile compounds that have a significant influence on cheese flavor development (33). Some of these compounds, such as 3-methylbutanal, 2-methylpropanal, isobutyric acid, and isovaleric acid, have structures similar to the BCAAs and therefore may be derived from BCAA catabolism. A recently published review more fully discusses BCAA catabolism by lactic acid bacteria and the implications for cheese flavor development (6).

Aminotransferases have been demonstrated to initiate the catabolism of aromatic amino acids and methionine under cheese-like conditions (11, 12, 13). These studies support the hypothesis that aminotransferase-initiated pathways are involved in the production of volatile compounds present in Cheddar cheese (33). Given the potential role of aminotransferases toward the formation of volatile flavor compounds in ripened cheese and their central role in cellular physiology, we have been interested in further characterizing aminotransferase-catalyzed pathways of *L. lactis*. This study focuses on the molecular characterization of *ilvE* from *L. lactis*, as well as the characterization of a strain in which this gene was inactivated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacteria and plasmids used in this study are listed in Table 1. All cultures were maintained at −80°C in 6.9% nonfat dry milk and 10% glycerol. *Escherichia coli* strains were routinely propagated in Luria-Bertani (29) broth at 37°C with aeration. M9 medium (14) was prepared containing all 20 common amino acids at 50 μg/ml except where stated otherwise. Additionally, M9 medium was supplemented with 20 μg of adenine, gua-

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TABLE 1. Bacterial strains and plasmids

Bacterial strains and plasmids	Description	Source or reference
E. coli		
DL39	*ilvE12 tyrB507 aspC13*	23
DH5α	F^- Δ*lacZ*ΔM15 Δ(*lacZYA-argF*)	GIBCO-BRL
L. lactis		
LM0230	Plasmid free; wild type	9
JLS450	LM0230; Δ*ilvE*	This study
Plasmids		
pTRKL2	6.4 kb; Em^r *lacZ*	27
pMOB	1.8 kb; Ap^r	Gold BioTechnology, St. Louis, Mo.
pJK550	55.0 kb; Lac^+ Prt^+ plasmid from *L. lactis* C20	34
pG^+host5	5.3 kb; Em^r; temperature-sensitive lactococcal integration vector	3
pSUW411	9-kb insert; $IlvE^+$; pTRKL2 derivative	This study
pSUW451	6-kb insert; $IlvE^+$; pMOB derivative	This study
pSUW452	2-kb insert; $IlvE^+$; pMOB derivative	This study
pSUW453	2-kb insert; $IlvE^+$; pTRKL2 derivative	This study
pSUW454	1.65-kb insert; $IlvE^-$; pMOB derivative	This study
pSUW455	1.65-kb insert; $IlvE^-$; pG^+host5 derivative	This study

nine, uracil, and xanthine per ml. *L. lactis* LM0230 and derivatives were routinely propagated in M17 broth with 0.5% glucose (M17-G) or lactose (M17-L) (31) at 30°C except where stated otherwise. Defined media (DM) for lactococcal strains were prepared based on the medium described by Jensen and Hammer (19), using the salt solution described by Jenness and Koops (18). Modifications included substituting α-ketoisocaproate (KIC), α-keto-β-methylvalerate (KMV), α-ketoisovalerate (KIV), or 4-methylthio-2-ketobutyric acid (KMBA) (100 μg/ml, each) for Leu, Ile, Val, and Met, respectively. These α-keto acids are the biosynthetic precursors to these amino acids. For growth studies in milk, pasteurized skim milk was aliquoted into sterilized glassware, steamed for 20 min, incubated for 2 h at 30°C, and steamed again for 20 min. Agar plates were prepared by adding 1.5% (wt/vol) granulated agar (Difco Laboratories, Detroit, Mich.) to liquid medium. For *E. coli*, erythromycin (EM) and ampicillin (AP) were used at final concentrations of 500 and 60 μg/ml, respectively. For *L. lactis*, EM was used at a final concentration of 5 μg/ml.

Molecular biology techniques. Recombinant DNA and plasmid isolation techniques for *E. coli* strains were performed as described by Sambrook et al. (29). Plasmid isolation from lactococci was conducted as described by Anderson and McKay (2). Calf intestinal alkaline phosphatase (Promega), T4 DNA ligase (GIBCO-BRL) and restriction enzymes (GIBCO-BRL) were used as recommended by the manufacturer. All DNA primers were synthesized by GIBCO-BRL. *L. lactis* transformations were performed as described by Holo and Nes (17). *E. coli* transformations were done with a Gene Pulser by following the manufacturer's instructions (Bio-Rad Laboratories, Richmond, Calif.). DNA fragments were purified from agarose gels by using the Qiagen Gel Extraction Kit (Qiagen, Inc., Santa Clarita, Calif.). Products from PCRs were purified using the Qiagen PCR purification kit (Qiagen, Inc.). Probe synthesis for Southern hybridization was performed using the Genius Nonradioactive Nucleic Acid Labeling and Detection System (Boehringer Mannheim, Indianapolis, Ind.). PCR subcloning of the *ilvE* gene was accomplished using the primers 5′-CGG CGGATCCCAACATTGATTTGATGTTC-3′ and 5′-CGAGGTCGACAGAA AATCCCTGTATTGAAT-3′. The primers were designed with a restriction site for *Bam*HI or *Sal*I (underlined), respectively, at the 5′ end. PCR was performed using the PCR Elongase Kit (GIBCO-BRL). The reactions were performed using a DNA Thermal Cycler 480 (The Perkin-Elmer Corp., Norwalk, Conn.) at 94°C for 30 s, 50°C for 90 s, and 68°C for 2 min in 35 cycles. The amplified product was digested with *Bam*HI and *Sal*I and ligated to similarly digested pMOB or pTRKL2.

Cloning of *ilvE* gene. A genomic library of *L. lactis* LM0230 constructed in pTRKL2 (E. G. Dudley and J. L. Steele, submitted for publication) was transformed into *E. coli* DL39. Cells were plated onto M9 agar medium lacking Leu. Plates were incubated at 37°C for 1 day. Colonies growing on plates lacking Leu were picked into Luria-Bertani broth containing EM. Aminotransferase activity was confirmed by enzyme assays.

Preparation of CEs. Ten-milliliter portions of overnight cultures of *E. coli* or 650 ml of log-phase *L. lactis* were harvested by centrifugation and washed twice with Bis-Tris (pH 6.5). The pellet was resuspended in 2.5 ml of Bis-Tris (pH 6.5), and 1 g of 150- to 212-μm-diameter glass beads was added. Cells were shaken on a Red Devil 5410 paint mixer for 5 min and placed on ice for 10 min before shaking them again for 5 min. After centrifugation the supernatant was collected and used as cell extracts (CEs) for the enzyme assays.

Aminotransferase enzyme assays. All components for the enzyme assay were purchased from Sigma. When screening *E. coli* derivatives for IlvE activity, Leu aminotransferase activity was determined using a modification of the method of Collier and Kohlhaw (7). The reaction mixture contained the following in 1 ml: CE, 100 mM MOPS (morpholine propanesulfonic acid; pH 6.5), 40 mM L-glutamic acid, 4 mM sodium α-ketoisocaproic acid, 1 mM NaN_3, 40 mM NH_4Cl, 0.2 mg of NADH, 0.25 mM pyridoxal-5-phosphate, and 0.4 U of bovine liver glutamate dehydrogenase. The specific activity was calculated as micromoles of NADH oxidized min^{-1} mg of $protein^{-1}$, using the molar extinction coefficient of NADH at 339 nm (6,220 M^{-1} cm^{-1}). Quantitative assays for lactococcal IlvE activity with each of the 20 common amino acids were done at 30°C as described by Gao et al. (12) using the colorimetric L-glutamic acid assay kit of Boehringer Mannheim. Specific activities were calculated as micromoles of formazan formed min^{-1} mg of $protein^{-1}$, using the extinction coefficient of formazan at 492 nm (19.9 $mmol^{-1}$ cm^{-1}). The protein concentration was determined using the Micro Protein Determination kit of Sigma and bovine serum albumin as the protein standard.

DNA sequencing. DNA templates for sequencing reactions were purified using a modified alkaline lysis-polyethylene glycol precipitation protocol (Applied Biosystems, Inc., Foster City, Calif.). Cycle sequencing reactions were performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). DNA sequence determination was conducted by the University of Wisconsin-Madison Biotechnology Center using an ABI model 370/3 automated sequencer. Sequences were analyzed using the GCG sequence analysis package (Genetics Computer Group, Inc., Madison, Wis.). Searches for protein sequences similar to the putative IlvE sequence were performed using the BLAST network service (1).

RNA methods. Total RNA was isolated by using the RNeasy kit (Qiagen). An internal *ilvE* fragment of 800 bp was amplified and used to synthesize a digoxigenin-labeled probe with the Genius system (Boehringer Mannheim) for Northern hybridization. The nucleotide sequence of the primers were 5′-GGTCCCA CTTCTGTTTCA-3′ and 5′-ACCTTTTCGTTATATCGC-3′. RNA molecular weight markers, solutions, and reagents used in Northern hybridizations were utilized according to the procedure supplied by the manufacturer. Mapping of the 5′ end of the *ilvE* transcript was conducted by using the 5′ rapid amplification of cDNA (5′ RACE) kit (version 2.0; GIBCO-BRL) as described by Chen and Steele (5). The first-strand cDNA synthesis was performed with the primer 5′-TTGGTCTGGACGAAAAAGTT-3′. Nested amplification of first-strand cDNA was carried out with primer 5′-AGGTAAGTTTCGATAACT-3′ and the anchor primer supplied with the kit.

Construction of an LM0230 derivative lacking IlvE activity. A PCR fragment containing an internal 450-bp in-frame deletion of *ilvE* was amplified using pSUW452 as the template via inverse PCR using the primers 5′-P-TCTTTGTT GTATTTAGCTGA-3′ and 5′-P-AATAAACATTTCAGTTGAAAC-3′. The resulting product (pSUW454) was self-ligated and transformed into *E. coli* DL39. Transformants were plated on Luria-Bertani plates containing AP and M9 media lacking Leu to confirm the Leu auxotrophy. The in-frame deletion was confirmed by sequencing pSUW454 using the primer 5′-GGTCCCACTTCTGTTTCA-3′. This fragment was subcloned in pG^+host 5 (3), designated pSUW455, and used to construct an $IlvE^-$ derivative of *L. lactis* LM0230 by the double-crossover homologous recombination method of Biswas et al. (3). PCR was used to identify LM0230 derivatives containing the deleted version of *ilvE*.

Growth studies. *L. lactis* strains were propagated at 30°C in M17-L to exponential phase, washed twice in 0.85% NaCl, resuspended in the saline solution, and standardized to inoculate 100 ml of skim milk to an initial optical density at

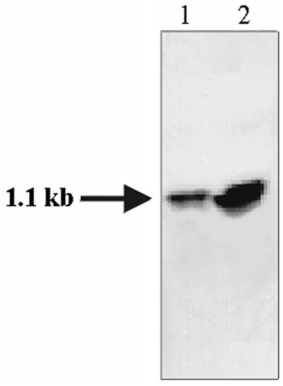

FIG. 1. Detection of *L. lactis* LM0230 *ilvE* transcript during growth in M17-G broth by Northern hybridization. Lanes: 1, total RNA from *L. lactis* LM0230; 2, total RNA from *E. coli* DH5α(pSUW411).

600 nm (OD_{600}) of 0.01. The inoculated skim milk was incubated at 30°C, and pH and OD_{600} were monitored. The OD was recorded as described previously (5). Growth studies in DM were performed by propagation of the strains at 30°C in M17-G, washing the cells twice with 0.85% NaCl and inoculating them into the appropriate DM at 0.1%. The growth of cultures was followed by monitoring the OD_{600}.

Nucleotide sequence accession number. The sequence for *ilvE* has been submitted to GenBank and assigned accession number AF169649.

RESULTS

Cloning of the gene encoding the branched-chain aminotransferase. A previously constructed genomic library of *L. lactis* LM0230 (Dudley and Steele, submitted) was transformed into *E. coli* DL39 and plated on M9 media lacking Leu. *E. coli* DL39 is unable to grow in the absence of Leu due to a mutation in *ilvE* (23). Twenty-one colonies capable of growth in the absence of Leu were isolated. Restriction digests of plasmids isolated from the 21 colonies revealed four distinct patterns (data not shown). Southern hybridization analysis using *Eco*RI-digested plasmids as probes suggested that all four plasmids contained overlapping chromosomal fragments (data not shown). The plasmid with the smallest insert (9 kb) was designated pSUW411. Additionally, a 6.0-kb *Sal*I-*Nru*I chromosomal fragment from pSUW411 was subcloned into pTRKL2, forming pSUW451. This construct was found by enzyme assays to encode IlvE activity.

DNA sequencing and analysis. The *ilvE* gene was further localized on pSUW451 by subcloning and DNA sequencing. A total of 2,040 bp of double-stranded sequence was obtained, and an open reading frame (ORF) of 1,023 nucleotides was identified which could encode a 340-amino-acid, 36.9-kDa polypeptide. BLAST searches indicated that the deduced amino acid sequence has 62% identity to IlvE of *H. influenzae* and a high degree of similarity to other IlvE homologs which belong to the class IV pyridoxal-phosphate-dependent ATase family. The lactococcal IlvE also has an amino acid sequence which matches the PROSITE aminotransferase class-IV pyridoxal-phosphate binding site; more information on this pattern can be found on the ExPASy Molecular Biology Server (http://www.expasy.ch/cgi-bin/nicedoc.pl?PDOC00618). The pyridoxal-phosphate binding site was tentatively identified as Lys_{184}. Downstream of the ORF a putative *rho*-independent transcriptional terminator with a ΔG of −14.5 kcal/mol was identified. The transcriptional start site was determined by 5′ RACE to be 23 bp upstream of the ATG start codon. Putative −10 and −35 promoter regions and a ribosome binding site were also identified upstream of the ORF. No signal peptide sequence was found in the N-terminal region of the deduced IlvE sequence. Northern blot hybridization analysis (Fig. 1) identified a single transcript of approximately 1.1 kb in LM0230 and *E. coli* DH5α(pSUW411). No band was observed in *E. coli* DH5α without pSUW411.

TABLE 2. Aminotransferase activity of the lactococcal IlvE in *E. coli* DL39

Substrate	Aminotransferase activity	
	Sp act ± SD[a]	% Relative activity[b]
Isoleucine	47 ± 2	100
Leucine	27 ± 7	57
Valine	25 ± 5	52
Methionine	2.4 ± 0.2	5.3
Phenylalanine	0.7 ± 0.1	1.5

[a] Specific activity ± the sample standard deviation. Specific activities are reported as micromoles of formazan formed $minute^{-1}$ $milligram\ of\ protein^{-1}$ from two independent experiments conducted in duplicate.
[b] The activity of IlvE with Leu was arbitrarily defined as 100% relative activity.

Substrate specificity assay. Substrate specificity of the cloned enzyme in terms of catabolism of amino acids was determined using CEs of *E. coli* DL39(pSUW452). The specific activity of the lactococcal IlvE against amino acid substrates was in descending order: Ile > Leu > Val > Met > Phe. No activity was detected against any of the other 14 amino acids examined (Table 2). No activity with any amino acid was observed with CEs from DL39.

Construction and analysis of *ilvE* mutant strain in *L. lactis* LM0230. An LM0230 derivative containing a 450-bp in-frame deletion in *ilvE* was constructed and designated JLS450. The ability of JLS450 to grow in DM lacking a specific amino acid but containing its α-ketoacid biosynthetic precursor was compared to the isogenic $IlvE^-$ strain LM0230 (Table 3). While JLS450 was unable to grow in DM when Ile or Val were replaced with KMV or KIV, respectively, LM0230 was capable of growth under these conditions. However, JLS450 grew like LM0230 when Met was replaced by KMBA; a 10-h lag in growth, relative to LM0230, was observed when Leu was replaced with KIC. To confirm that the inability of the mutant to grow under the conditions described above was due to the lack of IlvE activity, pSUW453 was introduced into JLS450. Growth of JLS450(pSUW453) was indistinguishable from the wild type in all of the media examined (data not shown). A plasmid

TABLE 3. Growth of *L. lactis* LM0230 and its $IlvE^-$ derivative, JLS450, in DM

DM containing:	Growth of *L. lactis* strains[a]	
	LM0230	JLS450
All amino acids	+	+
All amino acids, KMV, KIC, KIV, and KMBA	+	+
No Leu, Ile, Val, and Met	−	−
KMV, KIC, KIV, KMBA; no Leu, Ile, Val, and Met	+	−
No Leu	−	−
No Ile	−	−
No Val	−	−
No Met	−	−
KIC; no Leu	+	+[b]
KMV; no Ile	+	−
KIV; no Val	+	−
KMBA, no Met	+	+

[a] +, $OD_{600} \geq 1.0$ after 24 h; −, $OD_{600} < 0.3$ after 24 h.
[b] Growth after a 10-h lag period.

TABLE 4. Aminotransferase activities in CEs from *L. lactis* LM0230 and its IlvE$^-$ derivative, JLS450

Amino acid substrate	Relative activity (%) for[a]:	
	L. lactis LM0230	*L. lactis* JLS450
Isoleucine	100	<2
Leucine	100	43 ± 2.1
Valine	100	4.5 ± 2.1
Methionine	100	40 ± 9.9
Phenylalanine	100	76 ± 6.4

[a] The aminotransferase activity of *L. lactis* LM0230 on each amino acid substrate was defined as 100%.

(pJK550) containing genes for lactose utilization and the cell envelope-associated proteinase of *L. lactis* C20 (34), which are required for growth of lactococci in milk, was introduced by electroporation into LM0230 and JLS450. No differences in growth or acidification rates in milk were observed between derivatives with a wild-type or a deleted version of *ilvE* (data not shown). To characterize the contribution of the lactococcal IlvE to the catabolism of Val, Ile, Met, Leu, and Phe, enzyme assays with LM0230 and JLS450 CEs were performed. IlvE activity from CEs of logarithmically growing JLS450 in M17-G had <2, 4.5, 43, 40, and 76% the activity of LM0230 with Ile, Val, Leu, Met, and Phe, respectively (Table 4).

DISCUSSION

Catabolism of amino acids by lactococci is believed to have important physiological implications and to have an essential role in the development of cheese flavor. However, little is known concerning the amino acid catabolic pathways present in these organisms and the enzymes involved. Previous studies have reported aminotransferase activities in *L. lactis* strains capable of initiating the catabolism of Leu, Ile, Val, Met, Phe, and Tyr (11, 12, 13, 28, 35); however, the genes encoding these aminotransferases have not been identified. Therefore, the intent of this study was to characterize the gene encoding a branched-chain aminotransferase from *L. lactis*, to characterize its role in growth in milk, and to study its contribution to the ability of this organism to catabolize a variety of amino acids.

Sequence analysis of IlvE from *L. lactis* LM0230 revealed high identity with other branched-chain aminotransferase which belong to class IV family of aminotransferases. Northern blot analysis indicated the gene is transcribed monocistronically under logarithmic and stationary phases in rich media. In other microorganisms examined, *ilvE* was typically found in an operon with genes encoding other enzymes involved in the biosynthesis of BCAAs, and its product is required for the last step in the biosynthesis of Leu, Val, and Ile (32). The fact that lactococcal strains isolated from milk are typically auxotrophic for these amino acids (15, 19) suggests that this enzyme has other physiological roles besides the biosynthesis of amino acids in lactococci.

The enzyme was observed to have a broad specificity with activity on Ile, Leu, Val, Met, and Phe. Activity with Met is of particular interest since Met catabolism is known to be initiated by an aminotransferase in lactococci (11) and the conversion of Met to methanethiol is thought to be an essential reaction for the development of Cheddar cheese flavor (33). However, the lactococcal aromatic aminotransferase has also been demonstrated to have activity with Met (13, 35). The relative contribution of these two aminotransferases to the catabolism of Met in cheese remains to be elucidated.

Previous investigators have suggested that lactococci contain two branched-chain aminotransferases (10, 28). Engels (10) purified two enzymes from *L. lactis* subsp. *cremoris* B78 with activity on the BCAAs (AT-A and AT-B). The substrate specificity of AT-A (Ile, Leu, Val, Met, and Phe) is similar to the substrate specificity of the LM0230 IlvE. The specificity of AT-B was broader than AT-A and included Tyr and Trp in addition to the amino acids AT-A had activity with. However, the inability of JLS450 to grow in DM lacking Ile or Val but containing KMV or KIV, respectively, suggests that IlvE is the only lactococcal enzyme capable of transamination of KMV and KIV. Therefore, either *L. lactis* B78 encodes an aminotransferase not found in or weakly expressed in LM0230, or the preparation of AT-B may have been contaminated with AT-A. The ability of JLS450 to grow in DM when Leu or Met was replaced with KIC or KMBA, respectively, supports the biochemical evidence that the lactococcal aromatic aminotransferase has activity on these substrates (13, 35).

The physiological role of the lactococcal IlvE during growth in milk, if any, remains unclear. Recently, our lab has detected an enzyme in *L. lactis* which catalyzes the reduction of KIC, suggesting that BCAA catabolism may serve to regenerate NAD^+ in lactococci (S. Gao and J. L. Steele, unpublished results). Whether this pathway occurs in vivo remains to be determined. Additionally, the strain JLS450 as well as a lactococcal strain overexpressing *ilvE* will be useful in determining the involvement of IlvE in the production of volatile flavor compounds believed to derive from the BCAAs and Met.

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