APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2002, p. 4007–4014 Vol. 68, No. 8
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.8.4007–4014.2002

Cloning and Inactivation of a Branched-Chain-Amino-Acid Aminotransferase Gene from *Staphylococcus carnosus* and Characterization of the Enzyme

Søren M. Madsen,[1]* Hans Christian Beck,[2] Peter Ravn,[1] Astrid Vrang,[1] Anne Maria Hansen,[2] and Hans Israelsen[1]

Department of Lactic Acid Bacteria, Biotechnological Institute, Kogle Allé 2, DK-2970 Hørsholm,[1] and Department of Perception & Functionality, Biotechnological Institute, Holbergsvej 10, DK-6000 Kolding,[2] Denmark

Received 19 February 2002/Accepted 29 May 2002

***Staphylococcus carnosus* and *Staphylococcus xylosus* are widely used as aroma producers in the manufacture of dried fermented sausages. Catabolism of branched-chain amino acids (BCAAs) by these strains contributes to aroma formation by production of methyl-branched aldehydes and carboxy acids. The first step in the catabolism is most likely a transamination reaction catalyzed by BCAA aminotransferases (IlvE proteins). In this study, we cloned the *ilvE* gene from *S. carnosus* by using degenerate oligonucleotides and PCR. We found that the deduced amino acid sequence was 80% identical to that of the corresponding enzyme in *Staphylococcus aureus* and that the *ilvE* gene was constitutively expressed as a monocistronic transcript. To study the influence of *ilvE* on BCAA catabolism, we constructed an *ilvE* deletion mutant by gene replacement. The IlvE protein from *S. carnosus* was shown mainly to catalyze the transamination of isoleucine, valine, leucine, and, to some extent, methionine using pyridoxal 5′-phosphate as a coenzyme. The *ilvE* mutant degraded less than 5% of the BCAAs, while the wild-type strain degraded 75 to 95%. Furthermore, the mutant strain produced approximately 100-fold less of the methyl-branched carboxy acids, 2-methylpropanoic acid, 2-methylbutanoic acid, and 3-methylbutanoic acid, which derived from the BCAA catabolism, clearly emphasizing the role of IlvE in aroma formation. In contrast to previous reports, we found that IlvE was the only enzyme that catalyzed the deamination of BCAAs in *S. carnosus*. The *ilvE* mutant strain showed remarkably lower growth rate and biomass yield compared to those of the wild-type strain when grown in rich medium. Normal growth rate and biomass yield were restored by addition of the three BCAA-derived α-keto acids, showing that degradation products of BCAAs were essential for optimal cell growth.**

The gram-positive bacteria *Staphylococcus carnosus* and *Staphylococcus xylosus* are widely used in the meat industry for the manufacture of fermented meat products such as dried sausages. One role of these bacteria is in the formation of flavor compounds in the final product. Catabolism of aromatic amino acids (ArAAs), branched-chain amino acids (BCAAs), and methionine by different starter cultures is believed to play a major role in the formation of aroma and flavor compounds in fermented meat as well as in fermented dairy products (16, 18, 28).

In *S. carnosus*, leucine degradation gives rise to aroma compounds such as 3-methylbutanol acid, 3-methylbutanal acid, and 3-methylbutanoic acid (12, 15, 25). Based on the ability of *S. carnosus* to degrade leucine in the absence of an α-keto acid acceptor and pyridoxal 5′-phosphate, Larrouture et al. (12) suggested that both transamination and oxidative deamination reactions are involved in the deamination of leucine. The putative pathway for the catabolism of leucine is shown in Fig. 1. The described transamination and oxidative deamination reactions catalyze the initial step of leucine catabolism, which leads to α-ketoisocaproic acid. The metabolite, 3-methylbutanoic acid, may be formed either via the acyl-CoA intermediate (26) or the aldehyde (3, 15, 23, 24). Despite the importance of aroma formation in fermented meat products, no genetic studies on the amino acid catabolism in *S. carnosus* or *S. xylosus* have yet been described. In contrast, several studies using *Lactococcus lactis* have been done on gene cloning and mutant construction and for characterization of the genes encoding enzymes responsible for degradation of both ArAAs and BCAAs (1, 20, 27, 29). Although several enzymes in *L. lactis* may catalyze the first step in amino acid catabolism, only aminotransferases, which catalyze the transfer of an α-amino group from ArAAs and BCAAs to an α-keto acid acceptor using pyridoxal 5′-phosphate as a coenzyme, seem to be responsible for deamination of these amino acids (1, 20, 27, 29). Two recent studies with *L. lactis* showed that IlvE is responsible for approximately 90% of the total isoleucine and valine aminotransferase activity and also for 60% of the leucine and 38 to 60% of the methionine activity (1, 27). Recently, the genes encoding *ilvE* of *L. lactis* NCDO763 and LM0230 were cloned and sequenced, and expression studies showed that the BCAA aminotransferase of NCDO763 is repressed by free BCAAs, suggesting a biosynthetic role of the gene (27). However, dairy strains require BCAAs for growth and, therefore, the role of IlvE in *L. lactis* dairy strains is believed to be purely catabolic (27).

Another aminotransferase, the ArAA aminotransferase

* Corresponding author. Mailing address: Department of Lactic Acid Bacteria, Biotechnological Institute, Kogle Allé 2, DK-2970 Hørsholm, Denmark. Phone: (45) 45 16 04 44. Fax: (45) 45 16 04 55. E-mail: sma@bioteknologisk.dk.

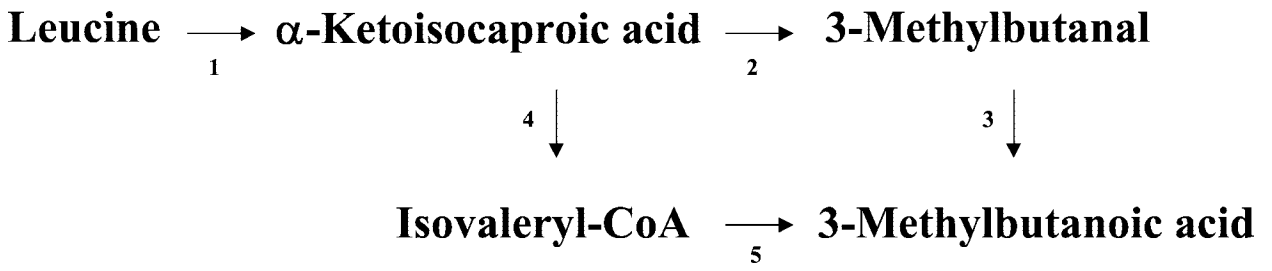

FIG. 1. Putative pathways of BCAA catabolism in *S. carnosus* as exemplified with leucine. Reaction 1 was catalyzed by BCAA aminotransferase, leucine dehydrogenase, or leucine oxidase; reaction 2 was catalyzed by branched-chain α-keto acid decarboxylase; reaction 3 was catalyzed by aldehyde dehydrogenase; reaction 4 was catalyzed by branched-chain α-keto acid dehydrogenase; and reaction 5 was catalyzed by acyl-CoA hydrolase or phosphate butyrate-CoA transferase and butyrate kinase.

(AraT) of *L. lactis* NCDO763, has been characterized biochemically (29), and the corresponding gene has been cloned (20). Inactivation of AraT results in a 90 to 95% decrease in aminotransferase activity towards phenylalanine, tyrosine, and tryptophan and a 50 and 25% decrease in activity towards methionine and leucine, respectively. Inactivation of AraT has minor effects on the aminotransferase activity towards valine and isoleucine.

In this work, we have initiated genetic and physiological studies of genes and gene products involved in the degradation of BCAAs in *S. carnosus*. Here we report the molecular cloning of the *ilvE* gene, which is involved in the degradation of BCAAs in *S. carnosus*, and the construction of an *ilvE* mutant strain and its use for analysis of amino acid catabolism and metabolite formation.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* strain DH10B was grown in Luria-Bertani broth or on agar plates at 37°C and supplemented with 100 μg of ampicillin/ml when appropriate. *S. carnosus* BioCarna Ferment S1 (Wisby Starter Cultures and Media, Niebüll, Germany) was grown at 30°C with shaking in B-T medium, which consisted of 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% $K_2HPO_4 \cdot 3H_2O$, and 0.5% glucose, and supplemented with 10 μg of chloramphenicol/ml when appropriate. α-Ketoisocaproate (KIC), α-keto-β-methylvalerate (KMV), and α-ketoisovalerate (KIV) obtained from Sigma Chemical Co. (St. Louis, Mo.) were used at 100 μg/ml.

Transformation and isolation of plasmid and chromosomal DNA. Plasmid DNA was introduced into competent *E. coli* cells (ElectroMAX DH10B from Life Technologies, Roskilde, Denmark) by electroporation as described by the manufacturer and into *S. carnosus* by protoplast transformation as described by Götz and Schumacher (7). Plasmid DNA was isolated from *E. coli* by using Jet Prep columns (Genomed, Bad Oeynhausen, Germany) and from *S. carnosus* as described by O'Sullivan and Klaenhammer (19), except that cells were incubated with 45 mg of lysozyme/ml at 37°C for 1½ h. Genomic DNA was prepared from 6 to 8 ml of an overnight culture of *S. carnosus* as described by Johansen and Kibenich (8), except that lysozyme (20 mg/ml) treatment was carried out for 1½ h. SDS was subsequently added, and samples were incubated at 37°C for 1/2 h and again at 65°C for 1/2 h.

DNA manipulation and sequencing. DNA was manipulated according to standard procedures (21). *Taq* polymerase (Life Technologies) was used for PCR unless otherwise noted. All primers used for PCR or DNA sequencing were obtained from DNA Technology A/S, Aarhus, Denmark. Plasmid DNA was sequenced on both strands by using an ALFexpress DNA sequencer (Amersham Pharmacia Biotech, Little Chalfont, England). DNA and deduced amino acid sequences were analyzed using the BlastN and BlastP programs available at www.ncbi.nlm.nih.gov/BLAST.

TABLE 1. Strains and plasmids

Bacterial strain or plasmid	Relevant characteristic(s) or DNA insert[a]	Reference or source
Bacterial strains		
S. carnosus		
BioCarna Ferment S1	Industrial flavor-producing strain, Nuc^-	Wisby[b]
PSM213	Strain S1 containing pPSM1064, Nuc^-, Cam^r	This study
PSM215	Strain S1 containing pPSM1064 integrated into the chromosome, wild-type *ilvE*, Nuc^+, Cam^r	This study
PSM217	Strain S1 containing a deletion in the *ilvE* gene, Nuc^-, Cam^s	This study
E. coli DH10B	*E. coli* cloning host strain	Life Technologies
Plasmids		
pBluescript KS II	*E. coli* cloning vector, Amp^r	Stratagene
pCR2.1	TA-cloning vector, Amp^r	Invitrogen
pBT2	*S. carnosus* integration vector, Ts replicon, Cam^r	6
pSMBI93	Vector containing *Usp45-nucB* gene cassette	Vrang et al., unpublished
pPRA26	pCR2.1 + 4.1-kb S1 chromosomal fragment containing the *ilvE* gene	This study
pPSM1058	pBT2 + 0.9-kb *Bam*HI-*Sal*I fragment containing the *Usp45-nucB* cassette from pSMBI93	This study
pPSM1060	pBluescript KS II + 0.8-kb *Bam*HI-*Sma*I Δ*ilvE* fragment	This study
pPSM1064	pPSM1058 + 0.8-kb *Bam*HI-*Kpn*I fragment with Δ*ilvE* gene from pPSM1060 fused to *Usp45-nucB*	This study

[a] Nuc^+, nuclease producer; Nuc^-, does not produce nuclease; Amp^r, ampicillin resistance; Cam^r, chloramphenicol resistance; Cam^r, chloramphenicol sensitivity; Ts, temperature sensitive.
[b] Wisby, Wisby Starter Cultures and Media.

Cloning of the *ilvE* gene. An internal *ilvE* fragment from *S. carnosus* was amplified by PCR using the degenerate primers P1-IlvE (5′ TTY GAR GGN YTN AAR GC 3′), used at a final concentration of 6 μM, and P4-IlvE (5′ ATN ACN GCN GCN GTN CC 3′), used at a final concentration of 0.6 μM, with Y being C or T; R being A or G; and N being A, C, G, or T. PCR amplifications were done by gradually decreasing the annealing temperature from 60°C to 45°C (6a). The resulting 0.7-kb PCR fragment was cloned into the pCR2.1 vector (Invitrogen, Carlsbad, Calif.). The remaining part of the *ilvE* gene and the adjacent regions were amplified by inverse PCR (17). In short, genomic DNA of *S. carnosus* was digested with *Eco*RI and religated in a large volume. PCR amplifications were carried out using primers P6-IlvE (5′ GAA GAA CGT CAT ATC TCT ATC G 3′) and P7-IlvE (5′ GCC TTC TAA TAG CTC TTC TTC 3′) and the Platinum *Pfx* DNA Polymerase (Life Technologies), with annealing temperatures being gradually decreased from 68 to 52°C. The obtained 4.1-kb PCR fragment was cloned into pCR2.1 by adding A overhangs to the PCR products prior to cloning, as recommended by Invitrogen. The resulting plasmid was named pPRA26.

Construction of a new nuclease reporter gene vector. The reporter gene cassette containing the Usp45 signal peptide from *L. lactis*, translationally fused to *nucB* from *Staphylococcus aureus*, was isolated on a 900-bp *Bam*HI-*Sal*I fragment from plasmid pSMBI93 (A. Vrang et al., unpublished data) and inserted into pBT2 (6) digested with *Bam*HI and *Sal*I. This resulted in plasmid pPSM1058.

Construction of an *ilvE* deletion mutant. An internal 277-bp (fragment B) deletion of *ilvE* was created using PCR. A 400-bp fragment corresponding to the 5′ end of the *ilvE* gene (fragment A) was amplified using *S. carnosus* genomic DNA as template and primers ilvE-N-term (5′ AAC CGG AAT TCA TGT CAG AAA AAG TAA AAT TTG AAA AAC GTG 3′) and ilvE-Δ1 (5′ CTT CAA TAT ATT TGC GTT CTA TTG TCC TTC GCC TTC AGG CAC CCA ATC AC 3′). A 408-bp fragment covering the 3′ end of the *ilvE* gene (fragment C) was amplified similarly, using primers P32-ilvE-*Sma*I (5′ TAG TCC CGG GCA ATT TTA ATA TTC TGG TAC TAC G 3′) and ilvE-Δ2 (5′ CCT GAA GGC GAA GGA CAA TAG AAC GCA AAT ATA TTG AAG AAG TGG GC 3′). Primers ilvE-Δ1 and ilvE-Δ2 were designed so that they contain DNA sequences complementary to each other. This allowed the two PCR fragments to anneal to each other after mixing. The overlapping sequences were extended using *Taq* polymerase, followed by PCR amplification using the outer primers ilvE-N-term and P32-ilvE-*Sma*I. The PCR product (~0.8 kb) was digested with *Eco*RI and *Sma*I and ligated to similarly digested pKS Bluescript II (Stratagene, La Jolla, Calif.), resulting in pPSM1060. This plasmid was digested with *Kpn*I and *Bam*HI, and the *ilvE* gene, containing an internal deletion, was inserted into the *Kpn*I-*Bam*HI-digested integration vector pPSM1058 (see Fig. 3). The resulting plasmid, pPSM1064, was introduced into *S. carnosus* protoplasts, and selection was performed at 30°C on B-T agar plates supplemented with chloramphenicol. The resulting strain, PSM213, was grown for approximately 20 generations in selective B-T medium at the nonpermissive temperature of 40°C. After appropriate dilution, the culture was plated on B-T agar plates containing chloramphenicol and incubated at 40°C. Colonies were subsequently screened for nuclease activity in an overlay assay essentially as described by Lachica et al. (11), except that 10 mM $CaCl_2$ and 0.1% DNA were used. A single clone (PSM215) that produced nuclease was inoculated in B-T medium containing KIC, KMV, and KIV and grown for approximately 50 generations at the permissive temperature of 30°C. The temperature was increased to 40°C for 10 generations to ensure loss of the excised integration vector. After appropriate dilution, the culture was spread on B-T agar plates containing the three α-keto acids and incubated overnight at 30°C. By replica plating to B-T agar plates supplemented with chloramphenicol and the three α-keto acids, a single chloramphenicol-sensitive clone was identified, which was named PSM217. PCR and DNA sequencing verified the presence of an internal deletion in the *ilvE* gene in the genome of strain PSM217.

Isolation of total RNA and Northern blotting. Total RNA was extracted from *S. carnosus* using the RNeasy Midi kit from Qiagen GmbH (Hilden, Germany) and modified as described by Bassias and Brückner (2). Ten micrograms of total RNA was separated on a 1% (wt/vol) agarose–0.66 M formaldehyde gel. The RNA was transferred to a membrane and baked for 2 h at 80°C. Prehybridization, hybridization with a radioactive DNA probe covering the *ilvE* gene, and washing conditions were as previously described (13). The *ilvE* probe was obtained by PCR using chromosomal DNA from *S. carnosus* as template and primers P27-ilvE (5′ GCA CCG ATC GAA CTA GAT CCA GCT GCA C 3′) and P32-ilvE-*Sma*I. Primer extension and labeling were done as recommended by the manufacturer of the Primer Extension System-AMV Reverse Transcriptase kit (Promega, Madison, Wis.). The oligonucleotide ilvE-PE 2 (5′ GCC AAC CGC CTT TTT CGC TAT CGT AGT C 3′) was used to prime cDNA synthesis. The primer extension product was analyzed on a denaturing 6% polyacrylamide gel by running it next to a sequencing ladder made by using the same primer and plasmid pPRA26 as the sequencing template.

Amino acid catabolism. The amino acid catabolism of *S. carnosus* and the *ilvE* mutant (PSM217) was examined by incubating whole cells in a reaction mixture containing 0.1 M phosphate buffer (pH 6.8), 20 mM α-ketoglutarate, 0.2 mM pyridoxal 5′-phosphate, and phenylalanine, tyrosine, tryptophan, methionine, leucine, valine, or isoleucine at a final concentration of 150 mg/liter. *S. carnosus* and the *ilvE* mutant were grown in a B-T medium containing KIC, KMV, and KIV. Cells were harvested in the late exponential phase by centrifugation (10,000 × *g* at 4°C), washed in a 0.9% sterile NaCl solution, and resuspended in the reaction mixture to a final optical density at 600 nm (OD_{600}) of ~12. Five-milliliter aliquots were transferred to 12-ml tubes and supplemented with the relevant amino acid. The tubes were incubated at 30°C with shaking at 250 rpm. One-milliliter aliquots were collected after 0 and 24 h of incubation. Cells were removed by centrifugation (20,000 × *g* at 4°C), and the supernatants were analyzed for the amount of the amino acid of interest by high-performance liquid chromatography (HPLC) as described below. The amount of the methyl-branched acids 2-methylpropanoic acid, 2-methylbutanoic acid, and 3-methylbutanoic acid, which are the major metabolites from the catabolism of valine, isoleucine, and leucine, respectively, was analyzed according to the levels of their corresponding methylesters by static headspace gas chromatography as previously described (3).

HPLC analysis. The amount of amino acids in the supernatant was analyzed by ion exchange HPLC. The equipment consisted of a BioLC pump, an ED50 electrochemical detector, and an AS50 auto sampler (Dionex, Austin, Tex.). The separation was performed on an AminoPAC PA 10 column (Dionex) equilibrated with a mixture of 76% deionized water and 24% 250 mM NaOH. The flow rate was 0.25 ml/min, and the volume injected was 25 μl. Amino acids were eluted with a linear gradient as described by Dionex (technical note # 50). The relevant amino acids (leucine, valine, isoleucine, methionine, phenylalanine, tryptophan, and tyrosine) were quantified by comparison with external standard curves made from a mixture of standards. Norleucine was used as internal standard.

Nucleotide accession number. The nucleotide sequence described here has been deposited in the EMBL database under accession number AJ279090.

RESULTS

Cloning and identification of ilvE in *S. carnosus*. Alignment of the protein sequences encoding BCAA aminotransferases (IlvE proteins) from *S. aureus*, *Bacillus subtilis*, *Haemophilus influenzae*, *Mycobacterium tuberculosis*, *Streptomyces coelicolor*, *Helicobacter pylori*, and *E. coli* showed several regions of high amino acid identity. Based on two highly conserved regions (region 1, encoding FEGLKA, and region 2, encoding GTAAVI), degenerate oligonucleotides were designed and used to PCR amplify an internal 0.7-kb fragment of the *ilvE* gene from the *S. carnosus* genome. The DNA sequence of this fragment was determined and used to design new primers, which allowed cloning of the remaining part of the *ilvE* gene by inverse PCR. Analysis of the deduced amino acid sequence revealed an open reading frame encoding a 359-amino acid polypeptide with homology to BCAA aminotransferases from other bacteria. BlastP searches showed that the protein was 80% identical to IlvE of *S. aureus*. Furthermore, the *S. carnosus* IlvE contained an amino acid motif that matched the PROSITE aminotransferase class-IV pyridoxal 5′-phosphate binding site (http://www.expasy.org/prosite), suggesting the presence of a pyridoxal 5′-phosphate-dependent aminotransferase. Analysis of the DNA sequence showed a consensus promoter region containing an extended −10 region (TGC-TATAAT) and a −35 region (TTGAAT) located upstream of the coding sequence of *ilvE*. A putative *rho*-independent transcription terminator with a ΔG of ~22 kcal/mol was found downstream of the *ilvE* coding sequence. Analysis of the DNA

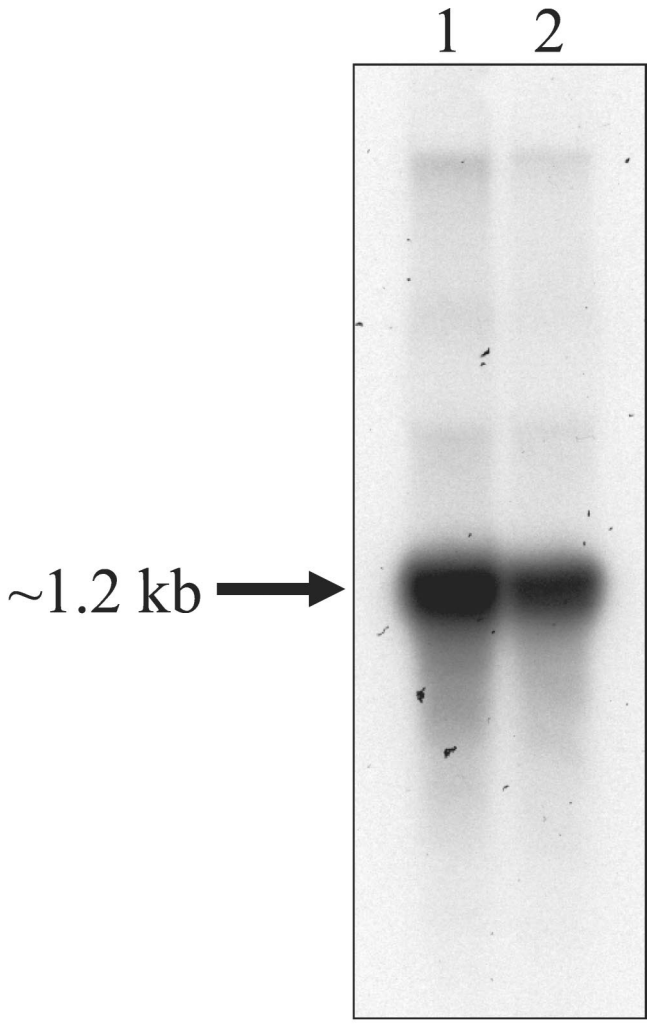

FIG. 2. Analysis of *ilvE* transcription in rich medium by Northern blot hybridization. Lanes: 1, total RNA from *S. carnosus*, isolated at an OD_{600} of ~1.3; 2, total RNA from *S. carnosus*, isolated at an OD_{600} of ~2.5. The probe covered an internal part of the *ilvE* gene.

sequence surrounding the *ilvE* gene showed no other genes involved in amino acid metabolism.

Using Northern blotting, a 1.2-kb transcript was identified both in the exponential growth phase and in the stationary phase (Fig. 2). This shows that *ilvE* was transcribed as a monocistronic mRNA, which agrees with the presence of a putative promoter upstream and a putative transcriptional terminator downstream of the coding sequence of *ilvE*. Primer extension analysis was used to map the 5′ end of the *ilvE* transcript to a G located 7 bp downstream of the −10 sequence (data not shown).

Development of a new integration vector and its use for construction of an *ilvE* mutant strain. Two conditionally active delivery vectors, pBT1 and pBT2, based on the temperature-sensitive replicon of pE194, have been developed for gene inactivation in *S. carnosus* and *S. xylosus* by Brückner (6). To facilitate identification of homologous recombination events in *S. carnosus* and *S. xylosus* leading to gene inactivation and to avoid construction of antibiotic-resistant mutant strains, one of these vectors, pBT2, was further developed in this work. A promoterless nuclease gene from *S. aureus* was cloned into pBT2, resulting in the new integration vector pPSM1058 (Fig. 3). During growth at the permissive temperature, pPSM1058 will exist as a freely replicating plasmid in *S. carnosus*. Furthermore, when gene fragments without promoter activity are cloned into the multiple cloning site of pPSM1058, the recombinant cells will not produce nuclease when maintained at the permissive temperature. However, plasmid replication ceases upon shift to the nonpermissive temperature and clones with a plasmid integrated through the homologous gene sequences present in the resident chromosome can be selected on chloramphenicol plates. In this case, the recombination event led to a transcriptional fusion of the nuclease reporter gene to the chromosomal promoter, located upstream of the *ilvE* gene. This resulted in the nuclease-producing strain PSM215, which was identified using a chromogenic substrate in an overlay plate assay. The expected homologous recombination was verified by Southern blot analysis (data not shown). Because it was shown that the homologous recombination event had occurred through fragment C, strain PSM215 was still wild type with respect to IlvE activity (Fig. 3D). An *ilvE* deletion mutant was subsequently obtained by growing PSM215 in rich medium at the permissive temperature without antibiotic selection but in the presence of the three α-keto acids. This allowed recombination between the two homologous A fragments present in strain PSM215, resulting in the *ilvE* mutant strain PSM217.

Characterization of the *ilvE* mutant strain and the transamination of BCAAs in *S. carnosus*. The role of IlvE in the catabolism of amino acids was studied by comparing the amino acid degradation by whole cells from *S. carnosus* and from the isogenic *ilvE* mutant strain PSM217. Cells were incubated in the reaction mixture containing α-ketoglutarate, pyridoxal 5′-phosphate, and the relevant amino acid. Samples were collected after 24 h of incubation, and the remaining amount of the added amino acid was determined by HPLC. The wild-type strain showed 75 to 95% degradation of the BCAAs, leucine, isoleucine, and valine, whereas only 2 to 4% percent degradation was observed in the *ilvE* mutant strain (Fig. 4). The degradation of phenylalanine and tryptophan was only slightly affected by *ilvE* inactivation, while degradation of methionine decreased from 88% in the wild-type strain to 50% in the *ilvE* mutant strain. Tyrosine degradation was similar in the two strains. We also found that the wild-type strain converted leucine, isoleucine, and valine stoichiometrically into 3-methylbutanoic acid, 2-methylbutanoic acid, and 2-methylpropanoic acid, respectively. The amounts of methyl-branched carboxy acids detected in the mutant strain were approximately 100-fold lower than in the wild-type strain (Table 2).

DISCUSSION

S. carnosus and *S. xylosus* are important starter cultures for production of essential flavor compounds, such as aldehydes, alcohols, esters, and carboxy acids in dried fermented sausages. Many of these compounds are derived from degradation of amino acids and provide the product with its unique taste and aroma. To our knowledge, no staphylococcal gene or enzyme involved in amino acid metabolism has yet been characterized at the genetic or biochemical level, although several similar

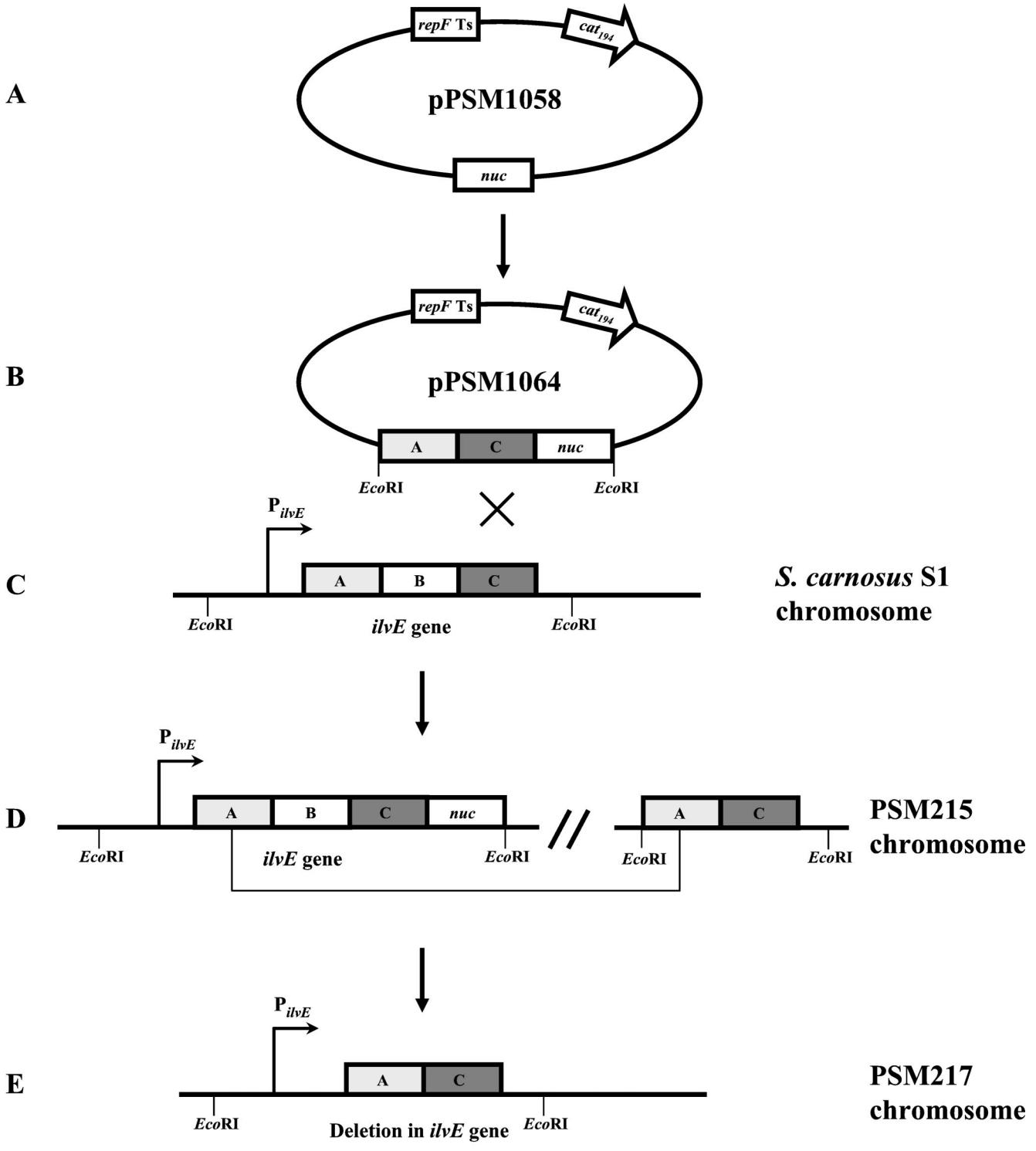

FIG. 3. Construction of an *ilvE* deletion mutant. (A) Plasmid pPSM1058 is a modified pBT2 integration vector and contains a promoterless nuclease reporter gene (*nuc*). The temperature-sensitive replicon from plasmid pE194 (*repF* Ts) and the chloramphenicol acetyltransferase gene from pC194 (cat_{194}) are also indicated. (B) The *ilvE* gene, containing an internal deletion, was inserted into pPSM1058, resulting in pPSM1064. The disrupted *ilvE* gene is divided into two parts, represented by fragments A and C for illustrative purposes. (C) Chromosomal structure of the *ilvE* gene from *S. carnosus*. The complete *ilvE* gene is divided into three parts, A, B, and C. (D) A single-crossover event between the homologous C fragments present in pPSM1064 and the *S. carnosus* S1 chromosome resulted in strain PSM215. Only part of the integrated plasmid structure is indicated. (E) A second crossover event between the two homologous A fragments present in the chromosome of strain PSM215 resulted in the *ilvE* deletion strain PSM217. The figure is not drawn to scale.

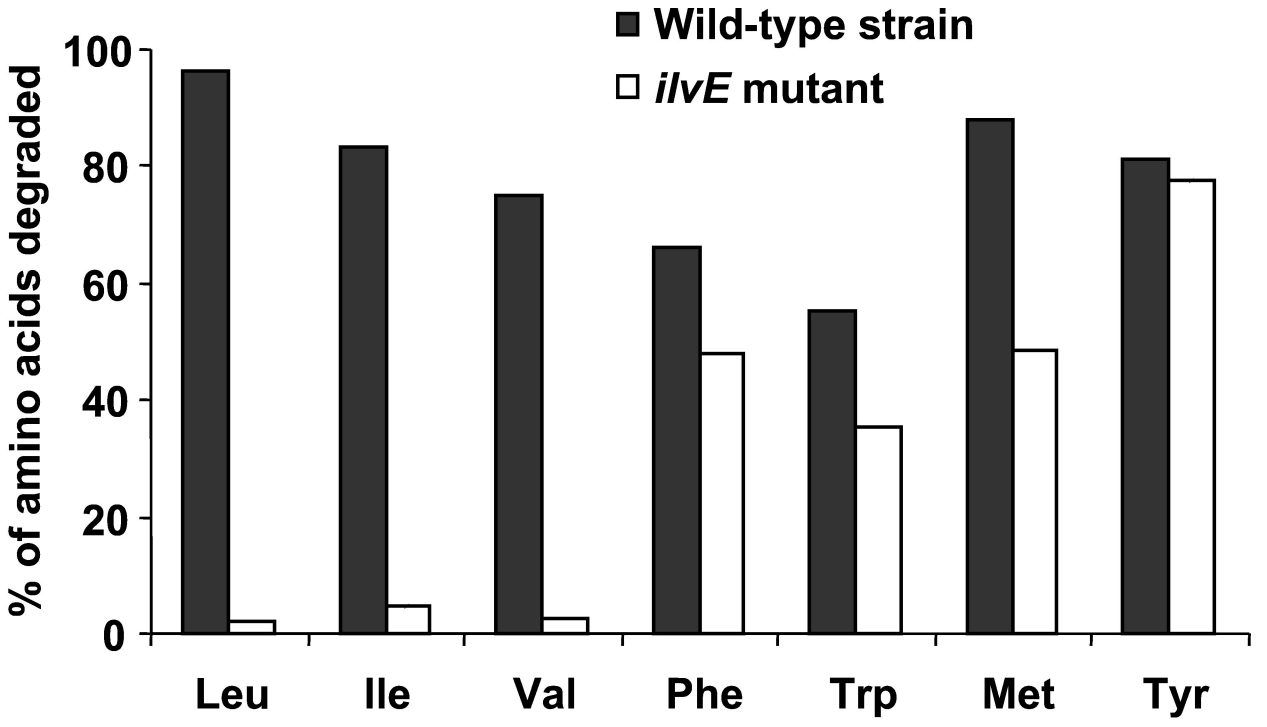

FIG. 4. Amounts of amino acids (in percentages) degraded by whole cells of *S. carnosus* and the isogenic *ilvE* mutant after incubation for 24 h in the reaction mixture containing the indicated amino acid, α-ketoglutarate, and pyridoxal 5′-phosphate. The amino acids studied were leucine (Leu), isoleucine (Ile), valine (Val), phenylalanine (Phe), tryptophan (Trp), methionine (Met), and tyrosine (Tyr). Values are means of duplicate determinations. Differences between samples were ≤6%.

genes and enzymes have been isolated and characterized in lactic acid bacteria. The knowledge obtained on lactococcal amino acid degradation has provided new opportunities for intensifying, directing, and controlling aroma formation in cheese, and similar approaches might also be applied to such meat starter cultures as *S. carnosus* and *S. xylosus*. Recently, it was reported that key sausage aroma compounds like 3-methylbutanal and 3-methylbutanoic acid are derived from catabolism of leucine (12, 15, 25). In many bacteria, the first degradation step towards these compounds is likely catalyzed by leucine dehydrogenase, leucine oxidase, or BCAA aminotransferase (14), while in *L. lactis*, only aminotransferases seem to be responsible for isoleucine, valine, and leucine catabolism (1, 20, 27, 28, 29). The aim of this study was to provide genetic and physiological evidence of the role of the IlvE enzyme in the degradation of BCAAs in *S. carnosus*.

We used degenerate DNA primers and inverse PCR to clone the complete *ilvE* gene from *S. carnosus*. The deduced amino acid sequence of IlvE showed highest identity to the IlvE proteins of *S. aureus*, *B. subtilis,* and *Bacillus halodurans*, all of which belong to the class IV family of pyridoxal 5′-phosphate-dependent aminotransferases.

TABLE 2. Percentages of Leu, Ile, and Val degraded to methyl-branched acids by whole cells from *S. carnosus* and the isogenic *ilvE* mutant[a]

Product	% Degradation by:	
	Wild type	*ilvE* mutant
3-Methylbutanoic acid	112	1.3
2-Methylbutanoic acid	76	<1
2-Methylpropanoic acid	89	<1

[a] Values are means of duplicate determinations. Differences between samples were ≤6%.

Transcriptional analysis by Northern blot hybridization showed constitutive expression of *ilvE* in rich medium. However, to reach firm conclusions on the regulation of *ilvE* expression, comprehensive studies in a chemically defined medium are required. Such studies were actually done in *L. lactis*, using a transcriptional fusion of the *ilvE* promoter to the *luxAB* reporter genes. The results showed that free BCAAs repress *ilvE* transcription (27). In the present study, a new reporter gene system, equally useful for promoter regulation studies, was developed and used for construction of an *ilvE* mutant. However, due to low expression levels, we found that the reporter construction needs to be optimized in order to analyze the regulation of the *ilvE* promoter. This optimization is presently in progress.

Previously, an efficient gene replacement system, pBT2, based on a temperature-sensitive replicon, has been developed for use in *S. carnosus* and *S. xylosus* (6). In this system, a chloramphenicol acetyltransferase gene (cat_{194}) is used for plasmid selection in staphylococci and a gene conferring erythromycin resistance (*ermB*) is used for recognition of gene inactivation. The use of this system results in a chromosomal structure in which the target gene is disrupted by the *ermB* gene cassette. However, we were unable to use this system directly, due to a background of erythromycin resistance in *S.*

carnosus. In addition, we wanted to develop a gene inactivation system which yields food-grade strains, harboring no antibiotic resistance genes in their chromosomes. To achieve this, the existing delivery vector and protocol were modified. First, an *ilvE* fragment containing an internal deletion was cloned directly into pBT2, thereby avoiding the use of the erythromycin resistance gene. However, efforts to force plasmid integration by single-crossover recombination using temperature shifts and chloramphenicol selection were unsuccessful, as all analyzed chloramphenicol-resistant colonies contained plasmid DNA. To detect homologous recombination into the chromosome, we therefore designed a new integration system, based on expression of a nuclease reporter and a plate assay. Using this system, we achieved a single-crossover recombination between the *ilvE* sequences present in the delivery vector and in the genome of *S. carnosus*. The food-grade *ilvE* deletion mutant was obtained by a second crossover event, demonstrating the usefulness of the system.

Although the importance of the *ilvE* gene product in the catabolism of amino acids into important flavor compounds is obvious, the physiological role of BCAA catabolism in *S. carnosus* is still unclear. We were not able to isolate *ilvE* mutants in rich medium without added α-keto acids, indicating that the α-keto acids are needed for optimal growth. Actually, the doubling time of the wild-type strain was 45 min, compared to 85 min for the *ilvE* mutant strain when grown without the three α-keto acids, showing that degradation of BCAAs is an important but not vital property of *S. carnosus*. The growth rate in the mutant strain was restored by addition of the three α-keto acids to the growth medium. In other organisms, the products of BCAA aminotransferases are used in the formation of fatty acids for cell membrane synthesis (9), in the biosynthesis of pantothenic acid (5), or in siderophore production (10). Ongoing work in our laboratories is aimed at providing evidence for the biological role of catabolism of BCAAs in *S. carnosus*.

We found that inactivation of the *ilvE* gene resulted in complete inhibition of the catabolism of Leu, Ile, and Val and in a reduction of Met, Trp, and Phe conversion. In *L. lactis*, inactivation of *ilvE* significantly reduces the catabolism of Ile and Val but does not affect the catabolism of Leu, Phe, Trp, Tyr, and Met (27). Interestingly, the BCAA aminotransferase of *S. carnosus* catabolized leucine much more efficiently than the corresponding enzyme in *L. lactis* (27). This dissimilarity in efficiency could be caused by the difference between the two IlvE proteins, which are only 38% identical.

Catabolism of the methyl-branched amino acids, leucine, isoleucine, and valine, leads to production of the important methyl-branched flavor aldehydes, 3-methylbutanal, 2-methylbutanal, and 2-methylpropanal, respectively. Leucine catabolism is of particular interest, since Leu is degraded to 3-methylbutanal, which is essential for the flavor of fermented sausages (4, 22). Larrouture et al. (12) concluded that both a transamination and an oxidative deamination reaction are involved in the catabolism of leucine in *S. carnosus*. In contrast, our results clearly showed that IlvE was the only enzyme that catalyzed the deamination of Leu, Ile, and Val in this organism. This conclusion is based on the observations that the *ilvE* mutant was unable to catabolize BCAAs and that the methyl-branched acids, 3-methylbutanoic acid, 2-methylbutanoic acid, and 2-methylpropanoic acid, which are the major products of BCAA catabolism, were not formed. A likely explanation for these contradictory results is that the intracellular pool of pyridoxal 5′-phosphate and α-ketoglutarate in resting *S. carnosus* cells is sufficient to catabolize BCAA.

In the present study, we characterized the *ilvE* gene from *S. carnosus* and constructed an *ilvE* mutant. This mutant strain could be useful in applied studies on model sausage minces, thereby increasing our understanding of BCAA catabolism in flavor development. In addition, knowledge about the genes involved in amino acid catabolism and how these genes are regulated could lead to better control of aroma formation in fermented meat products. We are presently working on the identification and characterization of other genes involved in BCAA catabolism in *S. carnosus*.

ACKNOWLEDGMENTS

This work was financed in part by The Danish Directorate for Development; Ministry of Food, Agriculture, and Fisheries (project BIOT 99-1).

We thank R. Brückner and Wisby Starter Cultures and Media for providing plasmid pBT2 and *S. carnosus* BioCarna Ferment S1, respectively; L. H. Stahnke and P. T. Olesen for important discussions; P. Smith and E. H. Hansen for excellent technical assistance; and B. Albrechtsen for critical reading of the manuscript.

REFERENCES

1. **Atiles, M. W., E. G. Dudley, and J. L. Steele.** 2000. Gene cloning, sequencing, and inactivation of the branched-chain aminotransferase of *Lactococcus lactis* LM0230. Appl. Environ. Microbiol. **66:**2325–2329.
2. **Bassias, J., and R. Brückner.** 1998. Regulation of lactose utilization genes in *Staphylococcus xylosus*. J. Bacteriol. **180:**2273–2279.
3. **Beck, H. C., A. M. Hansen, and F. R. Lauritsen.** 2002. Metabolite production and kinetics of branched-chain aldehyde oxidation in *Staphylococcus xylosus*. Enzyme Microb. Technol. **31:**94–101.
4. **Bérdague, J. L., P. Monteil, M. C. Montel, and R. Talon.** 1993. Effect of starter cultures on the formation of flavor compounds in dry sausage. Meat Sci. **35:**275–287.
5. **Brown, G. M., and J. M. Williamson.** 1987. Biosynthesis of folic acid, riboflavin, thiamine, and pantothenic acid, p. 521–538. *In* F. C. Neidhardt et al. (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
6. **Brückner, R.** 1997. Gene replacement in *Staphylococcus carnosus* and *Staphylococcus xylosus*. FEMS Microbiol. Lett. **151:**1–8.

6a. **Don, R. H., P. T. Cox, B. J. Wainwright, K. Baker, and J. S. Mattick.** 1991. 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res. **19:**4008.

7. **Götz, F., and B. Schumacher.** 1987. Improvements of protoplast transformation in *Staphylococcus carnosus*. FEMS Microbiol. Lett. **40:**285–288.
8. **Johansen, E., and A. Kibenich.** 1992. Characterization of *Leuconostoc* isolates from commercial mixed strain mesophilic starter cultures. J. Dairy Sci. **75:**1186–1191.
9. **Kaneda, T.** 1991. Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. Microbiol. Rev. **55:**288–302.
10. **Kingsley, R., W. Rabsch, M. Roberts, R. Reissbrodt, and P. H. Williams.** 1996. TonB-dependent iron supply in *Salmonella* by α-keto acids and α-hydroxyacids. FEMS Microbiol. Lett. **140:**65–70.
11. **Lachica, R. V. F., C. Genigeorgis, and P. D. Hoeprich.** 1971. Metachromic agar-diffusion methods for detecting staphylococcal nuclease activity. Appl. Microbiol. **21:**585–587.
12. **Larrouture, C., V. Ardaillon, M. Pépin, and M. C. Montel.** 2000. Ability of meat starter cultures to catabolize leucine and evaluation of the degradation products by using an HPLC method. Food Microbiol. **17:**563–570.
13. **Madsen, S. M., B. Albrechtsen, E. B. Hansen, and H. Israelsen.** 1996. Cloning and transcriptional analysis of two threonine biosynthetic genes from *Lactococcus lactis* MG1614. J. Bacteriol. **178:**3689–3694.
14. **Massey, L. K., J. R. Sokatch, and R. S. Conrad.** 1976. Branched-chain amino acid catabolism in bacteria. Bacteriol. Rev. **40:**42–54.
15. **Masson, F., L. Hinrichsen, R. Talon, and M. C. Montel.** 1999. Factors influencing leucine catabolism by a strain of *Staphylococcus carnosus*. Int. J. Food Microbiol. **49:**173–178.
16. **Montel, M. C., F. Masson, and R. Talon.** 1998. Bacterial role in flavour development. Meat Sci. **49:**111–123.

17. **Ochman, H., A. S. Gerber, and D. L. Hartl.** 1988. Genetic applications of an inverse polymerase chain reaction. Genetics **120:**621–623.
18. **Ordóñez, J. A., E. M. Hierro, J. M. Bruna, and L. de la Hoz.** 1999. Changes in the components of dry-fermented sausages during ripening. Crit. Rev. Food Sci. Nutr. **39:**329–367.
19. **O'Sullivan, D. J., and T. R. Klaenhammer.** 1993. Rapid mini-prep isolation of high-quality plasmid DNA from *Lactococcus* and *Lactobacillus* spp. Appl. Environ. Microbiol. **59:**2730–2733.
20. **Rijnen, L., S. Bonneau, and M. Yvon.** 1999. Genetic characterization of the major lactococcal aromatic aminotransferase and its involvement in conversion of amino acids to aroma compounds. Appl. Environ. Microbiol. **65:** 4873–4880.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Stahnke, L. H.** 1995. Dried sausages fermented with *Staphylococcus xylosus* at different temperatures and with different ingredient levels—part III. Sensory Evaluation. Meat Sci. **41:**211–223.
23. **Stahnke, L. H.** 1999. Volatile produced by *Staphylococcus xylosus* and *Staphylococcus carnosus* during growth in sausage minces. Part I. Collection and identification. Lebensm.-Wiss. Technol. **32:**357–364.
24. **Sugawara, Y., and S. Sakaki.** 1977. Purification and properties of aldehyde dehydrogenase from *Proteus vulgaris*. Biochim. Biophys. Acta **480:**343–350.
25. **Vergnais, L., F. Masson, M. C. Montel, J-L. Berdagué, and R. Talon.** 1998. Evaluation of solid-phase microextraction for analysis of volatile metabolites produced by *Staphylococci.* J. Agric. Food Chem. **46:**228–234.
26. **Ward, D. E., R. P. Ross, C. C. van der Weijden, J. L. Snoep, and A. Claiborne.** 1999. Catabolism of branched-chain α-keto acids in *Enterococcus faecalis*: the *bkd* gene cluster, enzymes, and metabolic route. J. Bacteriol. **181:**5433–5442.
27. **Yvon, M., E. Chambellon, A. Bolotin, and F. Roudot-Algaron.** 2000. Characterization and role of the branched-chain aminotransferase (BcaT) isolated from *Lactococcus lactis* subsp. *cremoris* NCDO 763. Appl. Environ. Microbiol. **66:**571–577.
28. **Yvon, M., and L. Rijnen.** 2001. Cheese flavor formation by amino acid catabolism. Int. Dairy J. **11:**185–201.
29. **Yvon, M., S. Thirouin, L. Rijnen, D. Fromentier, and J. C. Gripon.** 1997. An aminotransferase from *Lactococcus lactis* initiates conversion of amino acids to cheese flavor compounds. Appl. Environ. Microbiol. **63:**414–419.