Identification, Cloning, and Characterization of a *Lactococcus lactis* Branched-Chain α-Keto Acid Decarboxylase Involved in Flavor Formation

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The biochemical pathway for formation of branched-chain aldehydes, which are important flavor compounds derived from proteins in fermented dairy products, consists of a protease, peptidases, a transaminase, and a branched-chain α-keto acid decarboxylase (KdcA). The activity of the latter enzyme has been found only in a limited number of *Lactococcus lactis* strains. By using a random mutagenesis approach, the gene encoding KdcA in *L. lactis* B1157 was identified. The gene for this enzyme is highly homologous to the gene annotated *ipd*, which encodes a putative indole pyruvate decarboxylase, in *L. lactis* IL1403. Strain IL1403 does not produce KdcA, which could be explained by a 270-nucleotide deletion at the 3′ terminus of the *ipd* gene encoding a truncated nonfunctional decarboxylase. The *kdcA* gene was overexpressed in *L. lactis* for further characterization of the decarboxylase enzyme. Of all of the potential substrates tested, the highest activity was observed with branched-chain α-keto acids. Moreover, the enzyme activity was hardly affected by high salinity, and optimal activity was found at pH 6.3, indicating that the enzyme might be active under cheese ripening conditions.

Flavor formation in cheeses is mainly the result of catabolism of milk proteins, sugar, and lipids. Degradation of proteins into amino acids followed by conversion of these amino acids by *Lactococcus* spp. is very important for flavor formation in semihard types of cheese, like Gouda (for reviews see references 12, 25, 38, and 43). One of the predominant amino acid conversion routes is initiated by transaminases. Transamination of amino acids yields the corresponding α-keto acids, which do not have strong flavor properties (12, 13) but are the precursors of various aldehydes, alcohols, acids, and (thio-)esters. The latter compounds generally have much lower odor thresholds and strongly contribute to cheese flavor.

This is exemplified by the production of flavor compounds from leucine. Transamination of leucine results in the production of α-isocaproic acid (KICA). Interestingly, only a limited number of *Lactococcus lactis* strains are capable of decarboxylating this α-keto acid to 3-methylbutanal (36, 37). The decarboxylating activity is observed mainly in *L. lactis* strains with nondairy origins, whereas the industrial strains tested hardly showed this activity (36). Subsequently, (de)hydrogenation of 3-methylbutanal results in 3-methylbutanol or 3-methylbutyric acid, which also contributes to the flavor of cheese, but the odor thresholds for these compounds are higher than the odor threshold for 3-methylbutanal (24, 29). The rate-determining step in this route is the decarboxylation of KICA, and therefore, control of this enzyme offers good prospects for the design of starter cultures with tailored flavor production (37, 43).

The enzyme performing this reaction has not been identified. A number of thiamine pyrophosphate (TPP)-dependent α-keto acid decarboxylases have been identified in various organisms, and these enzymes include pyruvate decarboxylase (EC 4.1.1.1) (34), phenylpyruvate decarboxylase (EC 4.1.1.43) (4, 40), branched-chain 2-oxoacid decarboxylase (EC 4.1.1.72) (27), 2-oxoglutarate decarboxylase (EC 4.1.1.71) (28, 33), and indole-3-pyruvate decarboxylase (IPD) (EC 4.1.1.74) (18). In *L. lactis*, however, there has been only one report of partial purification of an α-keto acid decarboxylase (3), and in the genome of *L. lactis* IL1403 (8) a putative (partial) indole-3-pyruvate decarboxylase gene and a 2-oxoglutarate decarboxylase gene have been identified but pyruvate decarboxylase and branched-chain keto acid decarboxylase genes are absent. In the present paper we describe the identification and cloning of the gene encoding the branched-chain α-keto acid decarboxylase (*kdcA*) responsible for KICA conversion in *L. lactis* B1157. This gene was overexpressed in *L. lactis* by using the nisin-controlled overexpression system, and the gene product was characterized.

MATERIALS AND METHODS

Strains, plasmids, and media. All strains and plasmids used or constructed in this study are listed in Table 1. Lactococcal strains were grown in M17 broth (Difco, Detroit, Mich.) supplemented with lactose (LM17) or glucose (GM17) as an additional carbon source at a final concentration of 0.5% (wt/vol). If appropriate, chloramphenicol or erythromycin was added to the medium to a final concentration of 5 μg/ml. Strains were grown at either 28 or 40°C. The ability of strains to produce 3-methylbutanal was determined by static headspace gas chromatography (37) analysis after 24 h of growth (optical density at 600 nm, ~1.8) in the presence or absence of 2 mM KICA in 10-ml HS vials containing 3 ml of culture medium.

Escherichia coli was grown aerobically at 30°C on TY medium (Difco), which contained 200 μg of erythromycin per ml if appropriate.

DNA handling procedures. Before the protocols used for DNA isolation were performed, *L. lactis* cells were pretreated for 10 min with 2 mg of lysozyme per ml in THMS buffer at 37°C. Plasmid DNA was isolated with a Micro plasmid prep kit (Amersham/Pharmacia, Uppsala, Sweden), and total DNA was isolated with an AquaPure genomic DNA isolation kit (Bio-Rad, Veenendaal, The Neth-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Antibiotic resistance[a]	Reference(s) or source
Strains			
MC1061	*E. coli*		9
B1157	Branched-chain α-keto acid decarboxylase-positive *L. lactis* strain	Tct, Cm	5
NZ9000	MG1363 derivative; *pepN::nisRK*; standard host for NICE[b]		20
MG1363	Plasmid free, wild-type *L. lactis* strain, genome sequenced		14
IL1403	Wild-type *L. lactis* strain, genome sequenced		8
B2083	pGh9:ISSI integrated in the chromosome of B1157 at position 462 of the decarboxylase gene	Tet, Ery, Cm	This study
B2084	pGh9:ISS1 integrated in the chromosome of B1157 at position 995 of the decarboxylase gene	Tet, Ery, Cm	This study
B2085	NZ9000 (pNZ7500)	Ery	This study
Plasmids			
pGh9:ISS1	pWV01 carrying a thermosensitive replicon	Ery	22, 23
pNZ8148	Homologous *L. lactis* vector for NICE system	Cm	20
pNZ7500	pNZ8148 derivative containing a 1.8-kb fragment carrying the decarboxylase gene of B1157	Cm	This study
pNZ7501	pNZ8148 derivative containing a 1.5-kb fragment carrying the partial decarboxylase gene of B1157	Cm	This study
pNZ7502	pNZ8148 derivative containing a 1.4-kb fragment carrying the decarboxylase gene of IL1403 annotated as *ipd*	Cm	This study

[a] Tet, tetracycline, Cm, chloramphenicol; Ery, erythromycin.
[b] NICE, nisin-controlled expression.

erlands) used according to the manufacturer's protocols. *L. lactis* cells were transformed by electroporation as described previously (41). Agarose gel electrophoresis was performed as described by Sambrook et al. (30). DNA fragments were isolated from agarose gels by using a gel purification kit (Jetstar; Genomed, Bad Oeynhausen, Germany). Restriction enzymes, T4 ligase, and other DNA-modifying enzymes were purchased from Promega Corp. (Leiden, The Netherlands) or Invitrogen (Carlsbad, Calif.) and were used as recommended by the manufacturer. PCR was performed by using a *Pwo* polymerase PCR kit (Roche, Mannheim, Germany) and the primers listed in Table 2 with total DNA preparations as templates. Twenty-six thermal cycles with a Mastercycler (Eppendorf, Hamburg, Germany) were used with the 50-μl reaction mixtures. Each cycle started with 30 s at 95°C, which was followed by annealing for 30 s at 55°C and elongation for 3.5 min at 72°C. Sequencing was performed with a Thermo Sequenase Cy5.0 Dye terminator cycle sequencing kit (Amersham Pharmacia), and separation was performed with an Alf sequencer (Amersham Pharmacia).

Construction of a random mutant library. A random insertion mutant library was constructed by using the thermosensitive plasmid pGh9:ISS1 as described by Maguin et al. (22, 23). The pGh9:ISS1 plasmid was isolated from *E. coli*(pGh9:ISS1) and transformed into *L. lactis* B1157. Erythromycin-resistant, plasmid-containing transformants were selected at a permissive temperature, 28°C. Twenty-five milliliters of LM17 without erythromycin was inoculated (1%) with B1157(pGh9:ISS1), and the organism was grown at 28°C for 2.5 h; this was followed by transfer of the cultures to a nonpermissive temperature, 40°C, for 2.5 h to force random plasmid integration on the genome of strain B1157. More than 8,500 integrants were selected on LM17 agar containing 2 μg of erythromycin per ml; individual colonies were organized in 384-well microtiter plates containing LM17 with erythromycin and 20% glycerol, and the plates were frozen at −80°C. It was calculated that a library consisting of 8,000 mutants was needed so that 95% of the genes were represented by an integrant, assuming random insertion into the genome, a genome size of 2.3 Mb, and an average gene size of 880 kb (8). The frequency of integration was determined to be 2.4%, and the library was analyzed for the presence of random integrants by Southern blotting. For this purpose total DNAs of 20 strains from the insertion sequence (IS) library were isolated and cut with either EcoRI or HindIII. The fragments obtained were separated on a 0.7% agarose gel, blotted, and hybridized with HindIII-cut, digoxigenin-labeled pGh9:ISS1 (Roche) as the probe. Eighty percent of the colonies gave fragments that were different sizes.

For determination of the site of integration in 3-methylbutanal-negative clones B2083 and B2084, total DNA was digested with HindIII or EcoRI and subsequently ligated by using T4 ligase. Ligation mixtures were used to transform *L. Lactis* MG1363 that was grown at the permissive temperature (28°C). Plasmid sequencing was performed by using the four pGh9:ISS1-derived primers (Table 2). The HR and HF primers were used for the plasmids derived from HindIII-digested total DNAs, and the ER and EF primers were used for the plasmids originating from the EcoRI-digested total DNAs.

Identification of decarboxylase knockouts. Decarboxylase-negative clones in the mutant library were identified by fast screening of volatile compounds produced in miniaturized fermentations as described previously (36). Briefly, the method is based on the growth of individual mutants in a 96-well stainless steel

block containing 2-ml wells filled with 1 ml of LM17 with 2 mM KICA. The block is closed airtight, and after fermentation for 24 h at exactly 40°C, individual headspace samples (100 μl) were analyzed by using direct inlet mass spectrometry (Trace-MS; Interscience, Breda, The Netherlands) at a throughput rate of one sample per minute (36). The amount of 3-methylbutanal present in the sample was calculated from the response at $m/z = 58$.

Overexpression of the decarboxylase gene. To further analyze the function of the decarboxylase gene products, plasmids were constructed for nisin-dependent overexpression of the *ipd* gene from IL1403 (1,374 bp), the *kdcA* gene from B1157 (1,644 bp plus 166 bp), and a truncated *kdcA* gene from B1157 (1,569 bp, missing 75 bp) (Fig. 1). The fragments were obtained by PCR by using two primer pairs, primers IL1403-10fw and IL1403+1374R and primers IL1403-10fw and B1157+1793Rev (Table 2), with total DNAs of *L. lactis* IL1403 and *L. lactis* B1157, respectively. Thirty thermal cycles with the following steps were used: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 120 s. The PCR products obtained were digested with Nco1-XbaI and cloned into pNZ8148, resulting in plasmids pNZ7502 (containing IL1403 *ipd*) and pNZ7500 (containing the B1157 decarboxylase gene). The PCR product obtained from B1157 was also digested with SpeI and NcoI and cloned into pNZ8148, generating pNZ7501 (containing the B1157 decarboxylase gene lacking 75 bp at the 3′ terminus). The identities and integrities of the inserts cloned in pNZ8148 were confirmed by sequencing. All plasmids were introduced into strain NZ9000 (20) and used for nisin-controlled overexpression of the cloned (partial) genes by previously established procedures (10).

Colony PCR. *L. lactis* strains B1021, B1152, B1156, B1157, B1158, B1165, B1231, B1233, B1235, B1236, B1275, B1492, B2085, B234, B25, B26, B27, B28,

TABLE 2. PCR primers used in this study

Primer	Sequence[a]
Nis_292Rev	5′ AATTGAACGTTTCAAGCC
Nis_115fw	5′ CGGCTCTGATTAAATTCTG
IL1403-10fw	5′ GGAATG**C**catg**G**ATACAGTAGGAGATTACC
IL1403+1374Rev	5′ AGAA<u>TCTAGA</u>TCAACTATAAGAGTTCATATGAG
B1157+3fw	5′ GTATACAGTAGGACATTACC
B1157+623fw	5′ CAGTAGTGATTGCAGGACAC
B1157+1303Rev	5′ GTTGAAGTGAACCATCACC
B1157+1633Rev	5′ GCTCAGCAAATAATTTACCC
B1157+1793Rev	5′ GACTTC<u>TCTAGA</u>TTATATCTTGCTGGTT
pGh9:ISS1_ER	5′ CCGTTAAATCGACTGGCG
pGh9:ISS1_EF	5′ GACTTATCAGAAAACTTTGC
pGh9:ISS1_HR	5′ CGGTATCTACTGAGATTAAGG
pGh9:ISS1_HF	5′ GCGCGCGTAATACGACTC

[a] Bases that mismatch are indicated by boldface type; the restriction sites used for cloning are underlined; and the start of a gene is indicated by lowercase letters.
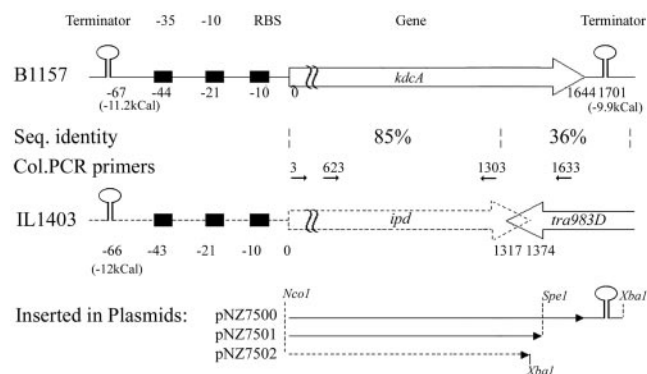
FIG. 1. Schematic representation of the DNA region carrying the *kdcA* gene in *L. lactis* B1157 and the *ipd* gene in strain IL1403. The fragments fused with pNZ8148 for overexpression in *L. lactis* NZ9000 are shown below the schematic diagrams. Between the two regions the sequence homologies (Seq. identity) and the positions of the PCR primers used for screening of *L. lactis* strains (Col.PCR primers) are indicated.

B31, B4, B40, B42, B48, B643, B697, B698, B700, B76, B77, B78, B78, B79, B80, B81, B83, IL1403, NZ9000(pNZ7502), NZ9000, and SK110, obtained from the NIZO culture collection, were grown overnight in 1 ml of GM17 broth. A 0.5-μl portion of a bacterial cell suspension was treated twice for 30 s in a microwave at 750 W. Four PCRs were performed for each strain by using the four possible combinations of primers B1157+3fw, B1157+623fw, B1157+1303Rev, and B1157+1633Rev (Table 2 and Fig. 1). Using these primers should have resulted in either four fragments (complete *kdcA* gene present), two fragments (truncated *kdcA* gene present), or no fragments (no *kdcA* gene present). Thirty thermal cycles with the following steps were used: denaturation at 95°C for 30s, annealing at 40°C for 30 s, and elongation at 72°C for 120 s.

Overproduction of decarboxylases. For overproduction of decarboxylases, an overnight culture of *L. lactis* NZ9000 harboring either pNZ7500, pNZ7501, or pNZ7502 was transferred (1%) to fresh medium (GM17) and grown until an optical density at 600 nm of 0.5 was reached. The cells were induced with 0.2 or 2 ng of nisin ml^{-1} and grown for another 2 h. The cells were then harvested, and the cell extracts (CFEs) were prepared as described below. These extracts were used for the enzymatic assays and protein analysis.

Preparation of CFEs. Cells were harvested by centrifugation and washed twice in 25 mM sodium citrate buffer (pH 6.5). The cells were disrupted by the Fast Prep method (Bio 101, Savant, Holbrook, N.Y.) twice for 30 s. The supernatant after centrifugation at $20,000 \times g$ for 15 min was used as the CFE. All steps were performed at temperatures below 6°C.

Protein analysis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with a 12% polyacrylamide gel in a mini Protean II system (Bio-Rad) was used to analyze the production of the protein. Protein concentrations were determined in triplicate by using a BCA protein assay reagent kit obtained from Pierce Inc. (Rockford, Ill.). A 30-μl sample was mixed with 240 μl of work reagent. After incubation at 37°C for 20 min, the optical density at 562 nm was measured with a Spectra max (Molecular Devices, Sunnydale, Calif.). Triplicate measurements were obtained independently.

KdcA assay. The α-keto acid decarboxylase assay with KICA was performed as described previously (37). In essence, an enzyme sample was incubated for 3 h at 30°C in a closed 10-ml HS vial at pH 6.3 (50 mM sodium citrate) with 10 mM KICA as the substrate and with 1 mM MgCl$_2$ and 0.1 mM TPP as cofactors. After 3 h the reactions were quenched by lowering the pH to between 2 and 3 with 6 N HCl. The production of 3-methylbutanal was quantified by static headspace gas chromatography as described previously (37). Gas chromatography enabled detection of the aldehyde of interest only, and a CFE of strain MG1363 was used as a negative control. The conversion rate was calculated by dividing the amount of product formed (in micromoles) by the reaction time (in minutes) and the protein content of the sample (in milligrams).

Characterization of KdcA. For the characterization experiments CFE of overexpressing strain B2085 was used in the KdcA assay, and the pH was varied between 4 and 9 by using sodium citrate, sodium phosphate, and Tris buffers. NaCl was added at concentrations ranging from 0.05 to 1.1 M. The substrate specificity of the decarboxylase was determined by measuring the decreases in the specific substrates by reverse-phase high-performance liquid chromatography

or ion-exchange high-performance liquid chromatography as described previously (35). The reaction mixture described above was used. The amount of cell extract of strain B2085 was adjusted for each substrate used; our aim was conversion of about 30% in 3 h. The following substrates were used at a final concentration of 10 mM (all substrates were obtained from Sigma, Zwijndrecht, The Netherlands, or Fisher, Landsmeer, The Netherlands) (2-oxo = α-keto): 2-oxo-4-methylpentanoic acid (α-ketoisocaproic acid or KICA), 2-oxo-3-methylpentanoic acid, 2-oxo-3-methylbutanoic acid, 2-oxohexanoic acid, 2-oxopentanoic acid, 2-oxobutanoic acid, pyruvic acid, 4-methylthio-2-oxobutanoic acid, phenylpyruvic acid, indole 3-pyruvate, 3-(4-hydroxyphenyl)-2-oxopropanoic acid, 2-oxopentanedioic acid, 4-methylpentanoic acid, 3-methylbutanoic acid, and 2-methylpropanoic acid. The conversion rate was calculated by dividing the amount of substrate converted (in micromoles) by the reaction time (in minutes) and the protein content of the sample (in milligrams).

Sequence homology analysis and homology modeling. Homology relationships were established by using BLASTP (1) and TBLASTX (2), and multiple-sequence alignments were constructed with ClustalW (15). Homology modeling of KdcA and IPD of *L. lactis* IL1403 was performed with the program WHAT IF (39) by using the structure of yeast pyruvate decarboxylase (PDB code 1QPB) as a template. The 1QPB structure consists of a dimer with bound Mg^{2+} ions, TPP, and pyruvamide. The results of the modeling were visualized by using Protein Explorer (http://molvis.sdsc.edu/protexpl/frntdoor.htm; copyright 1998–2003 by Eric Martz).

Nucleotide sequence accession number. The sequence of the *kdcA* gene has been deposited in the GenBank database under accession number AY548760.

RESULTS

Identification of the branched-chain α-keto acid decarboxylase gene. Previous work indicated the importance of a branched-chain keto acid decarboxylase in the formation of flavor-active compounds by a number of *L. lactis* strains (36, 37). So far, no gene encoding a decarboxylase with activity toward branched-chain α-keto acids in *L. lactis* has been cloned, nor did the (preliminary) genome sequences of lactic acid bacteria reveal a clear candidate gene for this activity (8, 17; http://genome.jgi-psf.org/draft_microbes/laccr/laccr.home .html). In order to identify the decarboxylase gene, an *L. lactis* B1157 IS mutant library was constructed by using the thermosensitive plasmid pGh9:ISS*1*. The library was validated by Southern blotting and restriction analysis (results not shown). Screening of 2,500 clones of the IS mutant library by using direct inlet mass spectrometry resulted in identification of two clones that had lost the capacity to produce 3-methylbutanal from KICA. These strains were designated B2083 and B2084. The integration sites were identified by recovering the chromosomal region flanking the integrated pGh9:ISS1 plasmid. This was done by ligation of either HindIII- or EcoRI-digested chromosomal DNAs of these strains. The ligation mixtures were used to transform *L. lactis* MG1363, and the cloned genomic regions were analyzed by restriction analysis. The plasmids that were recovered from strains B2083 and B2084 contained 6.2- and 8-kb inserts, respectively, and in the case of EcoRI digestion, the plasmid recovered from strain B2083 contained a 9.1-kb insert. The cloned flanking genomic regions were sequenced with primers annealing to pGh9:ISS1. In strain B2083 the ISS*1* element integrated at position 453 of a 1,644-bp open reading frame, and in strain B2084 integration occurred at position 916 of the same open reading frame. The gene was designated the *kdcA* gene and was sequenced in both directions by primer walking. Upstream of this gene there are three regions that might function as a terminator (-67 bp, -11.2 kcal), a promoter (-44 to -21bp), and a ribosome

binding site (-10 bp). Downstream of the *kdcA* gene a putative terminator (-9.9 kcal) starting at bp 1701 was identified (Fig. 1).

Homology of KdcA to other TPP-dependent decarboxylases. A search with the BLASTP and TBLASTX programs in various protein and DNA databases showed that the gene product KdcA was homologous to indolepyruvate decarbobxylases and pyruvate decarboxylases found in various organisms. The highest sequence identity, 96%, was observed with the first 438 amino acids of the putative *ipd* gene product of *L. lactis* IL1403. The remaining C-terminal fragments of KdcA and the *ipd* gene product consisted of 109 and 19 residues, respectively, and did not show significant homology (Fig. 1). Moreover, in strain IL1403 the 3′ terminus of the *ipd* gene overlaps the IS element IS983O carrying the putative transposase gene *tra983D*, which is located downstream of the *ipd* gene (Fig. 1) (8). Typically, the *ipd* genes encode polypeptides consisting of approximately 550 amino acid residues (32). These data indicate that in IL1403 the *ipd* gene is disrupted due to the insertion of IS983O.

The amino acid sequences of the *ipd* gene product and KdcA were aligned with the sequences of two well-characterized and homologous keto acid decarboxylating enzymes, yeast pyruvate decarboxylase (Pdc1p_SacCe) and indolepyruvate decarboxylase of *Enterobacter cloacae* (IpdC_EntCl) (11, 31). KdcA, pyruvate decarboxylases, and indolepyruvate decarboxylases all depend on TPP and Mg^{2+} as cofactors. The levels of sequence identity observed were between 35 and 83%. Several regions were highly conserved, including the regions containing residues catalyzing the transition state intermediate during catalysis (Fig. 2) (21) and residues involved in binding of the substrate and cofactors (31). The alignment in Fig. 2 clearly demonstrates that several of the residues involved in these functions are conserved in KdcA and are absent in the *ipd* gene product of strain IL1403.

A structural determination by X-ray crystallography of yeast pyruvate decarboxylases and IpdC_EntCl of *E. cloacae* has shown that the three-dimensional structures of these enzymes are very similar (32). The high levels of homology enabled us to model KdcA and the *ipd* gene product against the three-dimensional crystal structure of Pdc1p_SacCe (data not shown). This template was chosen since it is the only known three-dimensional structure with bound substrate and cofactor molecules, which allowed us to model these molecules as well. In the model of KdcA most of the residues likely to be involved in binding of the substrate and cofactors and in stabilization of the transition state intermediate during catalysis are identical in the template and KdcA model structures. The model suggests that larger substrates could fit into the substrate binding region, but the possible orientation could not be modeled due to a lack of template structures containing larger substrates. The model of the *ipd* gene product clearly showed that several of these essential residues, such as the catalytic Glu477, are absent. These results strongly imply that this gene product is not functional as an α-keto acid decarboxylase.

Overexpression of the *kdcA* gene. The *kdcA* and *ipd* genes were overexpressed in *L. lactis* for further characterization of the gene products. The *kdcA* gene was cloned by PCR into pNZ8148, where it was under the control of the *nisA* promoter, resulting in plasmid pNZ7500. Analogously, the *ipd* gene of

strain IL1403 and the corresponding region of the *kdcA* gene from strain B1157 were cloned into pNZ8148, resulting in pNZ7501 and pNZ7502, in order to analyze whether the truncated gene products were inactive with KICA and could explain the decarboxylase-negative phenotype of strain IL1403. The plasmids were introduced into *L. lactis* strain NZ9000, which contains the *nisRK* signal transduction genes required for nisin-dependent inducible expression. The protein profiles of all recombinant strains and wild-type strains B2083, B2084, B1157, IL1403, and NZ9000 were analyzed by SDS-PAGE. Moreover, their abilities to produce 3-methylbutanal on GM17 were determined, and the KICA decarboxylase activity was quantified in CFEs of GM17-grown cultures. The results are shown in Fig. 3 and 4. Wild-type strain B1157 and the induced recombinant strain NZ9000(pNZ7500) produced similar levels of 3-methylbutanal. At a nisin concentration of 2 ng ml^{-1} strain NZ9000(pNZ7500) clearly produced an extra protein with an estimated molecular mass of 62 kDa, which is in agreement with the predicted mass deduced from the gene sequence (60.9 kDa). The decarboxylase activity in CFE of this induced strain was more than 30 times higher than the activity observed with the wild type, strain B1157 (Fig. 3). Although sequence analysis of plasmids of pNZ7501 and pNZ7502 confirmed that the cloning strategy was successful, no extra protein was detected in extracts of induced cultures of strains NZ9000(pNZ7501) and NZ9000(pNZ7501). The absence of a gene product in these cases was unexpected and may have been due to decreased stability of the mRNAs or rapid degradation of misfolded protein

Characterization of KdcA. The pH optimum, salt tolerance, and substrate specificity are important parameters of flavor-forming enzymes with respect to their potential activity under cheese ripening conditions. As shown in Fig. 5B, the optimal pH was 6.3, and there was a broad pH activity profile, which corresponded well with the characteristics of known pyruvate and indole-3-pyruvate decarboxylases described in other microorganisms (16, 19, 26). The enzyme activity was not negatively affected at high salinity; in fact, it was slightly increased (Fig. 5A). The highest activity was observed with branched-chain α-keto acids derived from valine, leucine, and isoleucine, but linear α-keto acids with four and six carbon atoms and the α-keto acids derived from methionine, phenylalanine, and tryptophan (indole 3-pyruvate) could also be converted by the enzyme (Table 3). The latter substrate was of particular interest, because it is the preferred substrate for indolepyruvate decarboxylases (31) which are highly homologous to KdcA. No activity was detected with the α-keto acid α-ketoglutarate or with branched-chain aliphatic acids. No activity was detected with any of the substrates in CFE of *L. lactis* MG1363, indicating that KdcA was responsible for decarboxylation.

Genetic organization of the *kdcA* gene region in decarboxylase-negative and decarboxylase-positive *L. lactis* strains. A PCR strategy was used to obtain preliminary results for the genetic organization of the *kdcA* gene region in 30 decarboxylase-negative and 12 decarboxylase-positive *L. lactis* strains, because only a few lactococcal genomes were available for comparison. We used four primer sets that allowed discrimination between the presence of the complete *kdcA* gene and the presence of a truncated gene comparable to the *ipd* gene in strain IL1403 (Fig. 1 and Table 2). The absence of

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KdcA_LacLa     1 ---MYTVGDYLLDRLHELGIEEIFGVPGDYNLQFLDQIISREDMKWIGNANELNASYMAD
Ipd_LacLa      1 ---MYTVGDYLLDRLHELGIEEIFGVPGDYNLQFLDQIISRKDMKWVGNANELNASYMAD
IpdC_EntCl     1 MRTPYCVADYLLDRLTDCGADHLFGVPGDYNLQFLDHVIDSPDICWVGCANELNASYAAD
Pdc1p_SacCe    1 -MSEITLGKYLFERLKQVNVNTVFGLPGDFNLSLLDKIYEVEGMRWAGNANELNAAYAAD
                               #  #*?                                      #

KdcA_LacLa    58 GYARTKKAAAFLTTFGVGELSAINGLAGSYAENLPVVEIVGSPTSKVQNDKFVHHTLAD
Ipd_LacLa     58 GYARTKKAAAFLTTFGVGELSAVNGLAGSYAENLPVVEIVGSPTSKVQNEGKFVHHTLAD
IpdC_EntCl    61 GYARCKGFAALLTTFGVGELSAMNGIAGSYAEHVPVLHIVGAPGTAAQQRGELLHHTLGD
Pdc1p_SacCe   60 GYARIKGMSCIITTFGVGELSALNGIAGSYAEHVGVLHVVGVPSISSQAKQLLLHHTLGN
                                                                       ??

KdcA_LacLa   118 GDFKHFMKMHEPVTAARTLLTAEN-ATYEIDRVLSQLLKERKPVYINLPVDVAAA--KAE
Ipd_LacLa    118 GDFKHFMKMHEPVTAARTLLTAEN-ATYEIDRVLSALLKERKPVYINLPVDVAAA--KAE
IpdC_EntCl   121 GEFRHFYHMSEPITVAQAVLTEQN-ACYEIDRVLTTMLRERRPGYLMLPADVAKK--AAT
Pdc1p_SacCe  120 GDFTVFHRMSANISETTAMITDIATAPAEIDRCIRTTYVTQRPVYLGLPANLVDLNVPAK

KdcA_LacLa   175 KPALSLE-KESSTTNTTEQVILSKIEESLKNAQKPVVIAGHEVISFGLEKTVTQFVSETK
Ipd_LacLa    175 KPSLPLK-KENPTSNTSDQEILNKIQESLKNAKKPIVITGHEIISFGLEKTVTQFISKTK
IpdC_EntCl   178 PPVNALTHKQAHADSACLKAFRDAAENKLAMSKRTALLADFLVLRHGLKHALQKVVKEVP
Pdc1p_SacCe  180 LLQTPIDMSLKPNDAESEKEVIDTILALVKDAKNPVILADACCSRHDVKAETKKLIDLTQ

KdcA_LacLa   234 LPITTLNFGKSAVDESLPSFLGIYNGKLSEISLKNFVESADFIILMGVKLTDSSTGAFTH
Ipd_LacLa    234 LPITTLNFGKSSVDETLPSFLGIYNGKLSEPNLKEFVESADFIILMGVKLTDSSTGAFTH
IpdC_EntCl   238 MAHATMLMGKGIFDERQAGFYGTYSGSASTGAVKEAIEGADTVLCVGTRFTDILTAGFTH
Pdc1p_SacCe  240 FPAFVTPMGKGSISEQHPRYGGVYVGTLSKPEVKEAVESADLIILVGALLSDFNTGSFSY

KdcA_LacLa   294 HLDENKMISLNIDEGIIFNKVVEDFDFRAVVSSLSELKG---IEYEGQYIDKQY--EEFI
Ipd_LacLa    294 HLNENKMISLNINEGKIFNERIQNFDFESLISSLLDLSG---IEYKGKYIDKKQ--EDFV
IpdC_EntCl   298 QLTPAQTIEVQPHAARVGDVWFTGIPMNQAIETLVELCK---QHVAGLMSSSSGAIPFPP
Pdc1p_SacCe  300 SYKTKNIVEFHSDHMKIRNATFPGVQMKFVLQKLLTNIADAAKGYKPVAVPARTPANAAV

KdcA_LacLa   349 PSSAPLSQDRLWQAVESLTQSNETIVAEQGTSFFGASTIFLKSNSRFIGQPLWGSIGYT-
Ipd_LacLa    349 PSNALLSQDRLWQAVENLTQSNETIVAEQGTSFFGASSIFLKPKSHFIGQPLWGSIGYT-
IpdC_EntCl   355 QPDGSLTQENFWRTLQTFIRPGDIILADQGTSAFGAIDLRLPADVNFIVQPLWGSIGYT-
Pdc1p_SacCe  360 PASTPLKQEWMWNQLGNFLQEGDVVIAETGTSAFGINQTTFPNNTYGISQVLWGSIGFTT
                               **#   *                          #  #

KdcA_LacLa   408 ---FPAALGSQIADKESRHLLFIGDGSLQLTVQEIGLSIREKLNPICFIINNDGYTVERE
Ipd_LacLa    408 ---FPAALGSQIADKESRHLLFIGDGSLQLTVQERKLQVQVSQ-PSSSHMNSYS------
IpdC_EntCl   414 ---LAAAFGAQTACPNRRVIVLTGDGAAQLTIQEIGSMLRDKQHPIILVLNNEGYTVERA
Pdc1p_SacCe  420 GATLGAAFAAEEIDPKKRVILFIGDGSLQLTVQEISTMIRWGLKPYLFVLNNDGYTIEKL
                                 ###                            #  ###*?

KdcA_LacLa   465 IHGPTQSYNDIPMWNYSKLPETFGATEDRVVSKIVRTENEFVSVMKEAQADVNRMYWIEL
Ipd_LacLa    448 ------------------------------------------------------------
IpdC_EntCl   471 IHGAEQRYNDIALWNWTHIPQALSLDPQSECWRVS--EAEQTADVLEKVAHHERLSLIEV
Pdc1p_SacCe  480 IHGPKAQYNEIQGWDHLSLLPTFGAKDYETHRVATTGEWDKLTQD-KSFNDNSKIRMIEI
                 *

KdcA_LacLa   525 VLEKEDAPKLLKKMGKLFAEQNK-
Ipd_LacLa        ------------------------
IpdC_EntCl   529 MLPKADIPPLLGALTKALEACNNA
Pdc1p_SacCe  539 MLPVFDAPQNLVKQAKLT------
                       *  *** *
```

FIG. 2. Amino acid sequences of the branched-chain α-keto acid decarboxylase of *L. lactis* strain B1157 (KdcA_LacLa; accession number gi:44921617), the putative indolepyruvate decarboxylase of *L. lactis* IL1403 (Ipd_LacLa; accession number gi:15673286 gi:44921617), indolepyruvate decarboxylase of *E. cloacae* (IpdC_EntCl; accession number gi 118333), and pyruvate decarboxylase of *Saccharomyces bayanu* (Pdc1p_SacCe; accession number SGDID S000004034). The ClustalW program was used to align the sequences. The numbers indicate the locations of the adjacent amino acid residues in each protein. The black shading indicates amino acids that are identical in at least three of the proteins; the gray shading indicates similar amino acids. The groups of amino acids that were considered similar were I, L, M, and V; A, G, and S; H, K, and R; D and E; N and Q; F, W, and Y; and S and T. Residues interacting with TPP in IpdC_EntCl are indicated by asterisks, and residues interacting with the substrate indolepyruvate are indicated by number signs (32). Residues essential for catalysis in Pdc1p_SacCe (21) are indicated by solid triangles.
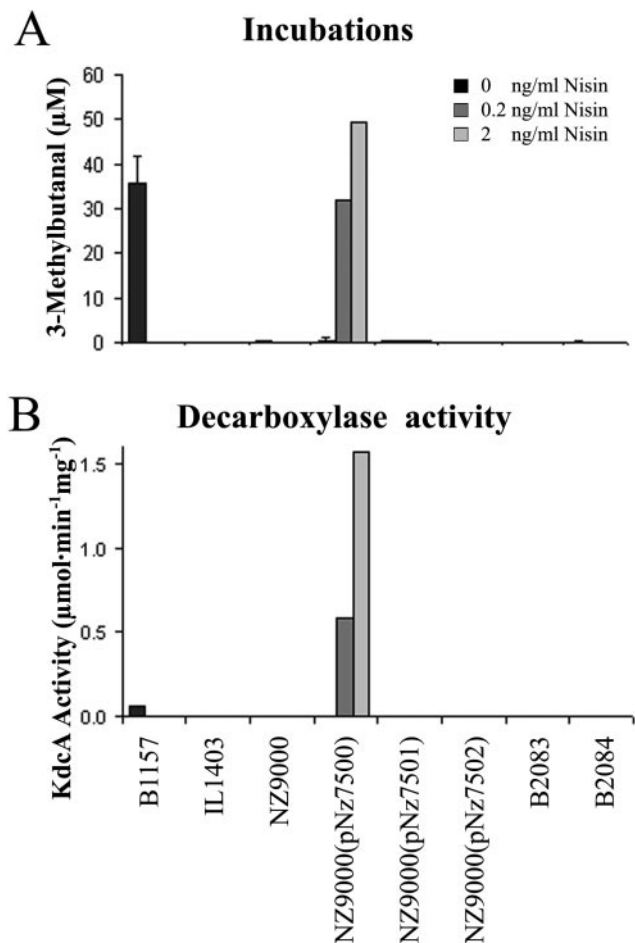
FIG. 3. (A) 3-Methylbutanal production during incubation in GM17. (B) KdcA activities of (nisin-induced) decarboxylase overexpression mutants, wild-type strain B1157, the cloning host *L. lactis* NZ9000, and the knockout mutants B2083 and B2084.
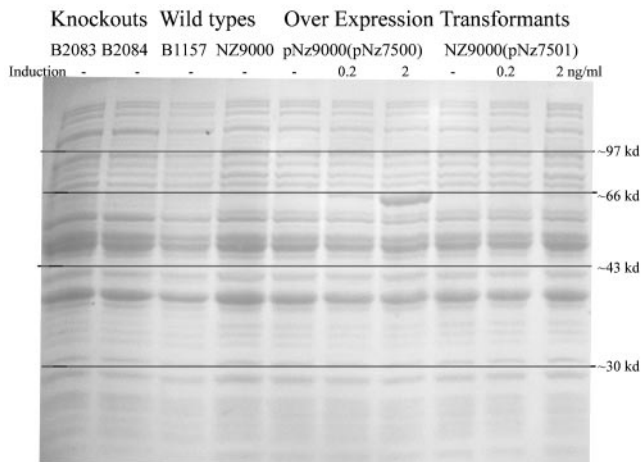

FIG. 4. SDS-PAGE of knockout mutants, wild-type strains, and decarboxylase overexpression recombinants. The overexpression recombinants were induced with 0, 0.2, and 2 ng of nisin A per ml. kd, kilodaltons.
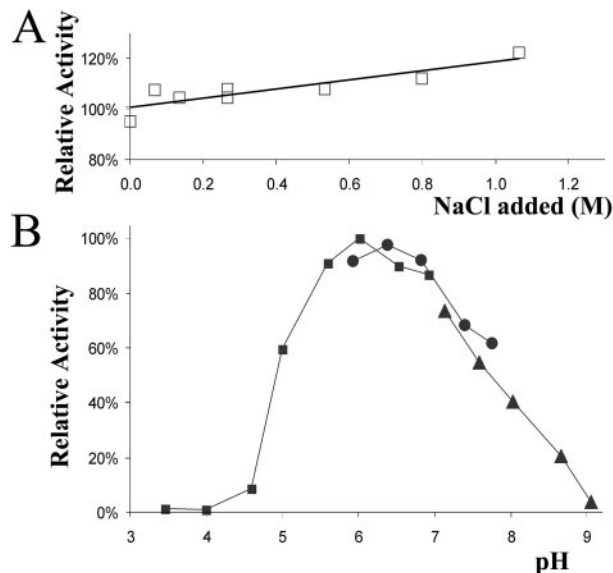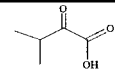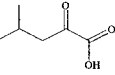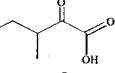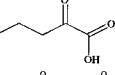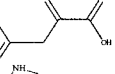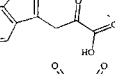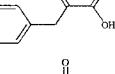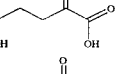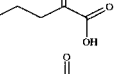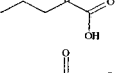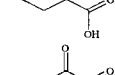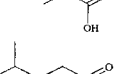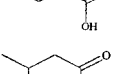

FIG. 5. (A) Effects of salt on the activity of KdcA. (B) Effects of pH on the activity of KdcA, measured in 50 mM sodium citrate (■), sodium phosphate (●), or Tris (▲) buffer with α-ketoisovaleric acid as the substrate.

amplification products clearly correlated with the absence of 3-methylbutanal formation and vice versa ($P > 0.95$, as determined by Student's t test). The non-3-methylbutanal producers could be divided into two categories. The largest group (~70%) did not give any PCR products, indicating absence of the *kdcA* gene. Strain SK11 belonged to this group, and the absence of the gene w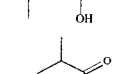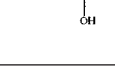as confirmed by searching the preliminary genome sequence (www.jgi.doe.gov and www.ncbi.nlm.nih.gov, assessed 17 July 2004). Strains in the second group (~30%) only yielded the two PCR products of the first fragment of the *kdcA* gene corresponding to the *ipd* sequence present in strain IL1403. None of the non-3-methylbutanal producers produced more than the first two PCR products; however, most (75%) of the 3-methylbutanal-producing strains produced three or four PCR products, indicating that a full-length gene was present. The majority of these strains do not have a history in dairy fermentations (wild isolates), while most of the non-3-methylbutanal-forming strains have a history of usage in the dairy industry. There was no clear correlation between the subspecies (*L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis diacetylactis*, and *L. lactis* subsp. *cremoris*) and the absence of the complete *kdcA* gene. However, the only *L. lactis* subsp. *cremoris* strain which was able to produce 3-methylbutanal in this experiment was B1157.

Occurrence of *kdcA* in lactic acid bacteria. The BLASTN and TBLASTX search of the newly identified *kdcA* gene against the (preliminary) genome sequences of dairy-related bacteria (www.jgi.doe.gov and www.ncbi.nlm.nih.gov, assessed 17 July 2004) only revealed very high similarity with the *ipd* annotated open reading frame of *L. lactis* IL1403, while in the other dairy-related bacteria, including *L. lactis* subsp. *cremoris*, *Lactobacillus casei*, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus gasseri*, *Lactobacillus johnsonii* NCC 533, *Lactobacillus delbrueckii*, *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293, and *Brevibacterium linens*, no or-

TABLE 3. Substrate specificity of KdcA

Substrate	Corresponding amino acid	Structure	Relative conversion rate[a]
3-Methyl-2-oxobutanoic acid	Valine		
4-Methyl-2-oxopentanoic acid	Leucine		
3-Methyl-2-oxopentanoic acid	Isoleucine		
4-Methylthio-2-oxobutanoic acid	Methionine		
2-Oxo-3-phenylpropanoic acid	Phenylalanine		
3-Indole-3-yl-2-oxopropanoic acid	Tryptophan		
3-(4-Hydroxyphenyl)-2-oxopropanoic acid	Tyrosine		
2-Oxopentanedioic acid	Glutamic acid		
2-Oxohexanoic acid			
2-Oxopentanoic acid			
2-Oxobutanoic acid			
2-Oxopropanoic acid			
Isohexanoic acid			
Isopentanoic acid			
Isobutanoic acid			

[a] Individual specific conversion rates were calculated from the amount of substrate converted (as determined by high-performance liquid chromatography), the reaction time, and the protein content of the CFE of strain NZ9000(pNz7500).

thologous gene was identified. Previously, we analyzed various strains of these species to determine their ability to produce 3-methylbutanal and the presence of the decarboxylase. The results showed that only a limited number of *Lactococcus* strains are able to produce 3-methylbutanal (5, 36, 37). It will be interesting to extend this analysis and elucidate whether KdcA-positive strains occur in other lactic acid bacterial species.

DISCUSSION

We cloned and sequenced the gene encoding KdcA of *L. lactis* strain B1157. KdcA has a broad substrate range and exhibits the highest activity with branched-chain α-keto acids. Therefore, it is best designated a branched-chain α-keto acid decarboxylase. The KdcA sequence was highly homologous to known indole pyruvate decarboxylase and pyruvate decarboxylase sequences, and characteristics like the pH optimum and molecular weight were also very similar. The structure of KdcA could easily be modeled by using yeast pyruvate decarboxylase as the template. The results indicate that KdcA is a new member of the pyruvate decarboxylase-indolepyruvate decarboxylase protein family. Moreover, they suggest that the mechanism of decarboxylation by kdcA is very similar to that in these enzymes, in which the TPP cofactor acts as an intramolecular

base catalyst and Asp29, His112, His113, and Glu462 are involved in stabilization of the transition state intermediate (21, 31).

Previously, Amarita et al. described the partial purification and characterization of an α-keto acid decarboxylase from *L. lactis* IFPL730 active on α-keto-4-methylthiobutyrate; also, this enzyme had the highest activity with the branched-chain α-keto acids (3). Despite the fact that the molecular mass of this enzyme was estimated to be 50 kDa, various other features are very similar to features of KdcA.

Our previous activity screening analyses indicated that most lactococci do not exhibit decarboxylase activity. Our PCR-based analysis of 30 decarboxylase-negative isolates indicated that in most of these strains *kdcA* is completely absent, as it is in strains SK11 and MG1363. A second group has a genotype similar to strain IL1403, which is characterized by the presence of a truncated and nonfunctional homologue. Most 3-methylbutanal-producing strains seem to contain the complete gene and do not have any history in industrial dairy fermentations.

Since the decarboxylase was active at pH 5.3 and at a high salinity, which are typical conditions for Gouda cheese ripening, the enzyme has the potential to play an important role in cheese flavor formation. This was also illustrated by the malty flavor of cheese made with strain B1157 (6). Overexpressing the decarboxylase hardly resulted in increased 3-methylbutanal production in fermentations (Fig. 3). A lack of the substrate KICA was most probably the reason for the absence of high 3-methylbutanal levels. The formation of KICA by transamination has previously been shown to be suboptimal for the production of Leu-derived flavor compounds under cheese ripening conditions (7, 42). A combination of transaminase stimulation and the decarboxylase overexpression mutant might result in a major increase in 3-methylbutanal formation and also in higher levels of 2-methylpropanal and 2-methylbutanal, which are derived from valine and isoleucine, respectively. Future research should focus on the impact of the use of these kinds of strains in model systems and cheese making in order to control flavor formation better.

ACKNOWLEDGMENTS

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