Cloning and Analysis of the *pepV* Dipeptidase Gene of *Lactococcus lactis* MG1363

MICHIEL A. HELLENDOORN, BLANDINE M. D. FRANKE-FAYARD,
IGOR MIERAU, GERARD VENEMA,* AND JAN KOK

*Department of Genetics, Groningen Biomolecular Sciences and
Biotechnology Institute, University of Groningen,
9751 NN Haren, The Netherlands*

The gene *pepV*, encoding a dipeptidase from *Lactococcus lactis* subsp. *cremoris* MG1363, was identified in a genomic library in pUC19 in a peptidase-deficient *Escherichia coli* strain and subsequently sequenced. PepV of *L. lactis* is enzymatically active in *E. coli* and hydrolyzes a broad range of dipeptides but no tri-, tetra-, or larger oligopeptides. Northern (RNA) and primer extension analyses indicate that *pepV* is a monocistronic transcriptional unit starting 24 bases upstream of the AUG translational start codon. The dipeptidase of *L. lactis* was shown to be similar to the dipeptidase encoded by *pepV* of *L. delbrueckii* subsp. *lactis*, with 46% identity in the deduced amino acid sequences. A PepV-negative mutant of *L. lactis* was constructed by single-crossover recombination. Growth of the mutant strain in milk was significantly slower than that of the wild type, but the strains ultimately reached the same final cell densities.

The starter bacterium *Lactococcus lactis* subsp. *cremoris* is widely used in cheese making to provide optimal conditions for curd formation and for the development of texture and flavor. Lactococci are organisms with multiple amino acid auxotrophies, and as a consequence, their growth to high cell densities in milk depends on their ability to efficiently degrade the milk protein casein (11). The peptides which are initially released from casein by the cell envelope-associated proteinase and translocated into the cell by the oligopeptide transport system Opp are broken down into amino acids by various intracellular peptidases. In recent years, many lactococcal peptidases have been isolated and characterized both biochemically and genetically (11, 12, 23, 25). Mutants lacking either PepX, PepO, PepT, PepN, PepC, PepF, or PepA have been constructed by gene disruption methods and analyzed for their ability to grow in milk. With the possible exception of PepA- and PepN-deficient mutants, no differences could be detected in growth rates and final cell densities between the various mutants and the wild-type strain (15, 22–25, 27). These observations indicate that none of these peptidases individually is essential for growth in milk. However, analysis of strains carrying multiple peptidase mutations showed that inactivation of several peptidases can lead to lower growth rates in milk, the general trend being that growth rates decrease when more peptidases are inactivated. A strain with mutations in five genes (*pepX*, *pepO*, *pepT*, *pepC*, and *pepN*) grew more than 10 times slower in milk than the wild-type strain did (24).

A dipeptidase from *L. lactis* subsp. *cremoris* Wg2 has been purified to homogeneity by van Boven et al. (39). The enzyme was shown to be a metallopeptidase which hydrolyzes a wide range of dipeptides but not tripeptides or tetrapeptides. Also, dipeptides with proline, histidine, glycine, or glutamate as the N-terminal amino acid or with proline in the second position are not degraded (39). Similar enzymes have been purified from *Lactobacillus delbrueckii* subsp. *bulgaricus* (43), from *Lactobacillus helveticus* SBT 2171 (34), and from *Lactobacillus sake* (28). From *Lactobacillus delbrueckii* subsp. *lactis*, the gene of a dipeptidase (*pepV*) has been cloned and sequenced (42). Dipeptidase genes *pepD* and *pepDA* have been cloned and sequenced from *Lactobacillus helveticus* 53/7 (41) and from *Lactobacillus helveticus* CNRZ32 (6), respectively. Dipeptidases are involved in the final breakdown of degradation products (dipeptides) produced by various other peptidases and could, thus, be important components of the casein degradation system in *L. lactis*. In this paper, we report the cloning, expression in *Escherichia coli*, and nucleotide sequence of the gene (*pepV*) of a dipeptidase from *L. lactis* subsp. *cremoris* MG1363. A *pepV*-negative mutant was constructed to assess the role of the dipeptidase in growth of *L. lactis* in milk.

MATERIALS AND METHODS

Strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown in TY medium (3) at 37°C with vigorous agitation or on TY medium solidified with 1.5% agar and containing 100 μg of ampicillin per ml or 100 μg of erythromycin per ml when needed. Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were used at concentrations of 1 mM and 0.004% (wt/vol), respectively. *L. lactis* was grown at 30°C in M17 broth (35) or on M17 medium solidified with 1.5% agar, both supplemented with 0.5% glucose. Erythromycin and X-Gal were added to 5 μg/ml and 0.008%, respectively.

To minimize possible lethality caused by high expression of heterologous peptidase genes in *E. coli*, a *pcnB* mutant of *E. coli* CM89 was made. This strain (CM89L) was created by P1 transduction of CM89 with a lysate made on *E. coli* MM38K26. Inactivation of the *pcnB* gene results in a reduced plasmid copy number of vectors based on the ColE1 replicon (21).

Molecular cloning, screening, and DNA sequencing. Molecular cloning techniques were performed essentially as described by Sambrook et al. (31). Plasmids from *L. lactis* were isolated by the method of Birnboim and Doly with the modifications described by Leenhouts et al. (18). Chromosomal DNA was isolated from *L. lactis* by the method described by Seegers et al. (33). DNA was introduced into *E. coli* and *L. lactis* by electrotransformation as described by Zabarovsky and Winberg (45) and Holo and Nes (10), respectively. A genome bank of *L. lactis* MG1363 in pUC19 constructed by Buist et al. (4) was used to isolate *pepV*. Double-stranded plasmid DNA was sequenced by the dideoxy chain termination method (32) with the T7 sequencing kit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) and universal and reverse pUC primers. The nucleotide sequence was completed by use of primers synthesized with an Applied Biosystems (Foster City, Calif.) 392 DNA/RNA synthesizer.

Nucleotide sequences were analyzed with the PC/Gene sequence analysis

* Corresponding author. Mailing address: Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands. Phone: 31 50 3632093. Fax: 31 50 3632348. E-mail: G.Venema@BIOL.RUG.NL.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype(s) or genotype(s)	Source or reference(s)
Strains		
L. lactis subsp. *cremoris*		
MG1363	Plasmid-free derivative of NCDO712	8
MG1363(pLP712)	Prt$^+$ Lac$^+$	8
MG::pPV5	*pepV*	This work
MG::pPV5(pLP712)	*pepV* Prt$^+$ Lac$^+$	This work
IM5(pLP712)	*pepN* Prt$^+$ Lac$^+$	24
IM7(pLP712)	*pepX pepN* Prt$^+$ Lac$^+$	24
E. coli		
NM522	*supE thi* Δ(*lac-proAB*) Δ*hsd5* (r$_K^-$ m$_K^-$) [F′ *proAB lacI^{q}Z*ΔM15]	9
CM89	*leu-9* Δ(*pro-lac*) *met thyA pepN102 pepA11 pepB1 pepQ10*	26
MM38K26	*argG6 asnA31* (or *asnB32*) *his-1 leuB6 metB1 pyrE gal-6 lacY1 xyl-7 supE44 bgl fhuA2 gyrA rpsL104 tsx-1 uhp* Δ*pcnB* Km^r	21
CM89L	CM89 derivative; *pcnB* Km^r	This work
EC1000	*repA$^+$* derivative of MC1000; Km^r	17
Plasmids		
pUC19	Ap^r	44
pORI28	Em^r; *ori$^+$* of pWV01; Rep$^-$	17
pDipLL	Ap^r; pUC19 with 3.6-kb MG1363 chromosomal *Sau*3A fragment carrying *pepV*	This work
pPV6	Ap^r; pUC19 with internal *Hin*dIII-*Pst*I fragment of *pepV*	This work
pPV5	Em^r; pORI28 with 570-bp *Hin*dIII-*Asp*718 fragment of pPV6	This work

program (version 6.8; Intelli Genetics Inc., Geneva, Switzerland). Protein homology searches in the SWISSPROT, PIR, and GenBank (release 23 September 1996) databases were carried out with the BLAST program (1). Protein sequence alignments were done with the PALIGN program of PC/Gene with the unitary matrix at standard settings. PROSITE (release 13.2) was used to find specific signature sequences (2).

RNA analysis. RNA was isolated from exponentially growing *L. lactis* cells at an optical density at 600 nm of 0.5 as described previously (38). Northern (RNA) hybridizations were done at 42°C in a buffer containing 50% formamide, 7% sodium dodecyl sulfate (SDS), 2% blocking reagent (Boehringer GmbH, Mannheim, Germany), 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate), 50 mM Na phosphate (pH 7), and 0.1% *N*-lauryl sarcosine. The 542-bp *Hin*dIII-*Pst*I fragment of pDipLL was labelled with [α-^{32}P]dCTP and used as a probe. A synthetic oligonucleotide (pepvTS, 5′-CCAGGGCCAAATGGATTT TCAGC) complementary to the mRNA from positions 136 to 158 was used for primer extension analysis. Twenty-five nanograms of primer was added to 5 μg of RNA in a reaction mixture containing dCTP, dGTP, dTTP, and [α-^{35}S]dATP. cDNA was synthesized with avian myeloblastosis virus reverse transcriptase (Boehringer). After 10 min of incubation at 42°C, excess cold dATP was added and incubation was prolonged for another 10 min at 42°C. The primer extension product was compared on a sequencing gel with the products of a sequence reaction with the same primer.

Construction of a *pepV* mutant. The internal *Hin*dIII-*Pst*I fragment of *pepV* was cloned in pUC19. This plasmid (pPV6) was digested with *Hin*dIII and *Asp*718, and the 570-bp fragment was ligated in the integration vector pORI28 (19). The ligation mixture was used to transform *E. coli* EC1000 (16). The proper plasmid, pPV5, was used to disrupt, by single-crossover integration, *pepV* in *L. lactis* MG1363, resulting in strain MG::pPV5. The proteinase-lactose plasmid pLP712 (8) was introduced into the mutant by electroporation. Colonies containing pLP712 were identified on LM17 agar plates containing 0.004% of the pH indicator bromocresol purple. Yellow Lac$^+$ colonies were purified and checked for proteinase production with the chromogenic substrate S2586 (MeO-Suc-Arg-Pro-Tyr-*p*NA; Chromogenix AB, Mölndal, Sweden) as described by Exterkate (7) with the following modifications. Cells of 1 ml of overnight culture in LM17 were washed with assay buffer (80 mM Tris [pH 7.0]) containing 5 mM CaCl₂ and resuspended in 300 μl of the same buffer containing 2 mM substrate S2586. Prt$^+$ cells produce a yellow color in the reaction mixture, whereas Prt$^-$ cells do not.

SDS-PAGE, Western blotting (immunoblotting), and immunodetection. Cells of *L. lactis* were harvested by centrifugation, resuspended in 10 mM Tris–1 mM EDTA (pH 7.4), and disrupted as described by van de Guchte et al. (40). The cell extract was used for analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by the protocol of Laemmli (14) with the Protean II minigel system (Bio-Rad Laboratories, Richmond, Calif.). The low-molecular-weight marker was obtained from Bio-Rad. Proteins separated by SDS-PAGE were transferred to a BA85 nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany) as described by Towbin et al. (37). Dipeptidase antigen was detected with monoclonal dipeptidase antibodies (13), diluted 1:5, and alkaline phosphatase-conju-

gated goat anti-mouse antibodies (Promega Corp., Madison, Wis.), as described in the manufacturer's instructions.

Dipeptidase and tripeptidase activity assay. Dipeptidase and tripeptidase activities in *E. coli* CM89L clones were measured by the L-amino acid oxidase assay (29). Samples were incubated for 20 min at 37°C in a well of a microtiter plate and subsequently analyzed in a Thermomax microplate reader (Molecular Devices Co., Menlo Park, Calif.) at 450 nm.

Nucleotide sequence accession number. The nucleotide sequence of *pepV* has been assigned the accession number U78036.

RESULTS

Cloning, sequencing, and analysis of the dipeptidase gene of *L. lactis*. To reduce the copy number of pUC plasmids (21) and, thus, to minimize possible problems of cloning peptidase genes, the *pcnB* gene of the *E. coli* multiple peptidase mutant CM89 was mutated (see Materials and Methods). A bank of genomic DNA of *L. lactis* MG1363 in pUC19 (4) was transferred to the resulting strain, CM89L. Approximately 2,400 transformants were assayed for dipeptidase activity. Plasmid DNA was isolated from the two isolates obtained which showed dipeptidase activity and subjected to restriction enzyme analyses. The plasmids had a common DNA fragment, and the one containing the smallest insert (pDipLL) was chosen for further characterization. Several subfragments of the insert in pDipLL were inserted into pUC19, and their nucleotide sequences were determined. Figure 1 shows part of the nucleotide sequence of the insert in pDipLL. It contains an open reading frame (ORF) of 1,614 bp which could encode a protein of 472 amino acids with a predicted molecular weight of 51,911. The amino acid composition of the deduced protein was almost the same as that determined for the dipeptidase purified from *L. lactis* Wg2 (39). By homology and mutation studies (see below), the ORF was identified as the dipeptidase gene *pepV*. *pepV* is preceded by a putative ribosome binding site which is complementary to the 3′ end of 16S rRNA of *L. lactis* with a Δ$G°$ of 12.8 kcal/mol (53.5 kJ/mol) (5, 36). Upstream of the ribosome binding site, a promoter-like structure, consisting of the −35 hexanucleotide TTGCAC, a spacing of 18 bp, and the −10 sequence TACAAT, is present. A
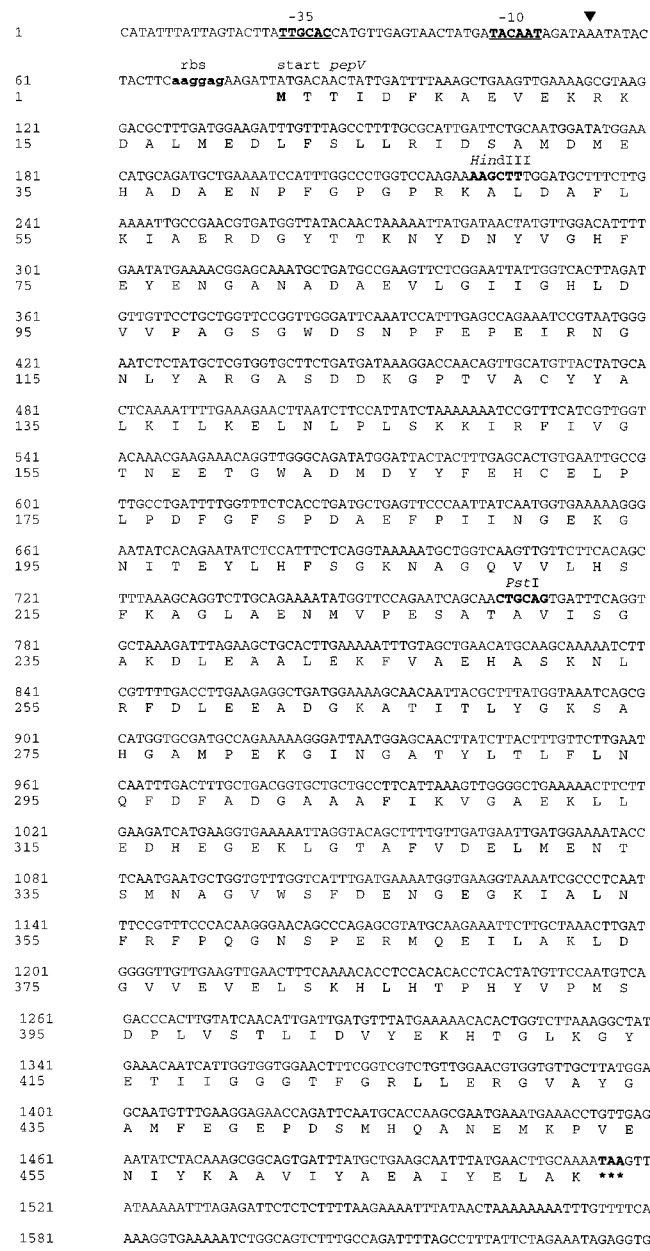
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                              -35                   -10           ▼
1      CATATTTATTAGTACTTATTGCACCATGTTGAGTAACTATGATACAATAGATAAATATAC

       rbs           start pepV
61     TACTTCaaggagAAGATTATGACAACTATTGATTTTAAAGCTGAAGTTGAAAAGCGTAAG
1                    M  T  T  I  D  F  K  A  E  V  E  K  R  K

121    GACGCTTTGATGGAAGATTTGTTTAGCCTTTGCGCATTGATTCTGCAATGGATATGGAA
15      D  A  L  M  E  D  L  F  S  L  L  R  I  D  S  A  M  D  M  E
                                           HindIII
181    CATGCAGATGCTGAAAATCCATTTGGCCCTGGTCCAAGAAAAGCTTTGGATGCTTTCTTG
35      H  A  D  A  E  N  P  F  G  P  G  P  R  K  A  L  D  A  F  L

241    AAAATTGCCGAACGTGATGGTTATACAACTAAAAATTATGATAACTATGTTGGACATTTT
55      K  I  A  E  R  D  G  Y  T  T  K  N  Y  D  N  Y  V  G  H  F

301    GAATATGAAAACGGAGCAAATGCTGATGCCGAAGTTCTCGGAATTATTGGTCACTTAGAT
75      E  Y  E  N  G  A  N  A  D  A  E  V  L  G  I  I  G  H  L  D

361    GTTGTTCCTGCTGGTTCCGGTTGGGATTCAAATCCATTTGAGCCAGAAATCCGTAATGGG
95      V  V  P  A  G  S  G  W  D  S  N  P  F  E  P  E  I  R  N  G

421    AATCTCTATGCTCGTGGTGCTTCTGATGATAAAGGACCAACAGTTGCATGTTACTATGCA
115     N  L  Y  A  R  G  A  S  D  D  K  G  P  T  V  A  C  Y  Y  A

481    CTCAAAATTTTGAAAGAACTTAATCTTCCATTATCTAAAAAAATCCGTTTCATCGTTGGT
135     L  K  I  L  K  E  L  N  L  P  L  S  K  K  I  R  F  I  V  G

541    ACAAACGAAGAAACAGGTTGGGCAGATATGGATTACTACTTTGAGCACTGTGAATTGCCG
155     T  N  E  E  T  G  W  A  D  M  D  Y  Y  F  E  H  C  E  L  P

601    TTGCCTGATTTTGGTTTCTCACCTGATGCTGAGTTCCCAATTATCAATGGTGAAAAGGG
175     L  P  D  F  G  F  S  P  D  A  E  F  P  I  I  N  G  E  K  G

661    AATATCACAGAATATCTCCATTTCTCAGGTAAAAATGCTGGTCAAGTTGTTCTTCACAGC
195     N  I  T  E  Y  L  H  F  S  G  K  N  A  G  Q  V  V  L  H  S
                                                 PstI
721    TTTTAAAGCAGGTCTTGCAGAAAATATGGTTCCAGAATCAGCAACCTGCAGTGATTTCAGGT
215     F  K  A  G  L  A  E  N  M  V  P  E  S  A  T  A  V  I  S  G

781    GCTAAAGATTTAGAAGCTGCACTTGAAAAATTTGTAGCTGAACATGCAAGCAAAAATCTT
235     A  K  D  L  E  A  A  L  E  K  F  V  A  E  H  A  S  K  N  L

841    CGTTTTGACCTTGAAGAGGCTGATGGAAAAGCAACAATTACGCTTTATGGTAAATCAGCG
255     R  F  D  L  E  E  A  D  G  K  A  T  I  T  L  Y  G  K  S  A

901    CATGGTGCGATGCCAGAAAAAGGGATTAATGGAGCAACTTATCTTACTTTGTTCTTGAAT
275     H  G  A  M  P  E  K  G  I  N  G  A  T  Y  L  T  L  F  L  N

961    CAATTTGACTTTGCTGACGGTGCTGCTGCCTTCATTAAAGTTGGGGCTGAAAAACTTCTT
295     Q  F  D  F  A  D  G  A  A  A  F  I  K  V  G  A  E  K  L  L

1021   GAAGATCATGAAGGTGAAAAATTAGGTACAGCTTTTGTTGATGAATTGATGGAAAATACC
315     E  D  H  E  G  E  K  L  G  T  A  F  V  D  E  L  M  E  N  T

1081   TCAATGAATGCTGGTGTTTGGTCATTTGATGAAAATGGTGAAGGTAAAATCGCCCTCAAT
335     S  M  N  A  G  V  W  S  F  D  E  N  G  E  G  K  I  A  L  N

1141   TTCCGTTTCCCACAAGGGAACAGCCCAGAGCGTATGCAAGAAATTCTTGCTAAACTTGAT
355     F  R  F  P  Q  G  N  S  P  E  R  M  Q  E  I  L  A  K  L  D

1201   GGGGTTGTTGAAGTTGAACTTTCAAAACACCTCCACACACCTCACTATGTTCCAATGTCA
375     G  V  V  E  V  E  L  S  K  H  L  H  T  P  H  Y  V  P  M  S

1261   GACCCACTTGTATCAACATTGATTGATGTTTATGAAAAACACACTGGTCTTAAAGGCTAT
395     D  P  L  V  S  T  L  I  D  V  Y  E  K  H  T  G  L  K  G  Y

1341   GAAACAATCATTGGTGGTGGAACTTTCGGTCGTCTCGTTGGAACGTGGTGTTGCTTATGGA
415     E  T  I  I  G  G  T  F  G  R  L  L  E  R  G  V  A  Y  G

1401   GCAATGTTTGAAGGAGAACCAGATTCAATGCACCAAGCGAATGAAATGAAACCTGTTGAG
435     A  M  F  E  G  E  P  D  S  M  H  Q  A  N  E  M  K  P  V  E

1461   AATATCTACAAAGCGGCAGTGATTTATGCTGAAGCAATTTATGAACTTGCAAAATAAGTT
455     N  I  Y  K  A  A  V  I  Y  A  E  A  I  Y  E  L  A  K  ***

1521   ATAAAAATTTAGAGATTCTCTCTTTTAAGAAAATTTATAACTAAAAAAAATTTGTTTTCA

1581   AAAGGTGAAAAATCTGGCAGTCTTTGCCAGATTTTAGCCTTTATTCTAGAAATAGAGGTG
       =====  ==========>    <==========  =====
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FIG. 1. Nucleotide sequence and deduced amino acid sequence of *pepV* of *L. lactis* MG1363. The −35 and −10 hexanucleotides are in boldface type and underlined. Facing arrows, inverted repeats; rbs, ribosome binding site; solid triangle, transcription start point; ∗∗∗, stop codon. The restriction enzyme sites used for construction of MG::pPV5 are indicated in boldface type.

16-bp inverted repeat (ΔG [25°C], 25.8 kcal/mol [−108 kJ/mol]) downstream of *pepV* could function as a transcription terminator.

To examine whether the putative promoter was active in vivo, a primer extension analysis was carried out. The results (Fig. 2) show that transcription started at an adenine residue 6 bp downstream of the −10 hexanucleotide. The size of the mRNA transcribed from *pepV* was determined by Northern blotting. A 1.7-kb transcript was detected in a total RNA preparation isolated from *L. lactis* MG1363 growing exponentially in GM17 broth (Fig. 3). Apparently, *pepV* is a monocistronic transcriptional unit which starts 24 bp upstream of the AUG
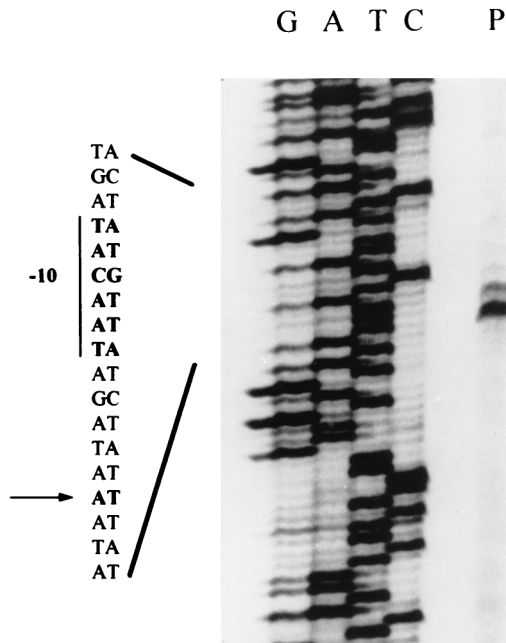

FIG. 2. Determination of the start point of transcription of *pepV*. Lane P contains the primer extension product obtained with RNA of *L. lactis* MG1363 and primer pepvTS. The sequence ladder (lanes G, A, T, and C) was obtained with the same primer on *pepV* template DNA. The sequences of the transcribed and sequenced strands are shown in the left margin. The −10 hexanucleotide is indicated in boldface type; the transcription start point is indicated with an arrow.

translational start codon and, most probably, stops at the transcription terminator immediately downstream of *pepV*.

PepV of *L. lactis* and PepV of *L. delbrueckii* subsp. *lactis* show extensive similarities. The SWISSPROT, PIR, and GenBank databases were screened for proteins and nucleotide sequences showing homology with the amino acid and nucleotide sequences of PepV and *pepV*, respectively. Extensive amino acid sequence similarity was found with PepV of *L. delbrueckii* subsp. *lactis* DSM 7290 (42). The two enzymes have 45.7%
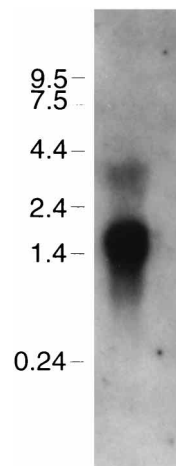

FIG. 3. Determination by Northern hybridization of the size of mRNA transcribed from *pepV*. RNA isolated from exponentially growing *L. lactis* MG1363 was used. The probe was an [α-^{32}P]dCTP-labeled *Hin*dIII-*Pst*I fragment of *pepV*. The sizes (in kilobases) of the RNA molecular mass markers are given in the left margin.

identical and 12.3% similar amino acids, supporting our conclusion that we have cloned the gene of a dipeptidase. Moreover, the enzymes are almost identical in size, since PepV of *L. lactis* MG1363 contains 472 amino acid residues and PepV of *L. delbrueckii* subsp. *lactis* consists of 470 residues. In the lactobacillar PepV, two regions which have homology to the two signature sequences of the ArgE/DapE/ACY1/CPG2/YscS family of proteins are identified (2). The first pattern contains a conserved histidine which could be involved in binding metal ions, and the second pattern contains a number of conserved charged residues. Both patterns are also present in PepV of *L. lactis* MG1363 (Fig. 4, boxes I and II). In Fig. 4, the amino acid sequences of both PepVs are aligned with the DapEs of *E. coli* and *Haemophilus influenzae*. Apart from the regions of similarity described above, two other highly similar stretches are present in the proteins (boxes III and IV).

Substrate specificity of PepV. The hydrolytic action of PepV on various peptides was examined in cell extracts of *E. coli* CM89L(pDipLL). Table 2 shows that the enzyme was active toward various dipeptides, whereas tripeptides were not hydrolyzed. Similar to the lactobacillar PepV, the lactococcal PepV was capable of hydrolyzing the unusual dipeptide carnosine (β-alanyl-leucine). After treatment of the cell extract with the metal-chelating agent EDTA (0.25 mM), PepV activity was completely inhibited (data not shown).

Construction and analysis of a *pepV* mutant. To investigate whether PepV is essential for growth of *L. lactis* in milk, the *pepV* gene was inactivated by the insertion of the integration vector pPV5 into the chromosome of *L. lactis* MG1363. The proper chromosomal location of the integrated plasmid in the resulting strain, MG::pPV5, was confirmed by Southern hybridization (data not shown). Subsequently, *prt* and *lac* genes were introduced into MG::pPV5 by electrotransformation of plasmid pLP712 (8).

Cell extracts of the various strains used in this study were subjected to SDS-PAGE and Western blotting with dipeptidase-specific antibodies (39). Whereas an approximately 50-kDa band representing PepV was present in *L. lactis* MG1363 and *E. coli* CM89L (pDipLL), it was absent in the mutant strain (Fig. 5).

Growth experiments in milk with MG1363(pLP712), the *pepN* mutant IM5(pLP712), the *pepN pepX* mutant IM7(pLP712), and the *pepV* insertion mutant MG::pPV5(pLP712) showed that all strains reached similar final cell densities. However, the growth rates of all the mutants (μ_{max} = 0.58) were significantly lower than that of the wild type (μ_{max} = 0.71). The growth rates in GM17 medium were similar for all strains (μ_{max} = 0.75). We conclude from this experiment that the dipeptidase is important but not essential for growth of *L. lactis* in milk.

DISCUSSION

We have cloned and sequenced the gene of a dipeptidase of *L. lactis*. This conclusion is based on the following observations. (i) Antibodies raised against the purified dipeptidase of *L. lactis* subsp. *cremoris* Wg2 (13) reacted with a protein of approximately 50 kDa encoded by the cloned lactococcal chromosomal fragment. (ii) The deduced amino acid composition of the cloned dipeptidase protein PepV was almost the same as that of the dipeptidase purified from *L. lactis* Wg2. (iii) The deduced amino acid sequence of the dipeptidase showed a high degree of similarity to PepV of *L. delbrueckii* subsp. *lactis* (42). (iv) The multiple-peptidase-negative *E. coli* CM89L carrying *pepV* on a plasmid displayed high dipeptidase activity. The deduced N-terminal amino acid sequence of PepV has no
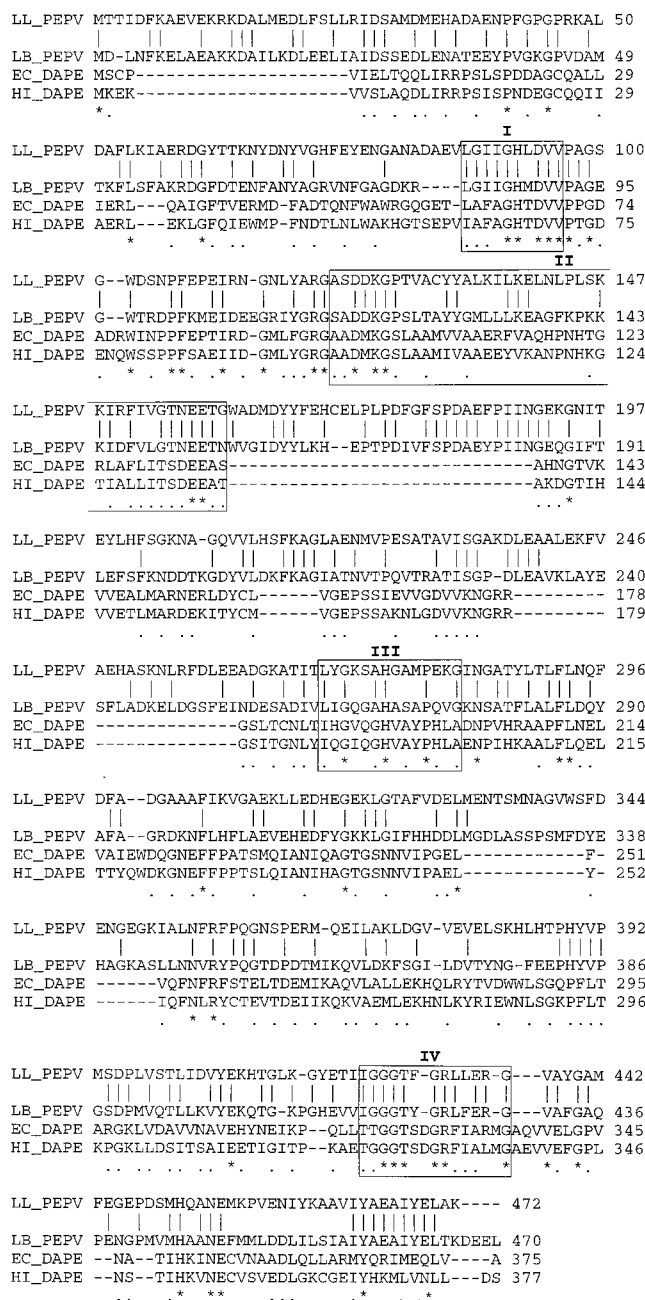

FIG. 4. Alignment of the amino acid sequences of the dipeptidases of *L. lactis* (LL_PEPV) and *L. delbrueckii* subsp. *lactis* (LB_PEPV), *E. coli* DapE (EC_DAPE), and *H. influenzae* DapE (HI_DAPE). Identical and similar amino acids in all four proteins are indicated by stars and dots, respectively. Regions of extensive similarity (I, II, III, and IV) are boxed. |, identical amino acids in the two dipeptidases.

obvious membrane-spanning domains, indicating that PepV is most probably located in the cytoplasm of *L. lactis*. This is in agreement with the immunological data of Laan et al. (13).

PepV of *L. lactis* is very similar to the dipeptidase PepV of *L. delbrueckii* subsp. *lactis* (42). The putative product of an incomplete ORF (ORF1) with unknown function upstream of the maltose transport gene (*malA*) of *Bacillus stearothermophilus* (20) also showed extensive amino acid sequence similarity (41.9% identical and 12.3% similar amino acids). Based on the

TABLE 2. Activity of *E. coli* CM89L(pDipLL) cell extracts
on different peptides[a]

Peptide	Hydrolysis[b]
Leu-Leu	+
Leu-Gly	+
Leu-Met	+
Leu-Ile	+
Phe-Leu	+
Phe-Gly	+
Ala-Ala	+
β-Ala-His	+
Leu-Pro	−
Phe-Pro	−
Val-Pro	−
Leu-Leu-Leu	−
Leu-Gly-Leu	−
Leu-Gly-Gly	−
Leu-Gly-Pro	−

[a] The plasmid-free *E. coli* CM89L strain did not break down any of these substrates.
[b] +, peptide hydrolyzed; −, no hydrolysis.

amino acid sequence similarities, ORF1 possibly represents a partially sequenced dipeptidase gene of *B. stearothermophilus* (20). Two regions of both PepVs which conform to the two signature sequences of the ArgE/DapE/ACY1/CPG2/YscS family of proteins were identified (Fig. 4). Thus, this places both dipeptidases in the M20 family of metallopeptidases (30). Apart from the two signature sequences, several of the proteins in this family have additional regions of sequence similarity. All members of the family except the PepT tripeptidases of *L. lactis* and *Salmonella typhimurium* and the ACYI proteins have the sequence [IL]-X-G-X(2)-[SAG]-H-X-[SAG]-X(1,2)-P-X(2)-[SAGT] [sequence shown in PROSITE database notation where either one of the amino acids in brackets is present in that position, X(n) is n number of X amino acids, and X(n,m) is either n or m number of X amino acids] (Fig. 4, box III). Moreover, a sequence previously identified in both PepTs and in CPG2 (23) is also present in the dipeptidases as well as in the DapEs (Fig. 4, box IV). These regions may represent other catalytically or structurally important domains in these proteins.

To assess the role of the dipeptidase in the proteolytic system of *L. lactis*, a mutant that lacked PepV activity was constructed. During growth in milk, no differences in final cell densities were observed between the wild type and the mutant

strain. The growth rate of the mutant was significantly lower than that of the wild type and was almost the same as the growth rate of the PepN mutant. Apparently, the missing dipeptidase activity can be replaced by another peptidase(s) present in the cell, but this peptidase(s) is not as efficient in degrading dipeptidase substrates as the dipeptidase itself. Thus, this is the second peptidase in *L. lactis* whose absence results in a clear growth effect. To better understand which peptidase(s) contributes to the replacement of the dipeptidase activity and to learn more about the relative importance of the dipeptidase for growth in milk, combinations of the *pepV* mutation with other peptidase mutations will be made.

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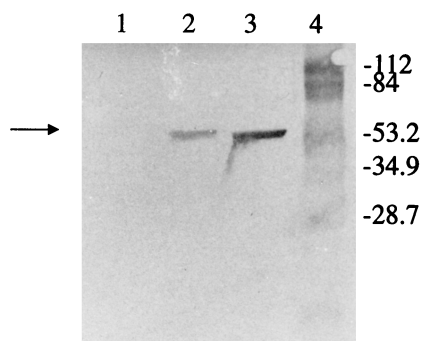
FIG. 5. Western blot analysis with monoclonal antibodies raised against purified dipeptidase of *L. lactis* Wg2. Lanes: 1, MG::pPV5; 2, MG1363; 3, *E. coli* CM89L(pDipLL); 4, standard molecular mass markers, the sizes of which are given in kilodaltons. The arrow indicates the position of PepV antigen.

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