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

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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 not 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 Omp 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, PepF, PepN, PepC, PepP, 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* 537/7 (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 ColEI 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 Leenhearts 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 electroporation 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 PCGene sequence analysis...
Cloning and Analysis of pepV of L. lactis MG1363

TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant phenotype(s) or genotype(s)</th>
<th>Source or reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. lactis subsp. cremoris</td>
<td>Plasmid-free derivative of NCD0712</td>
<td>8</td>
</tr>
<tr>
<td>MG1363</td>
<td>Prt+ Lac+</td>
<td>This work</td>
</tr>
<tr>
<td>MG1363(pLP712)</td>
<td>pepV Prt+ Lac+</td>
<td>This work</td>
</tr>
<tr>
<td>MG1363(pPV5(pLP712)</td>
<td>pepV Semi-prt Lac+</td>
<td>24</td>
</tr>
<tr>
<td>IM5(pLP712)</td>
<td>pepM PepN Prt+ Lac+</td>
<td>24</td>
</tr>
<tr>
<td>IM7(pLP712)</td>
<td>pepT PepM Prt+ Lac+</td>
<td>24</td>
</tr>
<tr>
<td>E. coli NM522</td>
<td>supE thi (lac-proAB) Dhsd5 (r6- m- ) [F' proAB lacPZΔM15]</td>
<td>9</td>
</tr>
<tr>
<td>CM89</td>
<td>leu-9 (pro-lac) met thy pepN102 pepA11 pepB1 pepQ10</td>
<td>26</td>
</tr>
<tr>
<td>MM38K26</td>
<td>argG6 asaA11 (asaA32) his-1 leuB6 metB1 pyrE gal-6 lacY1 xyl-7 supE44 bg1</td>
<td>21</td>
</tr>
<tr>
<td>CM89L</td>
<td>frhA2 gyrA3 papL104 tss- trace ΔpncB K10</td>
<td>This work</td>
</tr>
<tr>
<td>EC1000</td>
<td>repA+ derivative of MC89</td>
<td>17</td>
</tr>
</tbody>
</table>

Plasmids

pUC19 | Ap' | 44 |
| pOR128 | Em' ori' of pWW01; Rep- | 17 |
| pDipLL | Ap'; pUC19 with 3.6-kb MG1363 chromosomal Sau3A fragment carrying pepV | This work |
| pPV6 | pUC19 with internal HindIII-PstI fragment of pepV | This work |
| pPV5 | Em' ; pOR128 with 570-bp HindIII-Asp718 fragment of pPV6 | This work |

Construction of a pepV mutant. The internal HindIII-PstI fragment of pepV was cloned in pUC19. This plasmid (pPV6) was digested with HindIII and Asp718, and the 570-bp fragment was ligated into the integration vector pOR128 (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 carotenoid compound. The correct construct was then transferred to the resulting strain, CM89L. Approximately 2,400 transformants were assayed for dipeptidase activity. Plasmid DNA was isolated from the two isolates which obtained the correct pepV sequence 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 RNA of L. lactis with a ΔG° of 12.8 kcal/mol (5.3 kJ/mol) (5, 36). Upstream of the ribosome binding site, a promoter-like structure, consisting of the −35 hexanucleotide TTAGC, a spacing of 18 bp, and the −10 sequence TACAAT, is present. A
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 210 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 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%
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, *pir* 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 in milk of *L. lactis* MG1363(pLP712), the mutant IM5(pLP712), and IM7(pLP712) were significantly lower than that of the wild type (maximal growth rate of *L. lactis* MG1363 in milk, 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 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

![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 amino acids in all four proteins are indicated by stars and dots, respectively. Regions of extensive similarity (I, II, III, and IV) are boxed.](https://www.jb.asm.org/content/179/3/3413)
amino acid sequence similarities, ORF1 possibly represents a partially sequenced dipeptidase gene of *B. stea thermoophilus* (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 ACY1 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 III). Moreover, a sequence previously identified in both PepTs and in CPG2 (23) is also present in the dipeptidases as well as.

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 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.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-Leu</td>
<td>+</td>
</tr>
<tr>
<td>Leu-Gly</td>
<td>+</td>
</tr>
<tr>
<td>Leu-Met</td>
<td>+</td>
</tr>
<tr>
<td>Leu-Ile</td>
<td>+</td>
</tr>
<tr>
<td>Phe-Leu</td>
<td>-</td>
</tr>
<tr>
<td>Phe-Gly</td>
<td>-</td>
</tr>
<tr>
<td>Ala-Ala</td>
<td>-</td>
</tr>
<tr>
<td>β-Ala-His</td>
<td>-</td>
</tr>
<tr>
<td>Leu-Pro</td>
<td>-</td>
</tr>
<tr>
<td>Phe-Pro</td>
<td>-</td>
</tr>
<tr>
<td>Val-Pro</td>
<td>-</td>
</tr>
<tr>
<td>Leu-Leu-Leu</td>
<td>-</td>
</tr>
<tr>
<td>Leu-Gly-Leu</td>
<td>-</td>
</tr>
<tr>
<td>Leu-Gly-Gly</td>
<td>-</td>
</tr>
<tr>
<td>Leu-Gly-Pro</td>
<td>-</td>
</tr>
</tbody>
</table>

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

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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.