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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.

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 pCP10 mutant of E. coli CM89 was made. This strain (CM98L) was created by P1 transduction of CM89 with a lysate made on E. coli MM38K26. Inactivation of the pCP10 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 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 program.
and incubation was prolonged for another 10 min at 42°C. The primer extension analysis of RNA in a reaction mixture containing dCTP, dGTP, dTTP, and \( \text{primer extension analysis} \) was done with avian myeloblastosis virus reverse transcriptase of RNA from positions 136 to 158 was used for probe. A synthetic oligonucleotide (pepvTS, \( 5' \text{TCAGC} \)) was added to 5 \text{nanograms of primer}. 

Probe. A synthetic oligonucleotide (pepvTS, \( 5' \text{TCAGC} \)) complementary to the mRNA from positions 136 to 158 was used for primer extension analysis. Twenty-five nanograms of primer was added to 5 \text{nanograms of primer}. 

**Strains**

<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><strong>L. lactis subsp. cremoris</strong></td>
<td>Plasmid-free derivative of NCD0712</td>
<td>8</td>
</tr>
<tr>
<td>MG1363</td>
<td>Prt+ Lac+</td>
<td>8</td>
</tr>
<tr>
<td>MG1363(pLP712)</td>
<td>pepV</td>
<td>This work</td>
</tr>
<tr>
<td>MGCl pepV (pLP712)</td>
<td>pepV Prt+ Lac+</td>
<td>This work</td>
</tr>
<tr>
<td>IM5(pLP712)</td>
<td>pepN Prt+ Lac+</td>
<td>24</td>
</tr>
<tr>
<td>IM7(pLP712)</td>
<td>pepX pepN Prt+ Lac+</td>
<td>24</td>
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</table>

**E. coli**

<table>
<thead>
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<th>Relevant phenotype(s) or genotype(s)</th>
<th>Source or reference(s)</th>
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<tr>
<td>NM522</td>
<td>supE thi Dlac-proAB Dhsd5 (( \text{tr}^{-} ) ( \text{m}^{-} )) [F' proAB lacFZDAM15]</td>
<td>9</td>
</tr>
<tr>
<td>CM89</td>
<td>leu-9 Dpro-lac met thy-4 pepN102 pepA11 pepBI pepQ10</td>
<td>26</td>
</tr>
<tr>
<td>MM38K26</td>
<td>argG6 asnA1 (or asnB2) his-1 leuB6 metB1 pyrE gal-6 lacY1 xyl-7 supE44 bgl</td>
<td>21</td>
</tr>
<tr>
<td>fhuA2 glyA4 rpsL104 tss-1 uhp DpcnB Km'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM89L</td>
<td>CM89 derivative; pcnB Km'</td>
<td>This work</td>
</tr>
<tr>
<td>EC1000</td>
<td>repA+ derivative of pcnB Km'</td>
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**Plasmids**

<table>
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<tr>
<th>Plasmid</th>
<th>Relevant phenotype(s) or genotype(s)</th>
<th>Source or reference(s)</th>
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</thead>
<tbody>
<tr>
<td>pUC19</td>
<td>Ap'</td>
<td>44</td>
</tr>
<tr>
<td>pOR128</td>
<td>Em'; ori' of pWV01; Rep'</td>
<td>17</td>
</tr>
<tr>
<td>pDipLL</td>
<td>Ap'; pUC19 with 3.6-kb MG1363 chromosomal Sau3A fragment carrying pepV</td>
<td>This work</td>
</tr>
<tr>
<td>pPV6</td>
<td>Ap'; pUC19 with internal HindIII-PstI fragment of pepV</td>
<td>This work</td>
</tr>
<tr>
<td>pPV5</td>
<td>Em'; pOR128 with 570-bp HindIII-Asp718 fragment of pPV6</td>
<td>This work</td>
</tr>
</tbody>
</table>

**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 in 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-pvP5. 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 following: Em; Chromogenix AB, Mölndal, Sweden) 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 dipetidase gene of L. lactis.** To reduce the copy number of pUC plasmids (21) and, thus, to minimize possible problems of cloning peptide genes, the pcnB gene of the E. coli multiple peptide 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 dipetidase activity. Plasmid DNA was isolated from the two isolates obtained which showed dipetidase 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 dipetidase purified from L. lactis Wg2 (39). By homology and mutation studies (see below), the ORF was identified as the dipetidase 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 \( \Delta G^0 \) of 12.8 kcal/mol (55.3 kJ/mol) (5, 36). Upstream of the ribosome binding site, a promoter-like structure, consisting of the -35 hexanucleotide TTGACG, a spacing of 18 bp, and the -10 sequence TACAAT, is present. A
16-bp inverted repeat ($\Delta G$ [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 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, ppr 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 pepV mutant IM8 (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 (μmax = 0.58) were significantly lower than that of the wild type (μmax = 0.71). The growth rates in GM17 medium were similar for all strains (μmax = 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 (maaA) of Bacillus stearothermophilus (20) also showed extensive amino acid sequence similarity (41.9% identical and 12.3% similar amino acids). Based on the
amino acid sequence similarities, ORF1 possibly represents a partially sequenced dipeptidase gene of *B. stea-thermophilus* (20). Two regions of both PepVs which conform to the two signature sequences of the ArgE/DapE/ACY1/CPG2/YseS 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 tripeptidase 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] 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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**REFERENCES**