Effect of X-Prolyl Dipeptidyl Aminopeptidase Deficiency on Lactococcus lactis

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The genetic determinant (pepXP) of an X-prolyl dipeptidyl aminopeptidase (PepXP) has recently been cloned and sequenced from both Lactococcus lactis subsp. cremoris (B. Mayo, J. Kok, K. Venema, W. Bockelmann, M. Teuber, H. Reinke, and G. Venema, Appl. Environ. Microbiol. 57:38-44, 1991) and L. lactis subsp. lactis (M. Nardi, M.-C. Chopin, A. Chopin, M.-M. Cals, and J.-C. Gripon, Appl. Environ. Microbiol. 57:45-50, 1991). To examine the possible role of the enzyme in the breakdown of caseins required for lactococci to grow in milk, integration vectors have been constructed and used to specifically inactivate the pepXP gene. After inactivation of the gene in L. lactis subsp. lactis MG1363, which is Lac- and Prt+, the Lac+ Prt+ determinants were transferred by conjugation by using L. lactis subsp. lactis 712 as the donor. Since growth of the transconjugants relative to the PepXP+ strains was not retarded in milk, it was concluded that PepXP is not essential for growth in that medium. It was also demonstrated that the open reading frame ORF1, upstream of pepXP, was not required for PepXP activity in L. lactis. A marked difference between metenkephalin degradation patterns was observed after incubation of this pentapeptide with cell extracts obtained from wild-type lactococci and pepXP mutants. Therefore, altered expression of the pepXP-encoded general dipeptidyl aminopeptidase activity may change the peptide composition of fermented milk products.

For proper growth in milk, lactococci need a complete and functional proteolytic system. The cell-envelope-associated protease is the first enzyme active in casein degradation (12, 13). This essential enzyme degrades casein to large proline-rich peptide fragments (23), which are likely to be further degraded to peptides and amino acids which can be taken up by the cells (26). Although a wide variety of peptidases in lactococci have been characterized (for a review, see reference 33), it is uncertain which of these are essential in the caseinolytic system.

The presence of an X-prolyl dipeptidyl aminopeptidase (PepXP; formerly designated X-PDAP) capable of releasing dipeptides from oligopeptides containing proline at the penultimate position has been demonstrated in a wide range of lactic acid bacteria (3). It has been suggested that PepXP could participate in the degradation of the oligopeptides generated by the protease, producing dipeptides that could be taken up by the di- and tripeptide uptake system (3, 31). Alternatively, the dipeptides might be produced intracellularly from internalized oligopeptides. Both the oligopeptide and the di- and tripeptide uptake systems have been proven to be essential for the growth of lactococci in milk (30, 35).

Recently, the genes specifying PepXP (pepXP) have been cloned and sequenced from both Lactococcus lactis subsp. cremoris P8-2-47 (20) and L. lactis subsp. lactis NCDO 763 (24). The availability of the pepXP gene has prompted us to examine the role of PepXP in casein degradation. A second open reading frame, ORF1, in opposite relative orientation to pepXP, appeared to be present in close proximity to pepXP on the lactococcal chromosome (20, 24). The putative ORF1 protein showed considerable amino acid similarity to integral membrane proteins, such as the Escherichia coli glycerol-facilitator protein (20). The putative transcription and translation signals of both pepXP and ORF1 were located in a small intergenic DNA fragment of 171 nucleotides.

In this article, we describe the construction of PepXP-negative mutants via replacement recombination and examined their growth in milk. Moreover, in chromosomal deletion mutants lacking both pepXP and ORF1, pepXP was introduced under the control of the lactococcal promoter P59 (38) to investigate whether ORF1 was involved in PepXP activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used are listed in Table 1. L. lactis was grown in M17 medium (Difco, East Molesey, United Kingdom) with 0.5% glucose (GM17) or lactose (LM17) at 30°C. E. coli was grown in TY broth (Difco, Detroit, Mich.) at 37°C, with shaking. Skim milk was obtained by dissolving skim milk powder (Oxoid Ltd., Basingstoke, United Kingdom) to a 10% (wt/vol) concentration in distilled water. Milk agar plates consisted of 10% skim milk and 1.5% agar. The composition of the chemically defined medium was as previously described (25). Ampicillin (50 µg/ml for E. coli), erythromycin (100 µg/ml for E. coli; 5 µg/ml for L. lactis), or chloramphenicol (10 µg/ml for E. coli) was added to the medium when required.

Growth and acid production. Cells were routinely grown in sterile skim milk (heated at 100°C three times) at 30°C and subcultured daily. For the estimation of cell counts and acid production, 75 ml of skim milk was inoculated with 0.5 to 1% exponentially growing bacteria. The pH was monitored continuously with a sterile pH electrode. For the estimation of cell numbers, samples were taken and the bacteria were...
plated on M17 agar. The rate of acid production was calculated by the equation (pH<sub>1</sub> - pH<sub>0</sub>)/(t<sub>1</sub> - t<sub>0</sub>).

**DNA manipulations and sequencing.** Chromosomal DNA was isolated from *L. lactis* by the method of Leenhouts et al. (15). Plasmids from *E. coli* were isolated as described by Birnboim and Doly (2). Plasmid DNA from *L. lactis* was isolated by using the same method with minor modifications (36). Restriction endonucleases and T4 DNA ligase were purchased from Boehringer (Mannheim, Germany) and used as recommended by the supplier, and Taq polymerase was purchased from Promega (Madison, Wis.). General procedures for DNA manipulations were essentially as described by Maniatis et al. (19). Oligonucleotide synthesis and polymerase chain reaction.

**Oligonucleotide synthesis and polymerase chain reaction.** Synthetic primers were synthesized on Synthesizer 380 A (Applied Biosystems, Inc., Foster City, Calif.) by the β-cyanoethyl phosphoramidite procedure (29). The synthesized primers were A (5’-GAG TTA ATC GTG GGG 3’) and B (5’-CAG CAG TTT CCT GGA 3’).

The conditions for the polymerase chain reactions were essentially those described by Gould et al. (7). Denaturation was for 1 min at 94°C, annealing was for 2 min at 50°C, and polymerization was for 2 min at 72°C. The cycle was repeated 30 times. The product was digested with *ClaI* and *XhoI* (the recognition sites for these enzymes on the primers are underlined above) and ligated with *AccI* and *BamHI*-digested pUC18 (39).

**Conjugation.** Surface mating conjugations were performed essentially as described by McKay et al. (21). Donor and recipient cultures, grown overnight in M17 medium at 30°C, were mixed in a 10:1 (vol/vol) ratio, and 0.1 ml of the mixture was streaked on GM17 agar. After overnight incubation at 30°C, cells were harvested and plated onto appropriate media.

**Southern hybridization.** DNA was transferred to GeneScreen Plus membranes (Dupont NEN, Boston, Mass.) as described by Maniatis et al. (19). Nonradioactive DNA probes were labeled with digoxigenin-dUTP by using a nonradioactive labelling and detection kit (Boehringer). Hybridization and immunological detection were done as recommended by the supplier.

**Plate assay for PepXP and enzyme activity in crude cell extracts.** Colonies were screened for PepXP activity by a plate staining procedure described by Miller and MacKinon (22). Colonies were overlaid with 0.5% (wt/vol) agarose in distilled water and then flooded with a mixture of 0.2 ml of 1-glycyl-1-prolyl-β-naphthylamide (Bachem, Budendorf, Switzerland) solution in N,N-dimethyl formamide and 5 ml of fast garnet GBC salt (Sigma Chemical Co., St. Louis, Mo.) in 0.2 M of Tris hydrochloride buffer (pH 7.0). After a few minutes of incubation at room temperature, PepXP-positive colonies stain dark red whereas negative colonies remain white.

Crude cell extracts were prepared from late-log-phase M17 broth cultures. Cells were harvested by centrifugation, washed twice in 0.1 M phosphate buffer (pH 7.0), and resuspended in a solution of 0.5 M sucrose in 50 mM Tris hydrochloride buffer (pH 7.5) containing 10 mg of lysozyme per ml. The cell suspension was incubated for 2 h at 37°C, washed twice with 0.5 M sucrose in 50 mM Tris hydrochloride buffer (pH 7.0), and resuspended in 30 mM of phosphate buffer (pH 7.0). Protoplasts were disrupted by sonication (Soniprep 150; MSE Scientific Instruments, Crawley, United Kingdom), and the resulting suspension was centrifuged at 80,000 x g for 20 min in an L-8-80 centrifuge (Beckman, Palo Alto, Calif.) to remove cell debris. PepXP activity in the

### TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or phenotypea</th>
<th>Reference or source</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>E. coli</em> JM103</td>
<td>F' traD36 lacI&lt;sup&gt;+&lt;/sup&gt;(lacZ)M15 proAB/endoA1 supE sbcBC thi-1 rpsL (Str') (lac-pro)</td>
<td>39</td>
</tr>
<tr>
<td><em>L. lactis</em> subsp. lactis</td>
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<td></td>
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<tr>
<td>IL1403</td>
<td>Plasmid-free PepXP&lt;sup&gt;+&lt;/sup&gt; strain</td>
<td>4</td>
</tr>
<tr>
<td>MG1363</td>
<td>Plasmid-free PepXP&lt;sup&gt;+&lt;/sup&gt; strain</td>
<td>6</td>
</tr>
<tr>
<td>BM13</td>
<td>PepXP&lt;sup&gt;+&lt;/sup&gt; mutant of IL1403 obtained by chemical mutagenesis</td>
<td>This work</td>
</tr>
<tr>
<td>ML336</td>
<td>Em&lt;sup&gt;+&lt;/sup&gt; PepXP&lt;sup&gt;+&lt;/sup&gt; MG1363 carrying an Em&lt;sup&gt;+&lt;/sup&gt; marker disrupting pepXP and ORF1</td>
<td>This work</td>
</tr>
<tr>
<td>BM331</td>
<td>Em&lt;sup&gt;+&lt;/sup&gt; PepXP&lt;sup&gt;+&lt;/sup&gt; MG1363 carrying an Em&lt;sup&gt;+&lt;/sup&gt; marker disrupting pepXP and ORF1</td>
<td>This work</td>
</tr>
<tr>
<td>NCD0 712</td>
<td>Wild-type strain carrying the 55-kb lac&lt;sup&gt;-&lt;/sup&gt; pr&lt;sup&gt;-&lt;/sup&gt; conjugative plasmid pLP712</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pUC18</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; 2.68-kb lacZa pBR322 derivative</td>
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<tr>
<td>pUC19E</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Em&lt;sup&gt;+&lt;/sup&gt; carrying the Em&lt;sup&gt;+&lt;/sup&gt; gene of pE194 (9) in the SmaI site</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>pBM330</td>
<td>5.4-kb partial XbaI fragment carrying pepXP and ORF1 in pUC18</td>
<td>20</td>
</tr>
<tr>
<td>pBM329</td>
<td>5.4-kb partial XbaI fragment carrying pepXP and ORF1 in pGKV2</td>
<td>20</td>
</tr>
<tr>
<td>pBM331</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Em&lt;sup&gt;+&lt;/sup&gt;, 8.5 kb, replacement of the 0.7-kb FspI-HindIII fragment for an Em&lt;sup&gt;+&lt;/sup&gt; gene</td>
<td>This work</td>
</tr>
<tr>
<td>pML336</td>
<td>Em&lt;sup&gt;+&lt;/sup&gt; 9.1 kb, carrying an Em&lt;sup&gt;+&lt;/sup&gt; gene inserted in the BglII site of pepXP</td>
<td>17</td>
</tr>
<tr>
<td>pGKV259</td>
<td>Em&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt;, 4.9 kb, pGKV210 derivative containing the lactococcal promoter P59</td>
<td>38</td>
</tr>
<tr>
<td>pGKV330</td>
<td>Em&lt;sup&gt;+&lt;/sup&gt;, 8 kb, pGKV259 derivative containing pepXP controlled by promoter P59</td>
<td>This work</td>
</tr>
<tr>
<td>pAMS100</td>
<td>Em&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt;, 8.1 kb, pAM81 derivative</td>
<td>R. Kiewiet (unpublished data)</td>
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**a** Ap<sup>+</sup>, resistance to ampicillin.
supernatant was measured as described by Miller and MacKinnon (22) by using L-alanyl-L-prolyl-p-nitroanilide (Bachem) as the substrate. One unit of activity was defined as the amount of enzyme catalyzing the formation of 1.0 pmol of p-nitroanilide min⁻¹ (the molar absorption coefficient of p-nitroanilide at 405 nm is 9.62 × 10⁵ M⁻¹ cm⁻¹). Protein concentrations were measured by the method of Lowry et al. (18).

**SDS-PAGE and immunoblotting.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done as described previously (14). The proteins from cell extracts were transferred to Immobilon-P membranes (Millipore Corp., Bedford, Mass.) with an electroblotter (Bio-Rad). The Immobilon-P membranes were saturated with 1% skim milk and incubated with polyclonal antibodies against PepXP (1/5,000) (24). After washing with 10 mM Tris hydrochloride (pH 8.0) containing 150 mM NaCl and 0.05% Tween 20 (TBST), the membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin serum (Sigma) diluted 1/1,000. Subsequently, the membranes were washed with TBST and the enzyme-antibody complex was visualized by incubating the membrane in carbonate buffer (0.1 M NaHCO₃, 1.0 mM MgCl₂ [pH 9.8]) containing 1% (vol/vol) nitroblue tetrazolium (15 mg/ml) in 70% di-methyl formamide and 1% (vol/vol) 5-bromo-4-chloro-3-indolylphosphate toluidinium (salt) (15 mg/ml) in 100% di-methyl formamide.

**Peptide hydrolysis and separation.** A mixture consisting of 10 µl of a cell extract of *L. lactis*, 30 µl of 50 mM Tris-maleate (pH 6.0), and 10 µl of 10 mM metenkephalin (Bachem) was incubated for 30 min at 40°C. The reaction was stopped by the addition of 60 µl of 1% (wt/vol) trichloroacetic acid. The composition of the resulting peptide mixture was analyzed by capillary electrophoresis with a standard 72-cm capillary on a 270A capillary electrophoresis unit (Applied Biosystems, Weiterstadt, Germany). Samples were injected for 15 s at 5 kV; for the subsequent electrophoresis, 20 mM citrate buffer (pH 5.2) was used and a voltage of 30 kV was applied. After separation, peptides were detected by measuring the A₂⁰₀. Metenkephalin hydrolysis products were identified by comparing their retention times with that of metenkephalin (Tyr-Gly-Gly-Phe-Met) and some of its subsequences: Gly-Gly, Tyr-Gly, Tyr-Gly-Gly, Phe-Met, Gly-Phe-Met, Gly-Gly-Phe-Met, Tyr, and Phe.

**RESULTS**

**Construction of pBM331.** The plasmid pBM330 contains a 5.5-kb partial XbaI chromosomal DNA fragment of *L. lactis* subsp. *cremoris* P8-2-47 specifying PepXP activity as has been described elsewhere (20). In addition to the pepXP gene, an oppositely oriented open reading frame with unknown function was present on the same chromosomal fragment (ORF1). To create stable and well-defined mutants for both pepXP and ORF1 by replacement recombination, a pUC-derived integration vector, pBM331, was constructed. Plasmid pBM331 (Fig. 1) was made by replacing the 0.7-kb FspI-HindIII fragment of pBM330 for a 1.1-kb PvuII-HindIII fragment from pUC9E containing an erythromycin resistance (Em') gene. The recombinant plasmid, pBM331, was obtained in *E. coli* by selection on ampicillin and erythromycin. In this way, the transcriptional and translational regulatory sequences and more than 30 codons of both pepXP and ORF1 were removed.

**Inactivation of the chromosomal pepXP gene and ORF1.** Since the pepXP genes from different lactococcal strains showed more than 99% nucleotide sequence identity (20, 24), it was feasible to use pBM331 as an integration vector in *L. lactis* subsp. *lactis* MG1363. Electrotransformation of the plasmid-free lactococcal strain MG1363 with covalently closed circular pBM331 DNA resulted in approximately 60 erythromycin-resistant CFU/µg of plasmid DNA. Integration of the plasmid via a single crossover (Campbell-like integration) will result in the original phenotype (PepXP⁺). However, integration via a double crossover (gene replacement) of lactococcal DNA flanking the Em' marker will produce a PepXP⁻ phenotype (Fig. 2). From 712 Em' colonies recovered after electrotransformation, only one showed a PepXP⁻ phenotype. This PepXP⁻ transformant obtained with plasmid pBM331, designated BM331, was analyzed by Southern hybridization. Chromosomal DNA of this strain, that of the recipient strain MG1363 and, as a control, that of strain ML336 in which the erythromycin resistance gene exclusively disrupts the coding region of pepXP but not that of ORF1 (designated MG33611 in reference 17) were digested with HindIII. The PvuII fragment of approximately 2.2 kb spanning part of the coding region of pepXP and the entire ORF1 was used as a probe. Figure 2 shows the results of these hybridizations, which indicate that a double crossover event had taken place in the pBM331-derived integrant and that the hybridizing bands in BM331 are identical to those of pBM331: in the mutant, the 2.2-kb pepXP-ORF1 PvuII fragment hybridized with a 2.5-kb fragment instead of with a 2.1-kb HindIII fragment in the wild type, the size shifts being due to the replacement of the 0.7-kb FspI-HindIII fragment by the 1.1-kb Em' gene in the mutant chromosome.

**Immunoblotting of extracts and PepXP activity.** Polyclonal antibodies raised against PepXP of *L. lactis* subsp. *lactis* were used to judge whether the PepXP-synthesizing capacity of the mutants obtained by replacement recombination had been lost. The results are shown in Fig. 3. PepXP was visualized in PepXP⁺ strains as a clear band of about 85 kDa, which was absent in both integrant mutants ML336 and BM331. A band of lower molecular weight was still visible in the extract of the undefined PepXP⁻ mutant BM13, obtained by chemical mutagenesis, that also lacked a cross-reacting band present in all other strains. Much more antigen is produced by BM13 (pBM329) apparently because the pepXP gene was inserted on a plasmid with a copy number of about 10 (37). Actually, in contrast to what was stated before (20), the PepXP activity correlated well with the gene dosage. In crude cell extracts of the *L. lactis* subsp. *lactis* BM13 strain carrying pBM329, the PepXP activity had increased 10-fold (3.920 U/mg of protein compared to 0.337 U/mg of protein in *L. lactis* IL1403 and 0.316 U/mg of protein in *L. lactis* subsp. *cremoris* P8-2-47). No activity could be detected in *L. lactis* subsp. *lactis* BM13, produced by chemical mutagenesis, or in the replacement mutants (*L. lactis* subsp. *lactis* ML336 and BM331).

**Construction of pGKV330.** To examine the possible involvement of ORF1 in the PepXP⁺ phenotype, the promoter region from which transcription of both pepXP and ORF1 is driven was replaced by the lactococcal promoter P59 in such a way that only pepXP can be expressed. To this purpose, two synthetic primers were used to synthesize, by the polymerase chain reaction, a 198-bp DNA fragment of the 5'-end of the pepXP gene, lacking the promoter region but carrying the putative ribosome binding site. A C.lai site was designed 10-bp upstream of this ribosome binding site (Fig. 1). This fragment was cloned in pUC18 and checked by DNA

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sequence analysis. An XbaI-BamHI fragment from one of the clones was isolated and ligated to a 2.5-kb XhoII-SalI fragment from pBM330 carrying the 3′-end of pepXP. BamHI and XhoII were the only compatible ends. After ligation, XbaI and SalI sticky ends were made blunt by treatment with Klenow enzyme. pGKV259, a pGKV210 derivative carrying a promoter-containing fragment PS9 from L. lactis subsp. cremoris Wg2 (38), was digested with XbaI, and blunt ends were produced by incubation with the Klenow enzyme. The two fragments were ligated, and the ligation mixture was used to transform competent cells of E. coli JM103 and for electrotransformation of L. lactis subsp. lactis BM13. After screening for PepXP activity by the enzymatic plate assay, several colonies of L. lactis had recovered the parental PepXP phenotype, and some colonies of E. coli had acquired PepXP activity. The new plasmid was designated pGKV330. In crude cell extracts of L. lactis strains carrying pGKV330, the PepXP activity was increased twofold (0.663 U/mg of protein compared to 0.337 U/mg of protein in L. lactis subsp. lactis IL1403). DNA sequence analysis of the BamHI-XhoII junction point of pGKV330 isolated from an L. lactis strain showed that the XhoII restriction enzyme site was restored as expected and that the region surrounding this site was identical to that of the published pepXP sequence.

ORF1 is not required for PepXP activity. Plasmid pGKV330 was introduced into L. lactis subsp. lactis BM331. In the construction of pGKV330, the Cm' gene of pGKV259 had been destroyed and partially replaced by the pepXP gene. Since, as a consequence of the presence of the erythromycin resistance determinant on the chromosome, BM331 is Em', we could not select for pGKV330 by using erythromycin. To introduce pGKV330 in L. lactis subsp. lactis BM331, a cotransformation procedure was used (10) with the selectable plasmid pAMS100, a pAMβ1 derivative (chloramphenicol resistant [Cm']) compatible with pGK-derived plasmids (11a). L. lactis subsp. lactis BM331 was electrotransformed with a mixture consisting of 5 µg of pGKV330 and 0.5 µg of pAMS100, and transformants were selected on agar plates containing 5 µg of chloramphenicol per ml. As a control, L. lactis subsp. lactis ML336, which carries an intact ORF1, was electrotransformed under the
same conditions with the same amount of the DNA mixture. Cm' transformants obtained from both electrotransformations were screened for PepXP activity by the enzymatic plate assay. In both cases, several PepXP+ colonies were obtained with a cotransformation frequency of 12%. All PepXP+ transformants examined contained pBM330 and pAMS100 (data not shown). Thus, despite the fact that the chromosomal ORF1 in L. lactis subsp. lactis BM331 (pGKV330) was truncated, and ORF1 had been deleted from the plasmid, the strain was PepXP+. This indicates that ORF1 is not required for PepXP activity.

Restoration of the Lac+ and Prt+ phenotypes by conjugation. Since the MG1363-derived integrant obtained with pBM331 lacked both the proteinase and lactose genes, this strain could not be used to examine the possible requirement of the pepXP gene for the cells to grow in milk. The lac and prt genes were introduced in strain BM331 by conjugation. L. lactis subsp. lactis NCDO 712 carrying the lac and prt genes on the 55-kb conjugative plasmid pLP712 (6) was chosen as the donor. L. lactis subsp. lactis ML336 and BM331 were used as recipients. Since the donor is Em+, only Lac+ transconjugants will produce normal-sized colonies on LM17-erythromycin plates, and only Lac+ Prt+ transconjugants will form large colonies on milk agar plates containing erythromycin if the milk cases can be used by the mutants for growth. The Lac+ Prt+ transconjugants formed normal-sized colonies on the milk agar plates and had not converted to a PepXP+ phenotype, as judged from the enzymatic plate assay. Apparently, the pepXP gene was not required for growth on milk-based agar plates.

Growth and acid production of PepXP- cells in milk. For the estimation of cell counts and acid production, 10% reconstituted skim milk was inoculated with 1% (vol/vol) exponentially growing cells of the transconjugants BM331 (pLP712) and ML336(pLP712). L. lactis subsp. lactis NCDO 712 was used as control. Table 2 shows the results of the growth experiments. The rate of acid production by the PepXP- mutants did not significantly differ from the wild-type strain L. lactis subsp. lactis NCDO 712. Furthermore, both mutated strains ML336 and BM331 produced the same number of cells as the wild-type strain after exponential growth (results not shown).

Peptide hydrolysis by PepXP+ cells. PepXP is capable of removing the N-terminal dipeptide Tyr-Gly from the pentapeptide metenkephalin (Tyr-Gly-Gly-Phe-Met). Metenkephalin was digested to the N-terminal dipeptide Tyr-Gly by PepXP+ cells of L. lactis subsp. lactis MG1363 and BM331.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rate of acid production (ΔpH/h)</th>
<th>SD (n = 4)</th>
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<tbody>
<tr>
<td>NCDO 712</td>
<td>0.34</td>
<td>0.070</td>
</tr>
<tr>
<td>ML336</td>
<td>0.34</td>
<td>0.040</td>
</tr>
<tr>
<td>BM331</td>
<td>0.33</td>
<td>0.080</td>
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* The values given are averages of four separate experiments.
lin was incubated with cell extracts of the wild-type strain *L. lactis* NCDO 712 and the PepXP-deficient lactococcal strains ML336 and BM331. The resulting peptide mixtures were analyzed by using capillary electrophoresis. Upon incubation of metenkephalin with the cell extract of the wild-type strain NCDO 712, degradation products specific for the action of the lactococcal endopeptidase PepO (Tyr-Gly-Gly and Phe-Met) (34), a general aminopeptidase (Gly-Gly-Phe-Met and Tyr), and PepXP (Tyr-Gly and Gly-Phe-Met) were found (Fig. 4b). However, when metenkephalin was incubated with a cell extract from either of the pepXP mutants, only the degradation products specific for the action of the endopeptidase and the general aminopeptidase were observed, whereas the peptides Tyr-Gly and Gly-Phe-Met could not be detected (Fig. 4a). These results clearly indicate that not only the PepXP activity but also the more general dipeptidyl aminopeptidase activity was eliminated upon disruption of the lactococcal pepXP gene.

**DISCUSSION**

It has recently been shown that foreign DNA can be integrated into the chromosome of *L. lactis* subsp. *lactis* by a Campbell-type recombination (5, 15, 16) as well as by a means of gene replacement technology (17). We have used pBM331, a pUC derivative unable to replicate in *L. lactis*, to specifically inactivate pepXP and ORF1 jointly in *L. lactis* subsp. *lactis* MG1363. In crude cell extracts of the resulting mutant BM331 and in those of the mutant strain ML336, in which only pepXP was mutated (17), no PepXP activity was detectable, indicating that pepXP had indeed been inactivated. The PepXP activity in strain BM331, carrying both a disrupted pepXP and ORF1, was restored upon introduction of plasmid pGKV330 carrying pepXP only, indicating that ORF1 is not required for PepXP activity.

By means of conjugation, the lac” and pr+ genes were introduced in the PepXP+ integrants. The resulting strains grew equally fast in reconstituted skim milk and coagulated the milk as quickly as the wild-type *L. lactis* subsp. *lactis* 712 did. Therefore, neither pepXP nor the adjacent ORF1 is essential for this bacterium to grow in milk.

The location of PepXP has been a matter of discussion: both intracellular (40) and extracellular (11) locations have been suggested. The pepXP gene product was apparently not subject to processing at the N terminus (20, 24). Immunoblotting of the proteins from membrane vesicles with polyclonal antibodies and immunogold labeling of intact cells and protoplasts have recently revealed that PepXP is located intracellularly (32). This intracellular location is in agreement with the present observation that enzyme is not essential for lactococci to grow in milk. The fact that PepXP− mutants grow in milk can be explained as follows: (i) the PepXP is not involved in the casein degradation cascade and, therefore, does not have an essential role in the removal of N-terminal X-prolyl dipeptides from casein fragments and/or (ii) in the mutant, the function of the PepXP is taken over by the combined action of a general aminopeptidase and a proline iminopeptidase (1).

As illustrated in Fig. 4, disruption of the pepXP gene does not impair efficient degradation of metenkephalin; however, in the absence of the enzyme, there is a marked difference in the resulting peptide profile. The present results therefore suggest that although the enzyme PepXP is not essential for the growth of lactococci in milk, altered expression of the pepXP gene may have a dramatic impact on the peptide composition of fermented milk products.

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**REFERENCES**

35. Tynkynen, S. Personal communication.