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Published in:
Applied and environmental microbiology

Publication date:
1993

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1993, p. 2049–2055
0099-2240/93/072049-07$02.00/0

Vol. 59, No. 7

Effect of X-Prolyl Dipeptidyl Aminopeptidase Deficiency on *Lactococcus lactis*

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Received 8 February 1993/Accepted 20 April 1993

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 proteinase 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 has 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 proteinase, 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 interjacent 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

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype[a]	Reference or source
Strains		
E. coli JM103	F' *traD36 lacI*^{q} (*lacZ*)M15 *proAB/endA1 supE sbcBC thi-1 rpsL* (Str^r) (*lac-pro*)	39
L. lactis subsp. *lactis*		
IL1403	Plasmid-free PepXP$^+$ strain	4
MG1363	Plasmid-free PepXP$^+$ strain	6
BM13	PepXP$^-$ mutant of IL1403 obtained by chemical mutagenesis	This work
ML336	Em^r PepXP$^-$ MG1363 carrying an Em^r marker in the coding sequence of the *pepXP* gene	17
BM331	Em^r PepXP$^-$ MG1363 carrying an Em^r marker disrupting *pepXP* and ORF1	This work
NCDO 712	Wild-type strain carrying the 55-kb *lac prt* conjugative plasmid pLP712	6
Plasmids		
pUC18	Ap^r 2.68-kb *lacZ*α pBR322 derivative	39
pUC19E	Ap^r Em^r pUC19 carrying the Em^r gene of pE194 (9) in the *Sma*I site	Laboratory collection
pBM330	5.4-kb partial *Xba*I fragment carrying *pepXP* and ORF1 in pUC18	20
pBM329	5.4-kb partial *Xba*I fragment carrying *pepXP* and ORF1 in pGKV2	20
pBM331	Ap^r Em^r, 8.5 kb, replacement of the 0.7-kb *Fsp*I-*Hin*dIII fragment for an Em^r gene in pBM330	This work
pML336	Ap^r Em^r, 9.1 kb, carrying an Em^r gene inserted in the *Bgl*I site of *pepXP*	17
pGKV259	Em^r Cm^r, 4.9 kb, pGKV210 derivative containing the lactococcal promoter P59	38
pGKV330	Em^r, 8 kb, pGKV259 derivative containing *pepXP* controlled by promoter P59	This work
pAMS100	Em^r Cm^r, 8.1 kb, pAMβ1 derivative	R. Kiewiet (unpublished data)

[a] Ap^r, resistance to ampicillin.

plated on M17 agar. The rate of acid production was calculated by the equation $(pH_{t1} - pH_{t0})/(t_1 - t_0)$.

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). *E. coli* cells were made competent and transformed by the protocols of Hanahan (8). *L. lactis* was electrotransformed with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) by the method of Leenhouts et al. (15). Nucleotide sequence determination was carried out by the dideoxy method of Sanger et al. (27) by using deoxyadenosine 5'-(α-[^{35}S]thio)triphosphate (Amersham, Buckinghamshire, United Kingdom).

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' GAA TTA ATC GAT GTT TAT TAC GGA GG 3') and B (5' CAG CAG TTG CTT GTA GAT CCG 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 *Cla*I and *Xho*II (the recognition sites for these enzymes on the primers are underlined above) and ligated with *Acc*I- and *Bam*HI-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 MacKinnon (22). Colonies were overlaid with 0.5% (wt/vol) agarose in distilled water and then flooded with a mixture of 0.2 ml of L-glycyl-L-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 × *g* for 20 min in an L8-80 centrifuge (Beckman, Palo Alto, Calif.) to remove cell debris. PepXP activity in the

supernatant was measured as described by Miller and MacKinnon (22) by using L-alanyl-L-prolyl-*p*-nitroanalide (Bachem) as the substrate. One unit of activity was defined as the amount of enzyme catalyzing the formation of 1.0 pmol of *p*-nitroanalide min^{-1} (the molar absorption coefficient of *p*-nitroanalide at A_{405} is 9.62×10^3 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 raised 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 $NaHCO_3$, 1.0 mM $MgCl_2$ [pH 9.8]) containing 1% (vol/vol) nitroblue tetrazolium (30 mg/ml) in 70% dimethyl formamide and 1% (vol/vol) 5-bromo-4-chloro-3-indolylphosphate toluidinium (salt) (15 mg/ml) in 100% dimethyl 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 A_{200}. 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 *Xba*I 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 *Fsp*I-*Hin*dIII fragment of pBM330 for a 1.1-kb *Pvu*II-*Hin*dIII fragment from pUC19E containing an erythromycin resistance (Em^r) 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^r marker will produce a $PepXP^-$ phenotype (Fig. 2). From 712 Em^r 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 *Hin*dIII. The *Pvu*II 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 *Pvu*II fragment hybridized with a 2.5-kb fragment instead of with a 2.1-kb *Hin*dIII fragment in the wild type, the size shifts being due to the replacement of the 0.7-kb *Fsp*I-*Hin*dIII fragment by the 1.1-kb Em^r 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 from 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 present 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* subsp. *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 *Cla*I site was designed 10-bp upstream of this ribosome binding site (Fig. 1). This fragment was cloned in pUC18 and checked by DNA

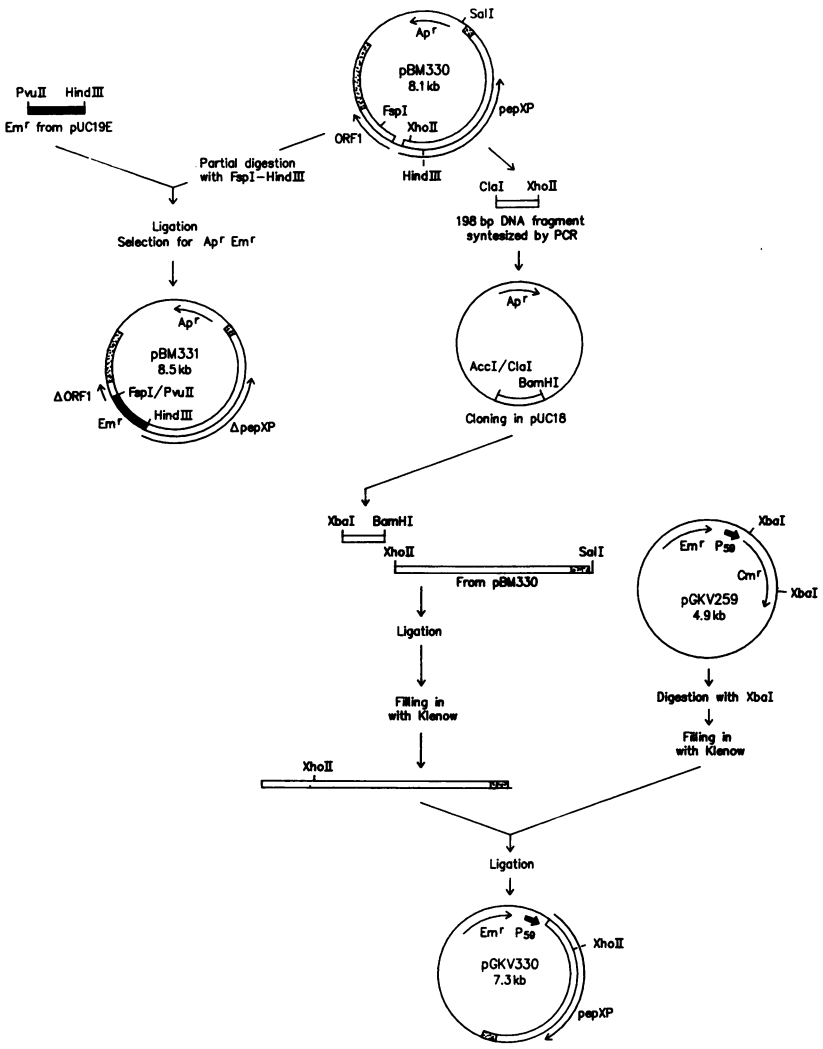

FIG. 1. Construction of pBM331 and pGKV330. Only relevant restriction sites for the construction and use of the recombinant vectors are given. The sizes of the fragments and plasmids are not to scale. The nucleotide sequence of the stippled areas is not known.

sequence analysis. An *Xba*I-*Bam*HI fragment from one of the clones was isolated and ligated to a 2.5-kb *Xho*II-*Sal*I fragment from pBM330 carrying the 3′-end of *pepXP*. *Bam*HI and *Xho*II were the only compatible ends. After ligation, *Xba*I and *Sal*I sticky ends were made blunt by treatment with Klenow enzyme. pGKV259, a pGKV210 derivative carrying a promoter-containing fragment P59 from *L. lactis* subsp. *cremoris* Wg2 (38), was digested with *Xba*I, 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 *Bam*HI-*Xho*II junction point of pGKV330 isolated from an *L. lactis* strain showed that the *Xho*II 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^r 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^r, 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^r]) 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

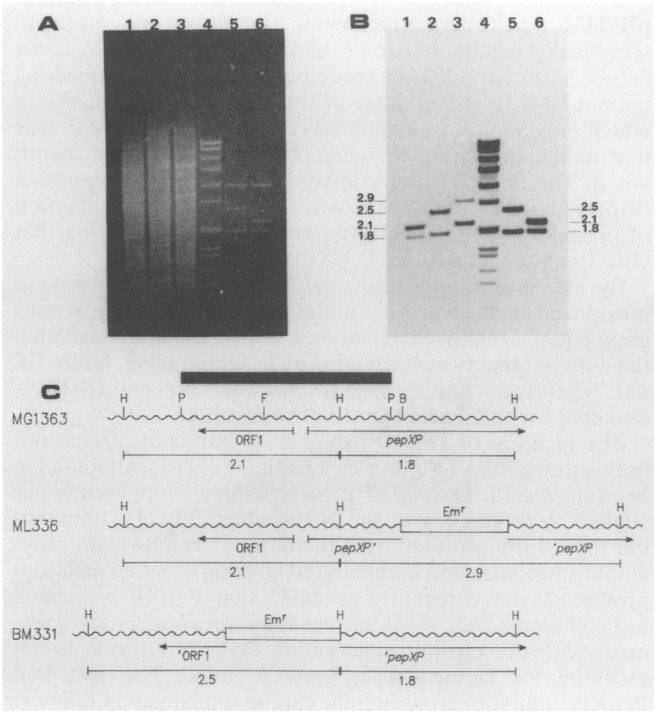

FIG. 2. Southern hybridization on chromosomal DNA of strains MG1363, ML336, and BM331. (A) DNA samples in an 0.8% agarose gel, digested in all cases with *Hin*dIII. Lanes: 1, total DNA of strain MG1363; 2, total DNA of strain BM331; 3, total DNA of strain ML336; 4, marker DNA from bacteriophage SPP1 (digested with *Eco*RI); 5, pBM331; 6, pBM330. The sizes of the *Eco*RI fragments of SPP1 DNA (in kilobases) are 8.0, 7.1, 6.0, 4.8, 3.5, 2.7, 1.9, 1.85, 1.5, 1.4, 1.15, and 1.0. (B) Autoradiogram of the Southern blot of the gel shown in panel A hybridized with the 2.2-kb *Pvu*II fragment of pBM330. The sizes of the hybridizing fragments are indicated in the margins. (C) Schematic representation of the relevant part of the chromosome of MG1363, ML336, and BM331. H, *Hin*dIII; P, *Pvu*II; F, *Fsp*I; B, *Bgl*I. The wavy lines indicate the chromosome, the arrows indicate the location of the genes, and the thin lines indicate the sizes of the fragments expected to hybridize with the probe, which is indicated by the bar. In the integration plasmid pML336, the 1.1-kb fragment containing the Em^r gene was inserted in the *Bgl*I site located in the *pepXP* gene. In plasmid pBM331, the 0.7-kb *Fsp*I-*Hin*dIII fragment was replaced by the 1.1-kb fragment carrying the Em^r gene.

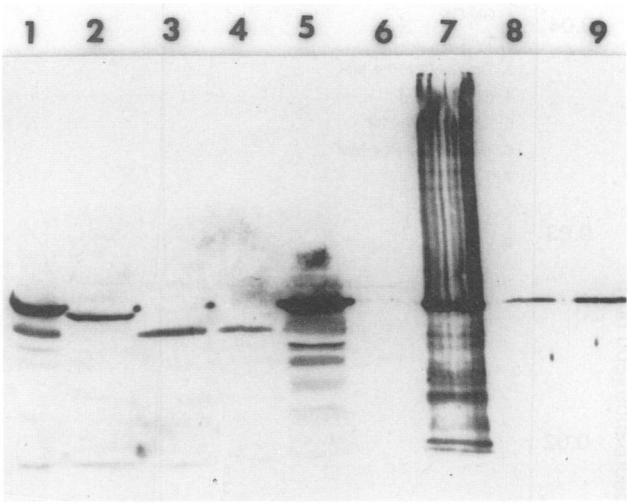

FIG. 3. SDS-PAGE and Western blot (immunoblot) analysis, using polyclonal antibodies raised against PepXP, of cell extracts of *L. lactis* subsp. *lactis* MG1363 (lane 1), *L. lactis* subsp. *lactis* BM13 (lane 2), *L. lactis* subsp. *lactis* ML336 (lane 3), *L. lactis* subsp. *lactis* BM331 (lane 4), *L. lactis* subsp. *lactis* BM13(pBM329) (lane 5), molecular weight markers (lane 6), *L. lactis* subsp. *cremoris* P8-2-47 (lane 7), and purified PepXP (lanes 8 and 9).

same conditions with the same amount of the DNA mixture. Cm^r 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^s, 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 caseins 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 pentapetide metenkephalin (Tyr-Gly-Gly-Phe-Met). Metenkepha-

TABLE 2. Rate of acid production of *L. lactis* strains in milk[a]

Strain	Rate of acid production (ΔpH/h)	SD (n = 4)
NCDO 712	0.34	0.070
ML336	0.34	0.040
BM331	0.33	0.080

[a] The values given are averages of four separate experiments.

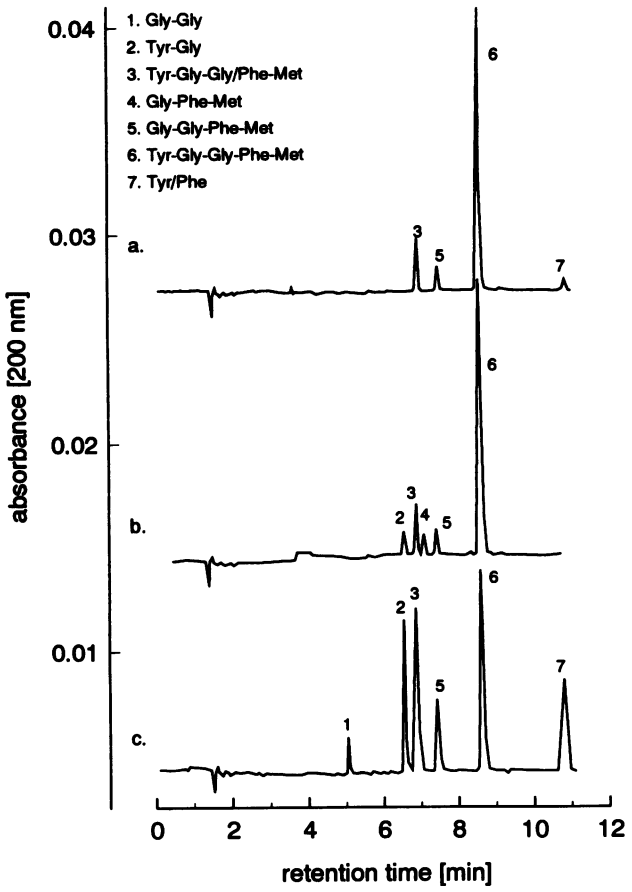

FIG. 4. Capillary electrophoresis of the degradation of metenkephalin (peak 6) by cell extracts of the PepXP$^-$ mutant BM331 (a) and the wild-type strain NCDO 712 (b). The third chromatogram (c) shows a standard peptide mixture consisting of metenkephalin and some of its subsequences, as specified in panel a.

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 *prt*$^+$ 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 the 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.

ACKNOWLEDGMENTS

Baltasar Mayo was the recipient of a grant from the Commission of the European Communities contract number 009138/1990.

Plasmid pAMS100 was a gift from Rense Kiewiet. The polyclonal antibodies raised against PepXP were a generous gift from Mari-Pierre Chapot-Chartier of Station de Recherches Laitières, Institute National de la Recherche Agronomique, Jouy-en-Josas, France. We thank Henk Mulder for preparing the figures.

REFERENCES

1. **Baankreis, R., and F. A. Extercate.** 1991. Characterization of a peptidase from *Lactococcus lactis* subsp. *cremoris* HP that hydrolyses di- and tripeptides containing proline or hydrophobic residues as the aminoterminal amino acid. Syst. Appl. Microbiol. **14**:317–323.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
3. **Casey, M. G., and J. Meyer.** 1985. Presence of an X-prolyl dipeptidyl peptidase in lactic acid bacteria. J. Dairy Sci. **68**: 3212–3215.
4. **Chopin, A., M.-C. Chopin, A. Moillo-Batt, and P. Langella.** 1984. Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. Plasmid **11**:260–263.
5. **Chopin, M.-C., A. Chopin, A. Rouault, and N. Galleron.** 1989. Insertion and amplification of foreign genes in the *Lactococcus*

lactis subsp. *lactis* chromosome. Appl. Environ. Microbiol. **55**:1769–1774.
6. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154**:1–9.
7. **Gould, S. J., S. Subramani, and I. E. Scheffler.** 1989. Use of the DNA polymerase chain reaction for homology probing: isolation of partial cDNA or genomic clones encoding the iron-sulfur protein of succinate dehydrogenase from several species. Proc. Natl. Acad. Sci. USA **86**:1934–1938.
8. **Hanahan, D.** 1985. Techniques for transformation of *Escherichia coli*, p. 100–135. *In* G. M. Glover (ed.), DNA cloning, vol. 1. IRL Press, Oxford.
9. **Hourinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. J. Bacteriol. **150**:804–814.
10. **Josephsen, J., and T. R. Klaenhammer.** 1990. Stacking of three different restriction modification systems in *Lactococcus lactis* by cotransformation. Plasmid **23**:71–75.
11. **Kiefer-Partsch, B., W. Bockelmann, A. Geis, and M. Teuber.** 1989. Purification of an X-prolyl dipeptidyl aminopeptidase from the cell wall proteolytic system of *Lactococcus lactis* subsp. *cremoris*. Appl. Microbiol. Biotechnol. **31**:75–78.

11a. **Kiewiet, R., J. Kok, J. F. M. L. Seegers, G. Venema, and S. Bron.** 1993. The mode of replication is a major factor in segregational plasmid instability in *Lactococcus lactis*. Appl. Environ. Microbiol. **59**:358–364.

12. **Kok, J.** 1990. Genetics of the proteolytic system of lactic acid bacteria. FEMS Microbiol. Rev. **87**:15–42.
13. **Laan, H., E. J. Smid, P. S. T. Tan, and W. N. Konings.** 1989. Enzymes involved in the degradation and utilization of casein in *Lactococcus lactis*. Neth. Milk Dairy J. **43**:327–345.
14. **Laemmli, U. K., and K. Faure.** 1973. Maturation of the head of bacteriophage T_4. DNA packaging events. J. Mol. Biol. **80**:575–590.
15. **Leenhouts, K. J., J. Kok, and G. Venema.** 1988. Campbell-like integration of heterologous plasmid DNA into the chromosome of *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. **55**:394–400.
16. **Leenhouts, K. J., J. Kok, and G. Venema.** 1990. Stability of integrated plasmids in the chromosome of *Lactococcus lactis*. Appl. Environ. Microbiol. **56**:2726–2735.
17. **Leenhouts, K. J., J. Kok, and G. Venema.** 1991. Replacement recombination in *Lactococcus lactis*. J. Bacteriol. **173**:4794–4798.
18. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Mayo, B., J. Kok, K. Venema, W. Bockelmann, M. Teuber, H. Reinke, and G. Venema.** 1991. Molecular cloning and sequence analysis of the X-prolyl dipeptidyl aminopeptidase gene from *Lactococcus lactis* subsp. *cremoris*. Appl. Environ. Microbiol. **57**:38–44.
21. **McKay, L. L., K. A. Baldwin, and P. M. Walsh.** 1980. Conjugal transfer of genetic information of group N streptococci. Appl. Environ. Microbiol. **40**:84–91.
22. **Miller, C. G., and K. MacKinnon.** 1974. Peptidase mutants of *Salmonella typhimurium*. J. Bacteriol. **120**:355–363.
23. **Monnet, V., W. Bockelmann, J.-C. Gripon, and M. Teuber.** 1989. Comparison of cell wall proteinases from *Lactococcus lactis* subsp. *cremoris* AC1 and *Lactococcus lactis* subsp. *lactis* NCDO 763. Appl. Microbiol. Biotechnol. **31**:112–118.
24. **Nardi, M., M.-C. Chopin, A. Chopin, M.-M. Cals, and J.-C. Gripon.** 1991. Cloning and DNA sequence analysis of an X-prolyl dipeptidyl aminopeptidase gene from *Lactococcus lactis* subsp. *lactis* NCDO 763. Appl. Environ. Microbiol. **57**:45–50.
25. **Poolman, B., and W. N. Konings.** 1988. Growth of *Streptococcus cremoris* in relation to amino acid transport. J. Bacteriol. **170**:700–707.
26. **Rice, G. H., F. H. C. Stewart, A. J. Hillier, and G. R. Jago.** 1978. The uptake of amino acids and peptides by *Streptococcus lactis*. J. Dairy Res. **45**:93–107.
27. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
28. **Simon, D., A. Rouault, and M.-C. Chopin.** 1985. Protoplast transformation of group N streptococci with cryptic plasmids. FEMS Microbiol. Lett. **26**:239–241.
29. **Sinha, N. D., J. Biernat, J. McManus, and H. Koster.** 1984. Polymer support oligonucleotide synthesis. XVIII. Use of β-cyanoethyl-N,N-dialkylamino-N-Morpholino phosphoramidite of deoxynucleotides for the synthesis of DNA fragments simplifying deprotection and isolation of the final product. Nucleic Acids Res. **12**:4539–4557.
30. **Smid, E. J., R. Plapp, and W. N. Konings.** 1989. Peptide uptake is essential for growth of *Lactococcus lactis* on the milk protein casein. J. Bacteriol. **171**:6135–6140.
31. **Smid, E. J., B. Poolman, and W. N. Konings.** 1991. Casein utilization by lactococci. Appl. Environ. Microbiol. **57**:2447–2452.
32. **Tan, P. S. T., M.-P. Chapot-Chartier, K. M. Pos, M. Rousseau, C.-Y. Boquien, J.-C. Gripon, and W. N. Konings.** 1992. Location of peptidases in lactococci. Appl. Environ. Microbiol. **58**:285–290.
33. **Tan, P. S. T., B. Poolman, and W. N. Konings.** 1993. Proteolytic enzymes of *Lactococcus lactis*. J. Dairy Res. **60**:271–288.
34. **Tan, P. S. T., K. M. Pos, and W. N. Konings.** 1991. Purification and charterization of an endopeptidase from *Lactococcus lactis* subsp. *cremoris* Wg2. Appl. Environ. Microbiol. **57**:3593–3599.

35. Tynkkynen, S. Personal communication.

36. **van der Lelie, D., J. M. B. M. van der Vossen, and G. Venema.** 1899. Effect of plasmid incompatibility on DNA transfer to *Streptococcus cremoris*. Appl. Environ. Microbiol. **54**:865–871.
37. **van der Vossen, J. M. B. M., J. Kok, and G. Venema.** 1985. Construction of cloning, promoter screening, and termination screening shuttle vectors for *Bacillus subtilis* and *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. **50**:540–542.
38. **van der Vossen, J. M. B. M., D. van der Lelie, and G. Venema.** 1987. Isolation and characterization of *Lactococcus lactis* subsp. *cremoris* Wg2-specific promoters. Appl. Environ. Microbiol. **53**:2452–2457.
39. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
40. **Zevaco, C., V. Monnet, and J.-C. Gripon.** 1990. Intracellular X-prolyl dipeptidyl peptidase from *Lactococcus lactis* subsp. *lactis*: purification and properties. J. Appl. Bacteriol. **68**:357–366.