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Genetic Characterization of *pepP*, Which Encodes an Aminopeptidase P Whose Deficiency Does Not Affect *Lactococcus lactis* Growth in Milk, Unlike Deficiency of the X-Prolyl Dipeptidyl Aminopeptidase

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We sequenced the *pepP* gene of *Lactococcus lactis*, which encodes an aminopeptidase P (PepP), and demonstrated that the X-prolyl dipeptidyl aminopeptidase PepX plays a more important role than PepP in nitrogen nutrition. PepP shares homology with methionine aminopeptidases and could play a role in the maturation of nascent proteins.

The proteolytic system of lactic acid bacteria is involved in two major events during the technological use of the bacteria. First, it ensures the nitrogen nutrition in hydrolyzing caseins (15). Second, during cheese ripening, it contributes to the development of flavor by releasing free amino acids which are precursors of aromatic compounds (42). In addition, although it is poorly documented, the proteolytic system of lactic acid bacteria is involved in more physiological and universal functions such as protein turnover, protein maturation, signal peptide processing, degradation of abnormal proteins, and inactivation of regulatory proteins (17).

Since milk contains only small amounts of free amino acids and small peptides, the optimal growth of lactococci in milk depends on the hydrolysis of caseins. This is accomplished by the primary action of an extracellular cell wall proteinase which hydrolyzes caseins into oligopeptides. The oligopeptides are transported into bacteria, where they are further hydrolyzed by intracellular peptidases (15). The high proline content of caseins limits the efficiency of the proteolytic system in releasing free amino acids. Most of the peptidases with general specificity are indeed unable to hydrolyze peptide bonds involving proline. Thus, the proline-specific peptidases already identified in lactococci probably play a major role in casein breakdown.

The X-prolyl dipeptidyl aminopeptidase (PepX; EC 3.4.14.5; able to release X-Pro dipeptides from the N-terminal end of peptide chains) is the most-studied proline-specific peptidase in lactococci. PepX of *Lactococcus lactis* was purified and characterized (4, 14, 18, 41, 43, 46), and its gene was cloned and sequenced (23, 32). In addition to PepX, an aminopeptidase P (PepP; EC 3.4.11.9) was purified and characterized in *L. lactis* (21, 24). This intracellular metalloenzyme releases N-terminal amino acids when proline is in the penultimate position. Moreover, it displays a specificity for X-Pro-Pro N termini, which constitutes an original property since it is not found in other aminopeptidases P already described.

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According to their complementary specificities, PepP and PepX were considered peptidases that are able to initiate the degradation of proline-containing peptides in two different pathways (5), but their relative importance in this degradation remains unknown.

To evaluate the role of the two proline-specific aminopeptidases, PepP and PepX, in nitrogen nutrition, we cloned and sequenced the *pepP* gene and constructed mutants negative for PepP and PepX as well as a $PepX^{-}$-$PepP^{-}$ double mutant. We demonstrated that, unlike PepX, PepP did not play a major role in nitrogen nutrition and probably had another specific function in bacteria.

The chromosomal gene *pepP* encodes an intracellular aminopeptidase P. We characterized the *pepP* gene encoding the lactococcal aminopeptidase P in *L. lactis* NCDO763 in a two-step procedure. First we identified the *pepP* gene; then we sequenced it and its flanking sequences. To identify the *pepP* gene, we purified PepP (21) and, after protein electrophoresis (16) and Western blotting (22), we determined its N-terminal sequence: Met-Arg-Ile-Glu-Lys-Leu-Lys-Val-Lys-Met-Leu-Thr-Glu-Asn-Ile-Lys-Ser-Leu-Leu-Ile-Thr-Asp-Met-Lys-Asn-Ile-Phe-Tyr-Leu-Thr (model 477A; Applied Biosystems, San Jose, Calif.). From the N-terminal sequence of PepP, we amplified with degenerate oligonucleotides a DNA fragment which was further cloned into pBluescript SK(+) (Table 1) and sequenced (373 DNA sequencer; Applied Biosystems). From this fragment, a 1.63-kb DNA sequence containing the whole *pepP* gene and two incomplete open reading frames (ORF1 and ORF3) was amplified by inverse PCR and sequenced. *pepP* encodes a protein with a molecular mass of 46 kDa, which is in accordance with the molecular mass of the purified protein PepP. Moreover, the N-terminal sequence of the protein deduced from the gene is identical to that sequenced from the protein, which shows that PepP is not subjected to any maturation at its N-terminal part. In addition, we did not find any typical hydrophobic sequence encoding a putative signal sequence which confirmed the intracellular location of PepP (21). A consensus ribosome-binding site (19) was found 6 bp upstream of the ATG start codon of *pepP* and was found to have a ΔG of -13.8 kcal/mol. In the whole nucleotide sequence, only one putative terminator structure was found downstream of ORF1. Close to the latter and upstream of *pepP*, a potential -10 extended promoter structure, fitting

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype or genotype	Source or reference
Strains		
L. lactis subsp. *cremoris*		
NCDO763	Wild-type strain	8
TIL46	2-kb plasmid-free derivative of NCDO763	29
MG1363	Plasmid-free derivative of NCDO712	11
TIL54	Tc^r, Em^r, derivative of TIL46 containing pTIL18B	This work
TIL55	Tc^r, $PepX^-$ derivative of TIL54	This work
TIL200	Ap^r, Em^r, $PepP^-$ derivative of TIL46	This work
TIL201	Ap^r, Em^r, Tc^r, $PepP^-$-$PepX^-$ derivative of TIL46	This work
Escherichia coli TG1		12
Plasmids		
pBluescript SK(+)	Ap^r, M13 ori,[a] pBr322 ori	Stratagene (La Jolla, Calif.)
pTAg	Ap^r, Km^r	R&D Systems
pG^+host4	Em^r, ori thermosensitive,[b] 3.8 kb	20
pIL253	Em^r, 4.9 kb	39
pTIL16	Ap^r, *pepX* in pBluescript SK(+), 5.2 kb	This work
pTIL18A	Ap^r, Tc^r, integration of Tc cassette in *pepX* (*Bgl*II site of pTIL16), 9.2 kb	This work
pTIL18B	Tc^r, Em^r, cloning the interrupted *pepX* gene in *Bam*HI and *Xho*I sites of pG^+host4, 11.8 kb	This work
pTIL101	Ap^r, *pepP* fragment in pTAg, 4.5 kb	This work
pTIL102	Ap^r, Em^r, *Sau*3A fragment of pIL253 in *pepP* (*Bam*HI site of pTIL101), 5.7 kb	This work
pTIL131	Ap^r, *pepP* N-terminal part in pBluescript SK(+), 3 kb	This work

[a] ori, origin of replication.
[b] Contains the *E. coli* thermosensitive origin of replication.

rather well with that observed in *Streptococcus pneumoniae* (37), was found. Another potential promoter was observed upstream of ORF3. The absence of a putative terminator downstream of *pepP* suggested that the gene encoding the aminopeptidase P belongs to an operon. *pepP* was similarly amplified from *L. lactis* NCDO763 and from *L. lactis* MG1363 (plasmid-free strain), which demonstrated its chromosome localization.

PepP belongs to the methionine aminopeptidase family. In order to identify similar proteins, the EMBL, GenBank, and DDBJ databases were screened with the deduced ORF1, PepP, and ORF3 amino acid sequences. PepP displays a significant homology with other aminopeptidases P, prolidases, and methionine aminopeptidases, which all belong to the M24 family of metallopeptidases (35). The highest homologies were found with potential aminopeptidase P from *Bacillus subtilis* (44% identity) (28), *Mycoplasma genitalium* (32% identity) (10), and *Haemophilus influenzae* (31% identity) (9) and with prolidase from *Lactobacillus delbrueckii* subsp. *lactis* (33% identity) (40). The highest homology with methionine aminopeptidases was obtained with those from *M. genitalium* (10), *Salmonella typhimurium* (30), and *B. subtilis* (31) (24 to 25% identity). PepP also showed homologies with creatinase from *Pseudomonas putida* (31% identity) (7), which has been shown to have a tertiary fold similar to that of the methionine aminopeptidase from *Escherichia coli* (2), although it is neither a peptidase nor a metal-dependent enzyme. The Asp 210, Asp 221, His 281, Glu 315, and Glu 329 residues were identified as potential metal ligands of the PepP protein of *L. lactis*, and the sequences around them are well conserved (Fig. 1).

Significant homologies were found for the proteins encoded by ORF1 (70-amino-acid sequence length) and ORF3 (39-amino-acid sequence length), although they were deduced from partial amino acid sequences. The protein encoded by ORF1 presents a significant homology with kasugamycin dimethyladenosine transferases of *B. subtilis* (45% identity) (33) and *Mycoplasma capricolum* (32% identity) (27). The ORF3 product displays a homology with the elongation factor P (EF-P) of *Corynebacterium glutamicum* (39.5% identity) (34) and the same putative factor of *B. subtilis* (44% identity) (28).

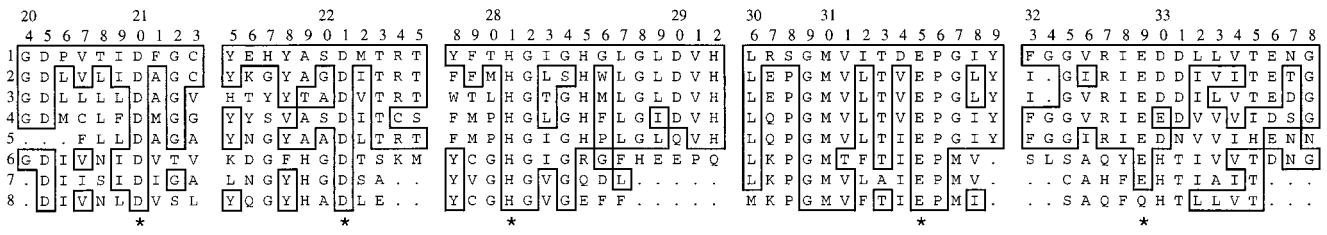

FIG. 1. Conservation of sequences around the cobalt ligands of the methionine aminopeptidase family (M24). Residues are numbered according to *L. lactis* aminopeptidase P. The cobalt ligands identified in *E. coli* methionine aminopeptidase (36) are indicated with asterisks. Residues identical to those in *L. lactis* aminopeptidase P are boxed. 1, *L. lactis* aminopeptidase P; 2, *E. coli* aminopeptidase P; 3, *Streptomyces lividans* aminopeptidase P; 4, human prolidase; 5, *E. coli* prolidase; 6, *E. coli* methionine aminopeptidase; 7, *B. subtilis* methionine aminopeptidase; 8, yeast methionine aminopeptidase.

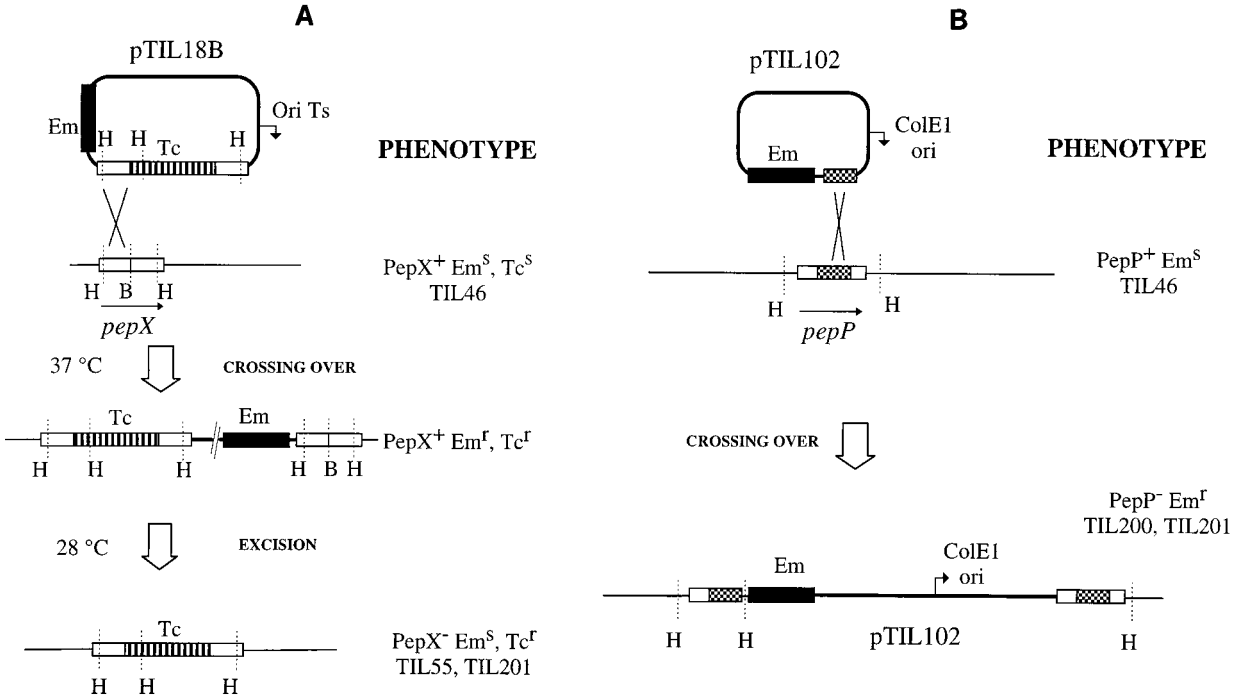

FIG. 2. Schematic representation of the integrations of the tetracycline resistance cassette into the *pepX* gene (A) and of the plasmid pTIL102 into the *pepP* gene (B). Both genes are indicated by empty bars, and the internal fragment of the *pepP* gene used for the integration is shown by a dotted bar. The arrows in plasmids pTIL18B and pTIL102 indicate the *E. coli* thermosensitive origin of pG$^+$host4 and the *E. coli* origin of replication of the pTAg plasmid, respectively. The relevant phenotypes of the clones are indicated on the right. H, *Hin*dIII restriction site; B, *Bgl*II restriction site.

The fact that a gene homologous to the *L. lactis* NCDO763 *pepP* gene was found in all the lactococcal strains tested, including those of plant origin, and in many other bacteria—especially *M. genitalium*, which possesses the smallest genome sequenced (10)—indicated that PepP could play a universal role in bacteria. The presence, downstream of *pepP*, of a gene coding for a protein homologous to an EF-P from *E. coli* (1) suggested a possible role of PepP during protein synthesis. Interestingly, in *B. subtilis*, the same organization was recently found: a gene encoding a protein 44% identical to PepP was followed by a gene encoding a protein 43% identical to the EF-P from *E. coli* (28).

PepP is widespread in *L. lactis* strains. Southern hybridization experiments under low-stringency conditions (20% formamide) (38) with a *pepP* probe revealed the presence of genes homologous to *pepP* in the seven lactococcal strains tested (*L. lactis* NCDO763, *Lactococcus lactis* subsp. *lactis* IL1403, *Lactococcus lactis* subsp. *cremoris* MG1363, Wg2, AM2, and E8, and *L. lactis* subsp. *lactis* CNRZ2118, of plant origin). However, no signal was visible for the other lactic acid bacteria tested: *Streptococcus thermophilus* CNRZ302, *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842, *Lactobacillus paracasei* subsp. *paracasei* CNRZ62, *Lactobacillus helveticus* CNRZ223, and *Lactobacillus plantarum* CNRZ1008.

Unlike PepX, PepP does not affect lactococcal growth in milk. To investigate whether PepP is important and/or complementary to PepX during growth of *L. lactis*, we constructed PepP, PepX, and a double PepP-PepX-negative mutant (Table 1). The integration vectors pTIL18B and pTIL102 were used to disrupt *pepX* and *pepP* genes in the TIL46 strain, respectively (Fig. 2). The disruptions of *pepP* and *pepX* were checked by Southern hybridization. The analysis of the plasmid profile of all mutated strains confirmed their *L. lactis* TIL46 origin (data not shown). The levels of PepP and PepX activities were measured in cell extracts of negative mutants and wild-type strains (50 mM TEA-HCl buffer, pH 7, 30°C). The increase in fluorescence (λ_{ex} = 320 nm, λ_{em} = 417 nm) due to hydrolysis of Lys(Abz)-Pro-Pro-pNa (Bachem) by PepP and the increase of absorbance (405 nm) due to hydrolysis of Ala-Pro-pNa by PepX were totally lost in PepP- and PepX-negative mutants, respectively. The maximal growth rates in M17 at 30°C, assessed by spectrophotometric measurements at 450 nm (CERES900; BioTek Instruments, Winooski, Vt.), were similar for the wild-type strain and PepP- and/or PepX-negative mutants. The maximal growth rates in milk at 30°C, assessed by enumeration with a spiral system (model DS; Spiral System, Cincinnati, Ohio), were 0.93 ± 0.09 h^{-1} for the wild type, 0.89 ± 0.07 for the PepP$^-$ mutant, 0.70 ± 0.15 for the PepX$^-$ mutant, and 0.68 ± 0.11 for the PepP$^-$-PepX$^-$ mutant. We observed that in milk the growth of the PepX-negative mutant was slightly but clearly affected, as already observed by Mierau et al. (25). In contrast, the PepP-negative mutant did not grow significantly slower than the wild-type strain. This indicated that PepP did not play a major role in the nitrogen nutrition of lactococci. This conclusion challenges the model of prolyl peptide degradation proposed by Booth et al. (5), who hypothesized that the X-Pro-Y-Z- peptides could be hydrolyzed either by PepX or by PepP. The absence of any effect of PepP deficiency both in the wild-type strain and in the PepX-negative mutant indicated that PepP is not involved in the degradation of casein-derived prolyl peptides during growth in milk. Consequently, we postulated that PepP had another function in lactococci.

PepP liberates N-terminal methionine. To investigate whether PepP is able to release the N-terminal methionine when proline is in the penultimate position, the peptides Arg-Pro-Pro-

Gly-Phe (the best substrate among those previously tested [21]), Met-Pro-Pro-Gly-Phe, and Met-Gly-Gly-Gly-Phe were synthesized (Applied Biosystems 432A synergy peptide synthesizer; Perkin-Elmer, San Jose, Calif.) and tested as substrates. They were incubated at 37°C in 50 mM TEA-HCl buffer (pH 7) (0.5 mM final concentration) with a partially purified fraction of aminopeptidase P (50% pure) containing none of the other already-characterized lactococcal peptidases. The release of Met from Met-Pro-Pro-Gly-Phe was twofold faster than that of Arg from Arg-Pro-Pro-Gly-Phe, which demonstrated that Met-Pro-Pro-Gly-Phe is a good substrate for PepP. The absence of hydrolysis of Met from Met-Gly-Gly-Gly-Phe confirmed the proline-specific property of PepP (21), as already shown with PepP from *E. coli* (44). Although the physiological role of N-terminal methionine hydrolysis from proteins is not completely understood, it is well established that the amino-terminal methionine is removed enzymatically after the initiation of the translation of intracellular proteins. Methionine is hydrolyzed by a methionine aminopeptidase (PepM), and the extent of methionine release depends on the nature of the second amino acid (13). PepM easily liberates methionine when a proline is in the penultimate position, but in vitro and in vivo experiments showed that a proline in the third position inhibits PepM activity (3, 13). PepP can help to increase the efficiency of release of the N-terminal methionine, as shown in in vivo experiments with PepP from *E. coli* (45). In an *E. coli* strain overproducing PepP and expressing human interleukin-6 (with a Met-Pro- N-terminal sequence), the rate of removal of the initial methionine is 99% compared to a rate of 85% in a strain in which the PepP is not overproduced. This observation demonstrated that the PepP could play a role in the maturation of nascent proteins. PepP never replaces PepM, since PepM is essential for bacterial cell growth (6, 26), but may play a role in the maturation of specific proteins.

Nucleotide sequence accession number. The GenBank, EMBL, and DDBJ nucleotide sequence accession number is Y08842.

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