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Cloning and Sequencing of the Gene for a Lactococcal Endopeptidase, an Enzyme with Sequence Similarity to Mammalian Enkephalinase

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The gene specifying an endopeptidase of *Lactococcus lactis*, named *pepO*, was cloned from a genomic library of *L. lactis* subsp. *cremoris* P8-2-47 in λEMBL3 and was subsequently sequenced. *pepO* is probably the last gene of an operon encoding the binding-protein-dependent oligopeptide transport system of *L. lactis*. The inferred amino acid sequence of PepO showed that the lactococcal endopeptidase has a marked similarity to the mammalian neutral endopeptidase EC 3.4.24.11 (enkephalinase), whereas no obvious sequence similarity with any bacterial enzyme was found. By means of gene disruption, a *pepO*-negative mutant was constructed. Growth and acid production of the mutant strain in milk were not affected, indicating that the endopeptidase is not essential for growth of *L. lactis* in milk.

Lactococci are the main component of starter cultures used in the dairy industry for the production of a variety of fermented milk products, such as cheese, butter, buttermilk, and yogurt.

Fast growth of lactococci is essential in fermentation of milk to achieve a rapid drop of pH to obtain optimal conditions for the action of rennet, for cheese ripening, and for prevention of growth of undesired organisms (10, 39, 60).

For rapid growth in milk, lactococci depend on the degradation of casein by means of their proteolytic system. This system is essential, because the pathways for the synthesis of several amino acids do not function in lactococci and because the concentrations of free amino acids and small peptides in milk are too low to sustain growth to high cell densities (49, 60).

The proteolytic activity of lactococci is furthermore involved in ripening of and flavor production in fermented milk products, especially cheese (39, 60).

At present, only one of the components of the proteolytic system has been shown to be essential in the breakdown cascade of casein: the cell envelope-associated proteinase. This enzyme is specified by the *prtP* gene that is located on a plasmid in most strains of *Lactococcus lactis* (20). The *prtP* gene product is initially synthesized as a pre-pro-enzyme. The product of a second gene, *prtM*, induces the autoproteolytic activation of the pro-proteinase. Mutants in *prtP* or *prtM* are unable to grow to high cell densities in milk (14, 20).

Mature PrtP generates large peptides from casein, which have recently been characterized elsewhere (5, 35, 36, 42, 71–73). However, our knowledge concerning the subsequent breakdown of these peptides is very limited. The general view is that this breakdown occurs in a cascade-like fashion in which several peptidases participate (50).

In the last decade, several laboratories have isolated and characterized a number of these enzymes, and considerable progress has been made. At this moment, at least nine peptidases with different specificities have been purified to homogeneity from lactococci (for recent reviews, see references 20 and 55). However, in only a few cases have the genetic determinants of these enzymes been isolated and characterized. These include the genes for aminopeptidase N (*pepN*) (52, 58, 64), aminopeptidase C (*pepC*) (6) and X-prolyl dipeptidyl aminopeptidase (*pepXP*) (34, 38).

Recently, a metalloendopeptidase has been isolated and purified from *L. lactis* subsp. *cremoris* Wg2 (56). The enzyme has a molecular weight of 70,000 and hydrolyzes peptides of different sizes, such as Met-enkephalin (5 amino acids) and the oxidized β-chain of insulin (30 amino acids). A neutral oligoendopeptidase with a comparable specificity, molecular weight, and inhibitory spectrum was recently isolated by Baankreis (3) and seems to be similar or even identical. Because an endopeptidase would be a likely candidate for cleavage of the large peptides generated from casein by the cell envelope-associated proteinase, we have given priority to the cloning and characterization of the gene specifying the endopeptidase of *L. lactis*. Once isolated, the gene could then be used to mutate the endogenous gene to examine the properties of the mutated strain, especially with respect to its capacity to grow in milk. Accordingly, the present paper describes the cloning of the endopeptidase gene and its sequence and shows that the enzyme is not essential for growth in milk.

MATERIALS AND METHODS

Strains, plasmids, and media. The strains and plasmids used are listed in Table 1. *Escherichia coli* strains were grown in TY medium at 37°C with vigorous agitation or in TY medium (43) solidified with 1.5% agar, which contained 100 μg of ampicillin or 100 μg of erythromycin per ml, when indicated. *L. lactis* strains were grown in M17 medium at 30°C as stand cultures or on M17 medium solidified with 1.5% agar (59) supplemented with 0.5% glucose or lactose. Erythromycin was added to 5 μg/ml. Growth experiments in milk were carried out in 10% reconstituted skim milk (Oxoid Ltd., London, England), which was steamed for 30 min on 2

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TABLE 1. Bacterial strains, bacteriophages, and plasmids

Strain, phage, or plasmid	Relevant phenotype or genotype	Source or reference
Strains		
L. lactis subsp. *cremoris* P8-2-47	Wild-type strain	2
L. lactis subsp. *cremoris* Wg2	Wild-type strain	40
L. lactis subsp. *lactis* NCDO712	Wild-type strain	12
L. lactis subsp. *lactis* MG1363	Plasmid-free derivative of NCDO712	12
L. lactis subsp. *lactis* MG1363 *pepO*	*pepO* (endopeptidase-negative mutant)	This work
L. lactis subsp. *lactis* MG1363 *pepO*(pLP712)	*pepO* Prt^+ Lac^+	This work
E. coli		
JM103	F′ *traD36 lacI*^{q} Δ(*lacZ*)*M15 proAB/endA1 supE sbcBC thi-1 rpsL* (Str^r) Δ(*lac-pro*) (P1) (r_K^+ m_K^+ r_{P1}^+ m_{P1}^+)	75
LE392	F^- *hsdR514* (r_K^- m_K^+) *supE44 supF58 lacY1* or Δ(*lacZY*)*6 galK2 galT22 metB1 trpR55*	32
Phages		
λEMBL3	*sbhI*λ*1° b189* <polycloning site *int29 ninL44 trpE* polycloning site> *KH54 chiC srI*λ*4° nin5 srI*λ*5°*	11
λENP81	λEMBL3 carrying lactococcal chromosomal DNA inserts with the endopeptidase gene	This work
λENP91	λEMBL3 carrying lactococcal chromosomal DNA inserts with the endopeptidase gene	This work
Plasmids		
pBluescript SK^+	Ap^r	Stratagene (La Jolla, Calif.)
pUC19	Ap^r	75
pUC7e	Ap^r Em^r; pUC7 carrying the *erm* gene of pE194 in the *Pst*I site	68
pMG60	Em^r, carrying a *lacZ* gene expressed with the lactococcal promoter P_{32}	67
pORI24	Tc^r *ori*$^+$ of pWVO1; integration vector which replicates only in strains providing *repA* in *trans*	22a
pENP01	Ap^r 3.8-kb *Eco*RI fragment	This work
pENP19	Ap^r 1.4-kb *Sal*I-*Eco*RI fragment	This work
pENP22	Ap^r 1.4-kb *Sal*I-*Eco*RI fragment	This work
pENP31	Ap^r 10-kb *Sal*I fragment	This work
pENP41	Ap^r 2.2-kb *Sal*I-*Eco*RI fragment	This work
pENP44	Ap^r 2.2-kb *Sal*I-*Eco*RI fragment	This work
pENP4401	Ap^r 4.6-kb *Eco*RI fragment	This work
pENP61	Ap^r 10-kb *Sal*I fragment	This work
pINT1924L	Em^r vector for gene disruption mutation of the endopeptidase gene	This work

successive days. For growth experiments, an overnight culture in reconstituted skim milk was diluted 50-fold into reconstituted skim milk and incubated at 30°C. Samples were taken at 30-min intervals, and the pH and optical density at 600 nm were determined.

Screening of the lactococcal genome library in phage λ. Chromosomal DNA of *L. lactis* subsp. *cremoris* P8-2-47 was isolated, digested with *Sau*3A, and ligated to λEMBL3 (11) digested with *Bam*HI. The ligation mixture was packed into phage heads (Packagene System; Promega Corporation, Madison, Wis.) and used to infect *E. coli* LE392 (34a). To detect phages carrying the gene of the endopeptidase, the phage bank was screened with antibodies raised against the purified endopeptidase (51, 56). To reduce the background, which was observed with the proteins of *E. coli*, the antibodies were preadsorbed with a cell extract of the host strain *E. coli* LE392.

Molecular cloning and DNA sequencing. Molecular cloning techniques were performed essentially as described by Sambrook et al. (46). Phage λ DNA was isolated according to the method described by Zabarovsky and Turina (76). Plasmids from *L. lactis* were isolated by the method described by Birnboim and Doly with the modifications described by Leenhouts et al. (25). Chromosomal DNA was isolated from *L. lactis* according to the method described by Leenhouts et al. (24). *E. coli* and *L. lactis* were transformed by electroporation as described by Zabarovsky and Winberg (77) and Holo and Nes (17), respectively. Conjugation in *L. lactis* was carried out following the protocol of Gasson and Davies (13).

Fragments of the endopeptidase gene, ranging from 250 to 900 bp, were cloned into pUC19 (75). Both strands were sequenced by the dideoxy chain termination method (47) with the T7 sequencing kit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) and double-stranded plasmid templates according to the manufacturer's instructions. The sequencing of larger fragments was completed by primer walking. Primers and oligonucleotides were synthesized with a 381A DNA Synthesizer (Applied Biosystems, Inc., Foster City, Calif.). The subsequences obtained were

aligned and analyzed with the PC/Gene sequence analysis program (IntelliGenetics, Inc., Geneva, Switzerland). Protein homology searches in the EMBL Data Library in Heidelberg, Germany, were carried out with the data bases SWISSPROT (release 21), PIR (release 32), EMBL (release 30), and GenBank (release 71) by means of the MAILFASTA program (41). Protein sequence alignment was carried out with the PALIGN program of PC/Gene by using the unitary matrix with standard settings.

Plasmid construction. To identify and further analyze the endopeptidase gene, various fragments of a phage clone were isolated and cloned into pBluescript SK$^+$ and pUC19.

The integration plasmid pINT1924L (see Fig. 5) was constructed as follows. An erythromycin resistance gene (from pUC7e [68]) was inserted into the *Sca*I site of the endopeptidase gene in the plasmid pENP19, yielding the plasmid pENP19e. The disrupted endopeptidase gene was then transferred to the integration plasmid pORI24 (22a). Subsequently, the tetracycline resistance gene of this plasmid was exchanged for a β-galactosidase expression unit from pMG60 (67).

Cell extracts and PAGE. Cell extracts of overnight cultures of *E. coli* and *L. lactis* were prepared as described by van de Guchte et al. (65). To obtain proteins encoded by the recombinant phages, the cells of 5 ml of an infected culture were collected by centrifugation, resuspended in 25 μl of 20 mM Tris (pH 7) solution, and mixed with 25 μl of 2× sample buffer (21), and the mixture was boiled for 5 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the protocol described by Laemmli (21) with the Protean II Minigel System (Bio-Rad Laboratories, Richmond, Calif.).

Western blotting and immunodetection. Proteins separated by SDS-PAGE were transferred to a BA85 nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by the method described by Towbin et al. (62). Endopeptidase antigen was detected with polyclonal endopeptidase antibodies (56) diluted 1:8,000 and alkaline phosphatase-conjugated goat anti-rabbit antibodies (Promega Corporation) according to the manufacturer's instructions.

Southern transfer and oligonucleotide hybridization. After agarose gel electrophoresis, DNA was transferred to GeneScreen Plus filters (Du Pont Co., NEN Research Products, Boston, Mass.) by the protocol of Southern, as modified by Chomczynski and Quasba (7). Instead of a buffer tank being used, the gel was placed on the bench, and transfer was carried out until the gel was dry (2 to 3 h).

From the N-terminal amino acid sequence of the endopeptidase (57), the following 17-mer degenerate oligonucleotide probe was synthesized: TT(T/C) GC(T/A) AC(T/A) GT(T/A) AAT GC. The probe was either labelled with the ECL oligonucleotide labelling and detection system (Amersham International plc, Amersham, United Kingdom) or the DIG oligonucleotide 3′-end-labelling kit (Boehringer GmbH, Mannheim, Germany) and hybridized according to the instructions of the manufacturer (for the ECL system). For use of the GeneScreen Plus filters with the DIG-labelled probes, 1% SDS was included in all buffers to prevent unspecific binding of the probe to the filter. Hybridization of the DIG-labelled probes was detected with the DIG luminescence detection kit (Boehringer GmbH).

Nucleotide sequence accession number. The *pepO* sequence has been assigned the GenBank accession number L04938.

RESULTS

Screening of the genome bank. A genome bank of *L. lactis* subsp. *cremoris* P8-2-47 in λEMBL3 was screened for plaques producing the endopeptidase with polyclonal antibodies. About 4,000 phages were plated, and the proteins in the plaques were transferred to nitrocellulose membranes and screened with antibodies raised against the purified endopeptidase. Seventeen plaques which gave a positive signal were isolated and used for further characterization. To determine which of these phages carried the endopeptidase gene, proteins from *E. coli* cells that were infected with the selected phage were subjected to SDS-PAGE and Western blotting and were analyzed with endopeptidase-directed antibodies. Only when *E. coli* cells were infected with the phages λENP81 or λENP91 was a protein with the same size as the endopeptidase detected (Fig. 1A).

Delineation of the endopeptidase gene. DNA was isolated from λENP81, a phage encoding a 70-kDa protein that reacted with the endopeptidase-directed antibodies. By single and double digestions, a restriction enzyme map of the lactococcal insert (14 kb) was constructed (Fig. 2). By using an oligonucleotide designed from the N-terminal amino acid sequence of the endopeptidase (57), fragments containing the 5′ end of the endopeptidase gene were identified and mapped (Fig. 2). Because the oligonucleotide specifically hybridized with particular fragments of the insert in λENP81 and because the endopeptidase-directed antibodies specifically reacted with a 70-kDa-protein in extracts of *E. coli* infected with λENP81, we conclude that the endopeptidase gene had been cloned.

To delineate the gene, various subclones of 1.4- to 10-kb fragments of the λENP81 insert were constructed and assayed for their capacity to produce the complete 70-kDa enzyme (Fig. 2). A 10-kb *Sal*I fragment and a 3.8-kb *Eco*RI fragment were isolated from λENP81 and subcloned into pBluescript SK$^+$, resulting in the plasmids pENP31, pENP61, and pENP01, respectively (Fig. 2). Proteins of *E. coli* cells harboring these plasmids were subjected to SDS-PAGE and Western blotting, and endopeptidase-directed antibodies were used for immunodetection. Only *E. coli* (pENP31) expressed the 70-kDa endopeptidase protein, whereas *E. coli*(pENP61) did not (Fig. 1B). *E. coli*(pENP01) produced a 30-kDa, truncated form of the endopeptidase (Fig. 1C). Furthermore, *E. coli* cells harboring different deletion derivatives of pENP31 produced truncated proteins of 35 and 65 kDa (pENP22 and pENP41, respectively) reacting with the antiendopeptidase antibodies (Fig. 1C; summarized in Fig. 2). From these data, we conclude that the endopeptidase gene is located on a DNA segment of approximately 2.5 kb starting at the *Sal*I site at 13.1 kb and pointing to the left (Fig. 2).

As documented in Fig. 1B and illustrated in Fig. 2, expression of the endopeptidase gene in the plasmids was dependent on the orientation of the gene relative to the *lac* promoter. This observation indicated that the endopeptidase gene on the 10-kb *Sal*I fragment in pENP31 and pENP61 lacked its own expression signals. The alternative explanation, that the promoter of the endopeptidase gene was not recognized by *E. coli*, could be ruled out, because *E. coli* infected with λENP81 does produce the full-size endopeptidase. Because the inserts in the plasmids pENP01 and pENP4401 direct the expression of a 30- and a 67-kDa protein, respectively, in the absence of the *lac* promoter (Fig. 2 and 1C), the promoter from which the endopeptidase gene is transcribed on λENP81 in *E. coli* and possibly in *L.*

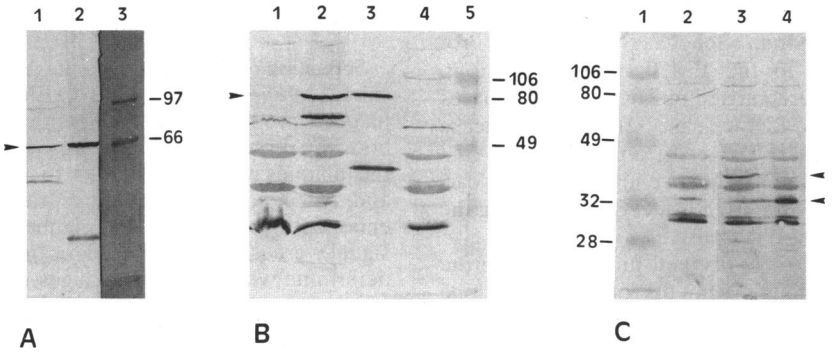

FIG. 1. Western blot analysis of proteins encoded by the phage clone λENP81 and various plasmid subclones for the presence of the endopeptidase or its truncated forms. The antibody preparation was a polyclonal serum against the purified endopeptidase (56). (A) Lanes: 1, *E. coli* infected with λENP81; 2, *L. lactis* P8-2-47; 3, standard molecular weight marker. The arrowhead indicates the position of the 70-kDa protein. (B) Lanes: 1, *E. coli*(pENP61); 2, *E. coli*(pENP31); 3, *L. lactis* P8-2-47; 4, *E. coli*(pUC19); 5, standard molecular weight marker. The arrowhead indicates the position of the endopeptidase. (C) Lanes: 1, standard molecular weight marker; 2, *E. coli*(pUC19); 3, *E. coli*(pENP22); 4, *E. coli*(pENP01). The upper and lower arrowheads indicate the 35- and 30-kDa truncated proteins specified by pENP22 and pENP01, respectively. Molecular sizes are given in kilodaltons.

lactis is located on the 2.3-kb *Sal*I-*Eco*RI fragment as it is in pENP01.

Sequencing of the endopeptidase gene. Several fragments of the insert in pENP31 extending from the *Sal*I site (2) (Fig. 2) were subcloned into pUC19 and sequenced by plasmid sequencing and primer walking. The nucleotide sequence of a contiguous piece of DNA sequenced on both strands is presented in Fig. 3 and shows the presence of an open reading frame (ORF) of 1,881 bp. This ORF could encode a protein of 627 amino acids with a molecular weight of 71,527. It is preceded by a putative ribosome-binding site which is complementary to the 3′ end of the lactococcal 16S rRNA (29) and has a ΔG of −12.8 kcal (ca. −53.5 kJ)/mol (61). The N-terminal amino acid sequence deduced from the nucleotide sequence was identical to that determined from the purified endopeptidase, except for the initiator methionine. Therefore, we conclude that this ORF encompasses the gene encoding the lactococcal endopeptidase. The gene was named *pepO*. Neither a typical bacterial leader peptide (74) nor possible membrane-spanning domains were present. Upstream of the endopeptidase gene, no consensus promoter sequence (66) was found. Since 127 bp upstream of the gene the stop codon of a second ORF which continues beyond the end of the sequenced fragment is present, it is likely that *pepO* is part of an operon and is transcribed from a distant promoter. This assumption is supported by the results of the subcloning experiments referred to above, which showed that the promoter for *pepO* lies beyond the *Sal*I site at 13.1 kb in λENP81.

Downstream of the endopeptidase gene and in the region between the two ORFs, inverted repeats are present (ΔG [25°C] = −14.4 kcal [ca. −60.2 kJ] and ΔG [25°C] = −8.4 kcal [ca. −35.1 kJ], respectively) (see Fig. 3). These inverted repeats might form rho-independent terminator-like structures. The functionality of these putative terminators remains unestablished.

The sequenced fragment has a G+C content of 34.8%, which is in the range of the G+C content of *L. lactis* (38.6%

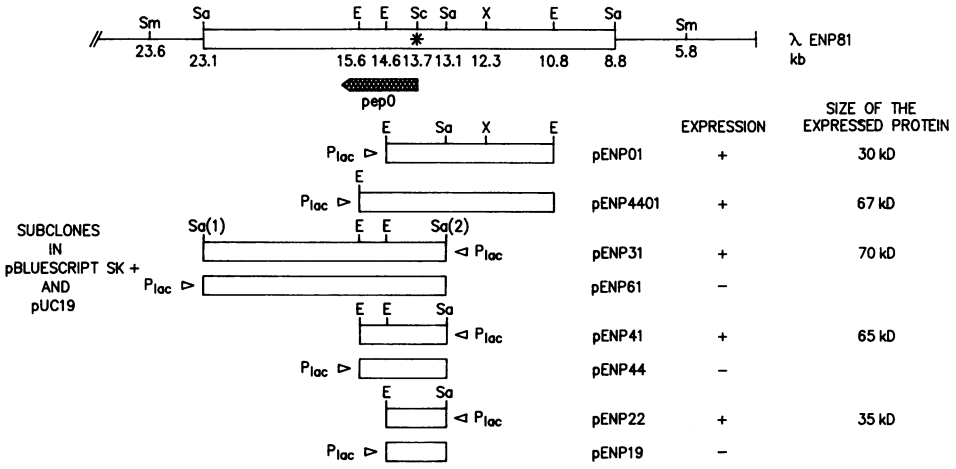

FIG. 2. Restriction enzyme map of λENP81, showing the location of the endopeptidase gene and expression of the endopeptidase gene in various subclones. *, the site of hybridization of the oligonucleotide probe derived from the N-terminal amino acid sequence of the purified endopeptidase; E, *Eco*RI; Sa, *Sal*I; Sc, *Sca*I; Sm, *Sma*I; X, *Xba*I; P_{lac}, *lac* promoter and its direction of transcription in pBluescript SK^+ or pUC19. The differences in size among the proteins produced in *E. coli*(pENP22) and *E. coli*(pENP01) on the one hand and *E. coli*(pENP4401) and *E. coli*(pENP41) on the other are due to an out-of-frame translational fusion in pUC19.

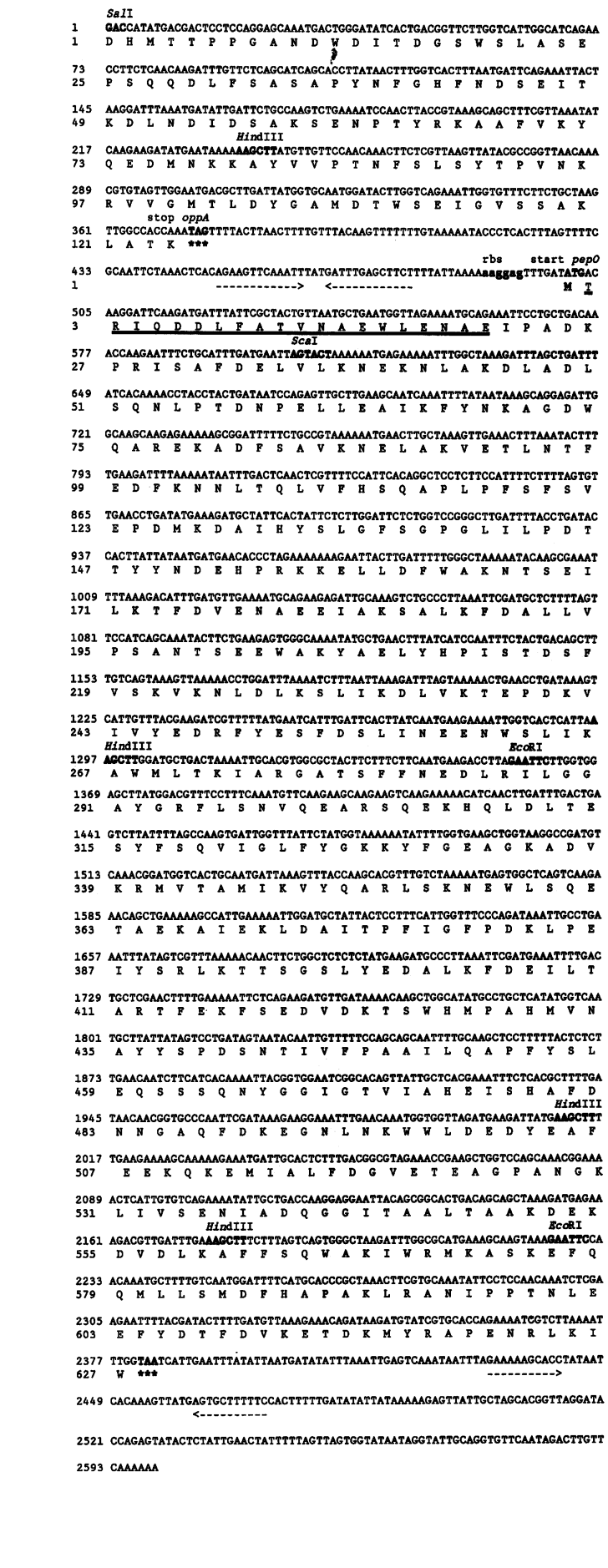

FIG. 3. Nucleotide sequence and deduced amino acid sequence of *pepO* and part of the preceding *oppA* gene of *L. lactis* P8-2-47. Facing arrows, inverted repeat; rbs, ribosome-binding site. The doubly underlined amino acid sequence was the same as that determined from the purified endopeptidase. A number of pertinent restriction enzyme sites are also indicated.

[37]), and the codon usage of the *pepO* gene was in agreement with that calculated by van de Guchte et al. (66) from several sequenced genes of *L. lactis*.

Homology comparisons. To examine whether PepO showed similarity to other proteins, the SWISSPROT, PIR, EMBL, and GenBank data bases were screened for homology with the amino acid and nucleotide sequences of PepO and *pepO*, respectively. Striking similarity was found to the neutral endopeptidase (NEP) (enkephalinase) EC 3.4.24.11 of mammalia (8, 9, 28, 30, 31, 48) (Fig. 4), but no obvious similarity to any bacterial enzyme was present. The overall identity of the endopeptidase of *L. lactis* with human NEP is 27.1%, whereas the similarity including conservative amino acid exchanges amounted to 37.3%. The highest degree of identity (71%) was within a stretch of 42 amino acids around the zinc-binding site of NEP. PepO contains the sequence

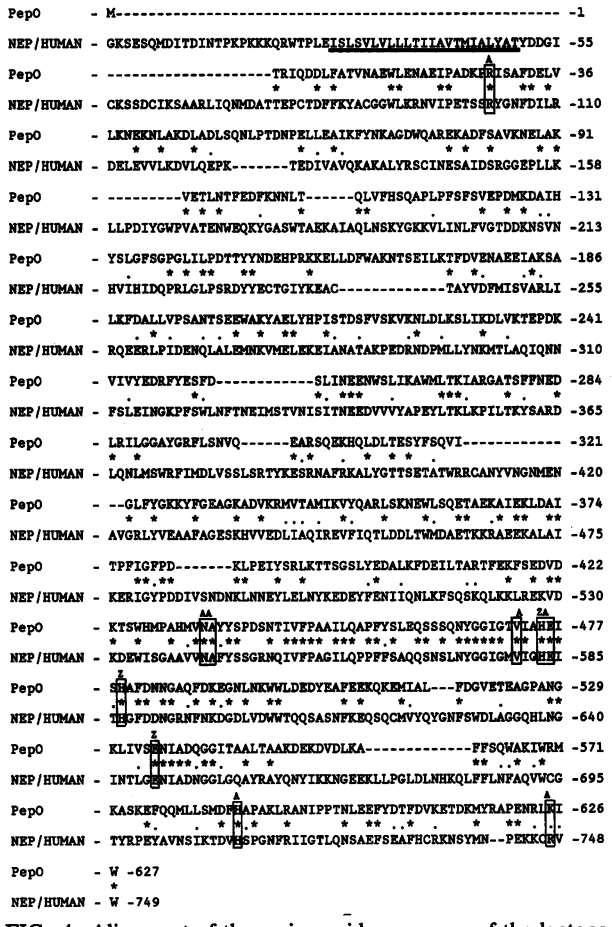

FIG. 4. Alignment of the amino acid sequences of the lactococcal endopeptidase and the human NEP. *, identical amino acids; •, similar amino acids; ═, membrane-spanning domain of NEP. Amino acids of the active site (A) or involved in Zn^{2+} binding (Z) of NEP are boxed.

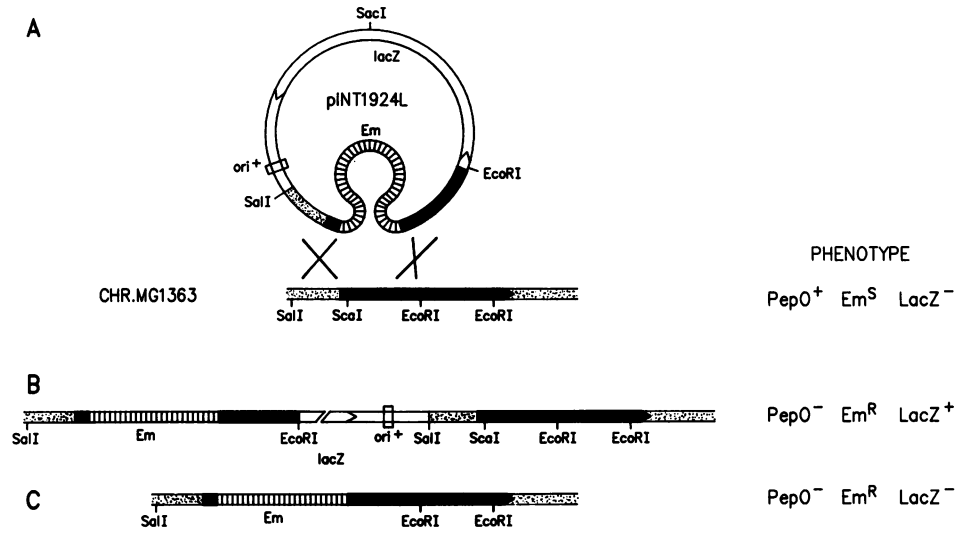

FIG. 5. Schematic representation of the integration plasmid pINT1924L. Black bar, *pepO* (endopeptidase gene); striped bar, Em^r (erythromycin resistance gene); empty bar, vector sequence including *lacZ* (β-galactosidase gene) expressed under control of the lactococcal promoter P_{32} and ori$^+$ (origin of replication of the lactococcal plasmid pWVO1); dotted bar, chromosome of *L. lactis*. Phenotypes of the relevant clones are shown on the right.

motive His-Glu-Xxx-Xxx-Glu, which is characteristic of zinc-dependent metalloproteinases and metallopeptidases (18, 27, 33). Furthermore, the putative active-site amino acids of NEP identified so far (4, 70) are identical to amino acids of PepO, except for one conservative change of Arg-747 in NEP into Lys-625 in PepO, indicating a possible role of these amino acids in the active site of the lactococcal enzyme.

Construction and analysis of endopeptidase-negative mutants of lactococci. To answer the question of whether the endopeptidase is required for growth in milk, a mutant lactococcal strain was constructed by disrupting the endopeptidase gene. The integration plasmid pINT1924L (Fig. 5) consisted of the 1.4-kb *Eco*RI-*Sal*I fragment (as present in pENP19; Fig. 2) of *pepO* disrupted at its *Sca*I site by an erythromycin resistance (Em^r) gene, a β-galactosidase gene expressed under the control of the lactococcal promoter P_{32} (69) and a plus origin of replication of the lactococcal plasmid pWVO1. This plasmid, which lacks the *repA* gene, can replicate only in a host strain which provides the replication protein in *trans* (23). *L. lactis* MG1363 was transformed with pINT1924L, and transformants were selected as erythromycin-resistant colonies. On M17 agar supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), two types of colonies were present. In cells forming white colonies, the Em^r gene was integrated in the *pepO* gene by double-crossover events in the *pepO* regions flanking the Em^r gene. Blue colonies had arisen from cells with Campbell-type integration in which crossover in only one of the flanking regions had occurred. A number of white colonies were selected and further analyzed by Southern hybridization. The results are presented in Fig. 6 and show that the size of a 3.8-kb *Eco*RI chromosomal DNA fragment has increased by 1 kb, as expected upon integration of the Em^r gene by a double-crossover event. The proteins produced by the transformant strains were subjected to SDS-PAGE and Western blotting, and the endopeptidase antigen was detected with specific antibodies. The lower 70-kDa band representing the endopeptidase was absent in the mutant strains, indicating that the disruption of *pepO* prevented the synthesis of full-length endopeptidase (Fig. 7). To examine whether the *pepO* gene was required for growth in milk, plasmid pLP712, carrying a gene specifying the cell envelope-associated proteinase of *L. lactis* NCDO712 (12), was conjugally transferred to *L. lactis* MG1363 and its *pepO*-negative derivative. Growth experiments in milk of the endopeptidase-proficient strain MG1363(pLP712) and its *pepO*-deficient mutant showed that the two strains did not significantly differ in cell densities and acidification rates over a period of 10 and 24 h after inoculation (Fig. 8). We

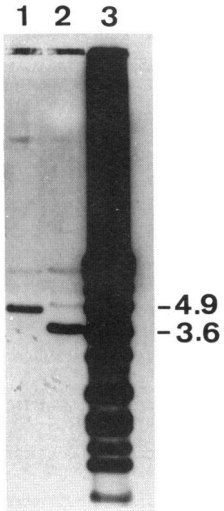

FIG. 6. Southern hybridization. Chromosomal DNAs of *L. lactis* MG1363 and a *pepO*-negative mutant of this strain were digested with *Eco*RI. Hybridization was carried out with the labelled plasmid pENP19 (Fig. 2). Lanes: 1, MG1363 *pepO*; 2, MG1363; 3, standard molecular weight marker. Molecular sizes (in kilobases) of relevant fragments are shown on the right.

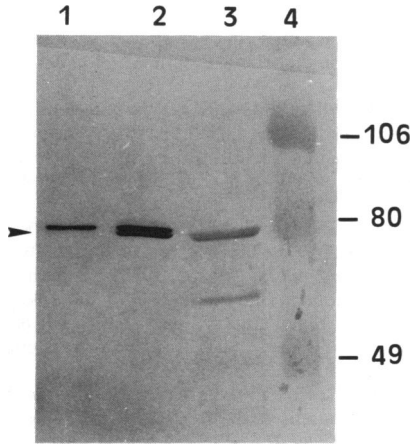

FIG. 7. Western blot analysis of a *pepO*-negative strain of *L. lactis* MG1363. Lanes: 1, MG1363 *pepO*; 2, MG1363; 3, *E. coli* (pENP31) (see Fig. 2); 4, standard molecular weight marker. Molecular sizes (in kilodaltons) are shown on the right. The arrowhead indicates the position of the endopeptidase. The antibodies used were raised against purified endopeptidase (56).

conclude from this experiment that the endopeptidase is not essential for growth of *L. lactis* in milk.

DISCUSSION

The following observations provide ample evidence that we have succeeded in cloning an endopeptidase gene of *L. lactis*: (i) antibodies directed against the purified endopeptidase of *L. lactis* bound to a 70-kDa protein specified by a lactococcal chromosomal DNA fragment cloned in phage λ, (ii) an oligonucleotide designed from the N-terminal amino acid sequence of the purified endopeptidase hybridized to specific fragments of the cloned lactococcal chromosomal fragment and was used to identify the 5′ end of the gene, (iii) the N-terminal amino acid sequence deduced from the nucleotide sequence of the endopeptidase gene was identical to that of the purified enzyme, and (iv) the deduced amino acid sequence of the endopeptidase showed a high degree of similarity to that of mammalian NEPs.

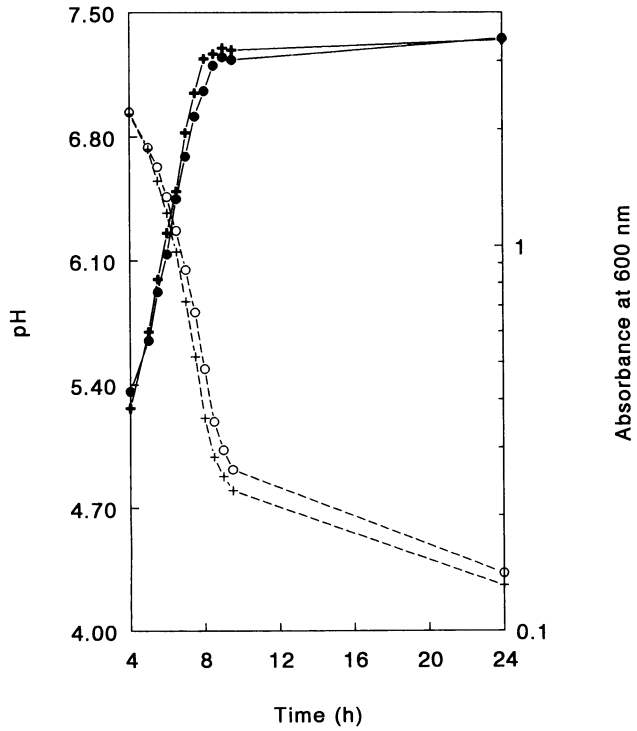

FIG. 8. Effect of a *pepO*-negative mutation on growth and acid production in milk. Growth (——) and acid production (– – –) are plotted against time. ++, MG1363; ○●, MG1363 *pepO*.

Recently, the genes for the binding-protein-dependent oligopeptide transport system (*opp*) from *L. lactis* SSL135 were characterized and sequenced elsewhere (63). Comparison of the nucleotide sequences of the genes of the *opp* operon with the sequence of the DNA fragment encompassing *pepO* of *L. lactis* P8-2-47 revealed that *pepO* is located immediately downstream of *oppA*, the last of the *opp* genes. Immediately upstream of the *oppA* gene, a promoter is present (62a), as was suggested by the results of the subcloning experiments with *E. coli*. The genetic linkage of an endopeptidase gene to the *opp* operon is unique for *L. lactis*. In the other binding-protein-dependent oligopeptide transport systems characterized (those of *E. coli*, *Salmonella typhimurium*, *Bacillus subtilis*, and *Streptococcus pneumoniae*), the gene order in the operons is mutually identical and different from that in *L. lactis* (1, 15, 16, 44). In *L. lactis*, *oppA* is the last of the *opp* genes, whereas in the other bacteria it is the first gene of the operons. The 127-bp region between *oppA* and *pepO* in *L. lactis* contains a weak rho-independent terminator. The region downstream of *oppA* in *S. typhimurium* is also followed by a long intercistronic region containing a rho-independent terminator-like structure (15), to which no function has yet been ascribed. In the deduced amino acid sequence of PepO, no typical prokaryotic signal sequence (74) was present. Furthermore, we did not detect possible membrane-spanning domains in the enzyme. This argues against an extracellular location of PepO or association of the enzyme with the outside of the cell envelope. This agrees with observations of Tan et al. (54) and Baankreis (3), who, on the basis of immunological and cell fractionation studies, have localized PepO and NOP, respectively, inside the cell. However, Tan et al. (54) found PepO in vesicle preparations. In these vesicles, the enzyme was resistant to proteinase K digestion. This indicates an association of the enzyme with the inner side of the cell membrane. The membrane association may be due to an interaction of PepO with components of the Opp system which would be consistent with *pepO* being part of the *opp* operon.

Comparison of the amino acid sequence of the endopeptidase with sequences in various data bases revealed homology to the mammalian NEP EC 3.4.24.11 (enkephalinase). NEP is a membrane-bound, zinc-dependent metalloendopeptidase, as indicated by the presence of a membrane-spanning domain, which is found at the surface of mammalian cells (27, 45). This enzyme is involved in degradation of various peptide hormones, including Met- and Leu-enkephalin (27, 53). NEP cleaves peptide bonds on the amino side of hydrophobic amino acids and is inhibited by phosphoramidon and thiorphan (26). Studies of substrate specificity and the effect of inhibitors show a remarkable similarity between PepO (3, 56) and NEP. At the primary-structure level, all amino acids which are presumably involved in zinc binding and in the formation of the active site in NEP are identical to those in PepO, except for one conservative change of

Arg-747 in NEP for Lys-625 in PepO. This includes Arg-102 in NEP (Arg-28 in PepO), a residue thought to be responsible for the dipeptidyl carboxypeptidase specificity and for the endopeptidase activity of NEP (4, 19). Interestingly, the lactococcal endopeptidase does not contain cysteine residues. This is in contrast to NEP, which carries 12 cysteine residues thought to stabilize the active enzyme by the formation of disulfide bridges (9, 53).

To assess the role of PepO in the proteolytic degradation of casein, a *pepO* mutant was constructed by gene disruption. No differences in growth kinetics, final cell densities, and pH values were observed after growth for 24 h in milk for the mutant and the parental strains. This indicates that the endopeptidase does not catalyze an essential step in the cascade of casein degradation. This observation is surprising, since mutations in the *opp* operon severely affect the growth of *L. lactis* in milk (63a). Apparently, the growth characteristics of these mutant strains can be solely ascribed to effects on the oligopeptide uptake system itself. One possible explanation for the dispensability of PepO is that its function can be taken over by one or several other peptidases present in the cell. Overlapping specificities of peptidases have been reported elsewhere for *E. coli* and *S. typhimurium* (22). Therefore, further characterization of *pepO* will be aimed at the construction of multiple mutations in the genes for peptidolytic enzymes to reveal and characterize possible overlapping specificities in the casein breakdown cascade.

Although PepO is not essential for growth of *L. lactis* in milk, it could be involved in cheese ripening. In pilot cheese-ripening experiments, Baankreis (3) observed that the endopeptidase is capable of releasing specific fragments from the α_{S1}-CN(f1-23) casein fragment, which is generated by the action of chymosin. Now that the *pepO* gene has been cloned, the possible contribution of the endopeptidase to cheese ripening and flavor production can be addressed in a rational way by using *pepO*-deficient mutants or strains overexpressing the *pepO* gene.

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REFERENCES

1. **Alloing, G., M.-C. Trombe, and J.-P. Claverys.** 1990. The *ami* locus of the gram-positive bacterium *Streptococcus pneumoniae* is similar to binding protein-dependent transport operons of gram-negative bacteria. Mol. Microbiol. **4:**633–644.
2. **Andressen, A., A. Geis, U. Krusch, and M. Teuber.** 1984. Plasmidmuster milchwirtschaftlich genutzter Starterkulturen. Milchwissenschaft **39:**140–143.
3. **Baankreis, R.** 1992. The role of lactococcal peptidases in cheese ripening. Ph.D. thesis. University of Amsterdam, The Netherlands.
4. **Beaumont, A., B. Barbe, H. Le Moual, G. Boileau, P. Crine, M.-C. Fournié-Zaluski, and B. P. Roques.** 1992. Charge polarity reversal inverses specificity of neutral endopeptidase-24.11. J. Biol. Chem. **267:**2138–2141.
5. **Bockelman, W., V. Monnet, A. Geis, M. Teuber, and J.-C. Gripon.** 1989. Comparison of cell wall peptidases from *Lactococcus lactis* subsp. *cremoris* AC1 and *Lactococcus lactis* subsp. *lactis* NCDO 763. Appl. Microbiol. Biotechnol. **31:**278–282.
6. **Chapot-Chartier, M.-P., M. Nardi, M.-C. Chopin, A. Chopin, and J.-C. Gripon.** 1993. Cloning and sequencing of *pepC*, a cysteine aminopeptidase gene from *Lactococcus lactis* subsp. *cremoris* AM2. Appl. Environ. Microbiol. **59:**330–333.
7. **Chomczynski, P., and P. K. Quasba.** 1984. Alkaline transfer of DNA to plastic membrane. Biochem. Biophys. Res. Commun. **122:**340–344.
8. **D'Adamio, L., M. A. Shipp, E. L. Masteller, and E. L. Reinherz.** 1989. Organization of the gene encoding common acute lymphoblastic leukemia antigen (neutral endopeptidase 24.11): multiple miniexons and separate 5′ untranslated regions. Proc. Natl. Acad. Sci. USA **86:**7103–7107.
9. **Devault, A., C. Lazure, C. Nault, H. Le Moual, N. G. Seidah, M. Chrétien, P. Kahn, J. Powell, J. Mallet, A. Beaumont, B. P. Roques, P. Crine, and G. Boileau.** 1987. Amino acid sequence of rabbit kidney neutral endopeptidase 24.11 (enkephalinase) deduced from a complementary DNA. EMBO J. **6:**1317–1322.
10. **Fox, P. F., and J. Law.** 1991. Enzymology of cheese ripening. Food Biotechnol. **5:**239–262.
11. **Frischauf, A.-M., H. Lehrach, A. Poustka, and N. Murray.** 1983. Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. **170:**827–842.
12. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:**1–9.
13. **Gasson, M. J., and F. L. Davies.** 1980. High-frequency conjugation associated with *Streptococcus lactis* donor cell aggregation. J. Bacteriol. **143:**1260–1264.
14. **Haandrikman, A. J., R. Meesters, H. Laan, W. N. Konings, J. Kok, and G. Venema.** 1991. Processing of the lactococcal extracellular serine proteinase. Appl. Environ. Microbiol. **57:**1899–1904.
15. **Hiles, I. D., M. P. Gallagher, D. J. Jamieson, and C. F. Higgins.** 1987. Molecular characterization of the oligopeptide permease of *Salmonella typhimurium*. J. Mol. Biol. **195:**125–142.
16. **Hogarth, B. G., and C. F. Higgins.** 1983. Genetic organization of the oligopeptide permease (*opp*) locus of *Salmonella typhimurium* and *Escherichia coli*. J. Bacteriol. **153:**1548–1551.
17. **Holo, H., and I. F. Nes.** 1989. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. Appl. Env. Microbiol. **55:**3119–3123.
18. **Jongeneel, C. V., J. Bouvier, and A. Bairoch.** 1989. A unique signature identifies a family of zinc-dependent metallopeptidases. FEBS Lett. **242:**211–214.
19. **Kim, Y.-A., B. Shriver, T. Quay, and L. B. Hersh.** 1992. Analysis of the importance of arginine 102 in neutral endopeptidase (enkephalinase) catalysis. J. Biol. Chem. **267:**12330–12335.
20. **Kok, J.** 1990. Genetics of the proteolytic system of lactic acid bacteria. FEMS Microbiol. Rev. **87:**15–42.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
22. **Lazdunski, A. M.** 1989. Peptidases and proteases of *Escherichia coli* and *Salmonella typhimurium*. FEMS Microbiol. Rev. **63:**265–276.

22a. **Leenhouts, K. J.** Unpublished data.

23. **Leenhouts, K. J., J. Kok, and G. Venema.** 1991. Lactococcal plasmid pWVO1 as an integration vector for lactococci. Appl. Environ. Microbiol. **57:**2562–2567.
24. **Leenhouts, K. J., J. Kok, and G. Venema.** 1990. Stability of integrated plasmids in the chromosome of *Lactococcus lactis*. Appl. Env. Microbiol. **56:**2726–2735.
25. **Leenhouts, K. J., J. Kok, and G. Venema.** 1989. Campbell-like integration of heterologous plasmid DNA into the chromosome of *Lactococcus lactis* subsp. *lactis*. Appl. Env. Microbiol. **55:**394–400.
26. **Le Moual, H., A. Devault, B. P. Roques, P. Crine, and G. Boileau.** 1991. Identification of glutamic acid 646 as a zinc-coordinating residue in endopeptidase-24.11. J. Biol. Chem. **266:**15670–15674.
27. **Lennarz, W. J., and W. J. Strittmatter.** 1991. Cellular functions of metallo-endoproteinases. Biochim. Biophys. Acta **1071:**149–158.
28. **Letarte, M., S. Vera, R. Tran, J. B. L. Addis, R. J. Onizuka,**

E. J. Quackenbush, C. V. Jongeneel, and R. R. McInnes. 1988. Common acute lymphocytic leukemia antigen is identical to neutral endopeptidase. J. Exp. Med. **168:**1247–1253.
29. **Ludwig, W., E. Seewaldt, R. Klipper-Bälz, K. H. Schleifer, L. Magrum, C. R. Woese, G. E. Fox, and E. Stackebrandt.** 1985. The phylogenetic position of *Streptococcus* and *Enterococcus*. J. Gen. Microbiol. **131:**543–551.
30. **Malfroy, B., W.-J. Kuang, P. H. Seeburg, A. J. Mason, and P. R. Schofield.** 1988. Molecular cloning and amino acid sequence of human enkephalinase (neutral endopeptidase). FEBS Lett. **229:** 206–210.
31. **Malfroy, B., P. R. Schofield, W.-J. Kuang, P. H. Seeburg, A. J. Mason, and W. J. Henzel.** 1987. Molecular cloning and amino acid sequence of rat enkephalinase. Biochem. Biophys. Res. Commun. **144:**59–66.
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **Matthews, B. W.** 1988. Structural basis of the action of thermolysin and related zinc peptidases. Acc. Chem. Res. **21:**333–340.
34. **Mayo, B., J. Kok, K. Venema, W. Bockelman, 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.
34a. **Mayo, B., and K. Venema.** Unpublished data.
35. **Monnet, V., W. Bockelman, 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. II. Specificity towards bovine β-casein. Appl. Microbiol. Biotechnol. **31:**112–118.
36. **Monnet, V., D. Le Bars, and J.-C. Gripon.** 1986. Specificity of a cell wall proteinase from *Streptococcus lactis* NCDO 763 towards bovine β-casein. FEMS Microbiol. Lett. **36:**127–131.
37. **Mundt, J. O.** 1986. Lactic acid streptococci, p. 1065–1066. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. Williams & Wilkins, Baltimore.
38. **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* NCDO763. Appl. Environ. Microbiol. **57:**45–50.
39. **Olson, N. F.** 1990. The impact of lactic acid bacteria on cheese flavor. FEMS Microbiol. Rev. **87:**131–148.
40. **Otto, R., W. M. de Vos, and J. Gravieli.** 1982. Plasmid DNA in *Streptococcus cremoris* Wg2: effect of pH on selection on chemostats of a variant lacking a protease plasmid. Appl. Environ. Microbiol. **43:**1272–1277.
41. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
42. **Reid, J. R., K. H. Ng, C. H. Moore, T. Coolbear, and G. G. Pritchard.** 1991. Comparison of β-casein hydrolysis by P_I and P_{III} type proteinases from *Lactococcus lactis* subsp. *cremoris*. Appl. Microbiol. Biotechnol. **36:**344–351.
43. **Rottlander, E., and T. A. Trautner.** 1970. Genetic and transfection studies with *Bacillus subtilis* phage SP50. J. Mol. Biol. **108:**47–60.
44. **Rudner, D. Z., J. R. LeDeaux, K. Ireton, and A. D. Grossman.** 1991. The *spo0K* locus of *Bacillus subtilis* is homologous to the oligopeptide permease locus and is required for sporulation and competence. J. Bacteriol. **173:**1388–1398.
45. **Sales, N., I. Dutriez, B. Maziere, M. Ottaviani, and B. P. Roques.** 1991. Neutral endopeptidase 24.11 in rat peripheral tissues: comparative localization by 'ex vivo' and 'in vitro' autoradiography. Regul. Pept. **33:**209–222.
46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
47. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
48. **Shipp, M. A., N. E. Richardson, P. H. Sayre, N. R. Brown, E. L. Masteller, L. K. Clayton, J. Ritz, and E. L. Reinherz.** 1988. Molecular cloning of the common acute lymphoblastic leukemia antigen (CALLA) identifies a type II integral membrane protein. Proc. Natl. Acad. Sci. USA **85:**4819–4823.
49. **Smid, E.** 1991. Physiological implications of peptide transport in *Lactococci*. Ph.D. thesis. University of Groningen, The Netherlands.
50. **Smid, E. J., B. Poolman, and W. N. Konings.** 1991. Casein utilization by lactococci. Appl. Environ. Microbiol. **57:**2447–2452.
51. **Snyder, M., S. Elledge, D. Sweetser, R. A. Young, and R. Davis.** 1989. λgt11: gene isolation with antibody probes and other applications, p. 309–329. *In* R. Wu, L. Grossman, and K. Moldave (ed.), Recombinant DNA methodology. Selected methods in enzymology. Academic Press, San Diego.
52. **Strøman, P.** 1992. Sequence of a gene (*lap*) encoding a 95.3-kDa aminopeptidase from *Lactococcus lactis* ssp. *cremoris* Wg2. Gene **113:**107–112.
53. **Tam, L.-T., S. Engelbrecht, J. M. Talent, R. W. Gracy, and E. G. Erdös.** 1985. The importance of disulfide bridges in human endopeptidase (enkephalinase) after proteolytic cleavage. Biochem. Biophys. Res. Commun. **133:**1187–1192.
54. **Tan, P. S. T., M.-P. Chapot-Chartier, K. M. Pos, M. Rousseau, C.-Y. Boquien, J.-C. Gripon, and W. N. Konings.** 1992. Localization of peptidases in lactococci. Appl. Environ. Microbiol. **58:**285–290.
55. **Tan, P. S. T., B. Poolman, and W. N. Konings.** 1992. The proteolytic enzymes of *Lactococcus lactis*. J. Dairy Res. **60:** 271–288.
56. **Tan, P. S. T., K. M. Pos, and W. N. Konings.** 1991. Purification and characterization of an endopeptidase from *Lactococcus lactis* subsp. *cremoris* Wg2. Appl. Environ. Microbiol. **57:**3593–3599.
57. **Tan, P. S. T., K. M. Pos, and W. N. Konings.** Unpublished results.
58. **Tan, P. S. T., I. J. van Alen-Boerrichter, B. Poolman, R. J. Siezen, W. M. de Vos, and W. N. Konings.** 1992. Characterization of the *Lactococcus lactis pepN* gene encoding an aminopeptidase homologous to mammalian aminopeptidase N. FEBS Lett. **306:**9–16.
59. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29:**807–813.
60. **Thomas, T. D., and G. G. Pritchard.** 1987. Proteolytic enzymes of dairy starter cultures. FEMS Microbiol. Rev. **46:**245–268.
61. **Tinoco, I., Jr., P. N. Borer, B. Dengler, M. D. Levine, O. C. Uhlenbeck, D. M. Crothers, and J. Gralla.** 1973. Improved estimation of secondary structure in ribonucleic acids. Nature (London) New Biol. **246:**40–41.
62. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
62a. **Tynkkynen, S.** Personal communication.
63. **Tynkkynen, S., G. Buist, E. Kunji, A. J. Haadrikman, J. Kok, B. Poolman, and G. Venema.** Unpublished data.
63a. **Tynkkynen, S., A. von Wright, and E.-L. Syväoja.** 1989. Peptide utilization encoded by *Lactococcus lactis* subsp. *lactis* SSL135 chromosomal DNA. Appl. Environ. Microbiol. **55:**2690–2695.
64. **van Alen-Boerrigter, I. J., R. Baankreis, and W. M. de Vos.** 1991. Characterization and overexpression of the *Lactococcus lactis pepN* gene and localization of its product, aminopeptidase N. Appl. Environ. Microbiol. **57:**2555–2561.
65. **van de Guchte, M., J. Kodde, J. M. B. M. van der Vossen, J. Kok, and G. Venema.** 1990. Heterologous gene expression in *Lactococcus lactis* subsp. *lactis*: synthesis, secretion, and processing of the *Bacillus subtilis* neutral protease. Appl. Environ. Microbiol. **56:**2606–2611.
66. **van de Guchte, M., J. Kok, and G. Venema.** 1992. Gene expression in *Lactococcus lactis*. FEMS Microbiol. Rev. **88:**73–92.
67. **van de Guchte, M., J. Kok, and G. Venema.** 1991. Distance-dependent translational coupling and interference in *Lactococ-*

cus lactis. Mol. Gen. Genet. **227:**65–71.
68. **van de Guchte, M., J. M. B. M. van der Vossen, J. Kok, and G. Venema.** 1989. Construction of a lactococcal expression vector: expression of hen egg white lysozyme in *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. **55:**224–228.
69. **van der Vossen, J. M. B. M., D. van der Lelie, and G. Venema.** 1987. Isolation and characterization of *Streptococcus cremoris* Wg2-specific promoters. Appl. Environ. Microbiol. **53:**2452–2457.
70. **Vijayaraghavan, J., Y.-A. Kim, D. Jackson, M. Orlowski, and L. B. Hersh.** 1990. Use of site-directed mutagenesis to identify valine-573 in the S'1 binding site of rat neutral endopeptidase 24.11 (enkephalinase). Biochemistry **29:**8052–8056.
71. **Visser, S., F. A. Exterkate, C. J. Slangen, and G. J. C. M. de Veer.** 1986. Comparative study of action of cell wall proteinases from various strains of *Streptococcus cremoris* on bovine α_{s1}-, β-, and κ-casein. Appl. Environ. Microbiol. **52:**1162–1166.
72. **Visser, S., A. J. P. M. Robbes, and C. J. Slangen.** 1991. Specificity of a cell-envelope-located proteinase (P_{III}-type) from *Lactococcus lactis* subsp. *cremoris* AM1 in its action on bovine β-casein. Appl. Microbiol. Biotechnol. **35:**477–483.
73. **Visser, S., C. J. Slangen, F. A. Extercate, and G. J. C. M. de Veer.** 1988. Action of a cell wall proteinase (P_I) from *Streptococcus cremoris* HP on bovine β-casein. Appl. Microbiol. Biotechnol. **29:**61–66.
74. **von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14:**4683–4690.
75. **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.
76. **Zabarovsky, E. R., and O. V. Turina.** 1988. Rapid isolation of λ phage DNA in micro- and macro-variants. Nucleic Acids Res. **16:**10925.
77. **Zabarovsky, E. R., and G. Winberg.** 1990. High efficiency electroporation of ligated DNA into bacteria. Nucleic Acids Res. **18:**5912.