

## Characterization and Overexpression of the *Lactococcus lactis pepN* Gene and Localization of Its Product, Aminopeptidase N

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The chromosomal *pepN* gene encoding lysyl-aminopeptidase activity in *Lactococcus lactis* has been identified in a  $\lambda$ EMBL3 library in *Escherichia coli* by using an immunological screening with antiserum against a purified aminopeptidase fraction. The *pepN* gene was localized and subcloned in *E. coli* on the basis of its expression and hybridization to a mixed-oligonucleotide probe for the previously determined N-terminal amino acid sequence of lysyl-aminopeptidase (P. S. T. Tan and W. N. Konings, Appl. Environ. Microbiol. 56:526-532, 1990). The *L. lactis pepN* gene appeared to complement an *E. coli* strain carrying a mutation in its *pepN* gene. High-level expression of the *pepN* gene in *E. coli* was obtained by using the T7 system. The overproduction of the 95-kDa aminopeptidase N could be visualized on sodium dodecyl sulfate-polyacrylamide gels and immunoblots. Cloning of the *pepN* gene on a multicopy plasmid in *L. lactis* resulted in a 20-fold increase in lysyl-aminopeptidase activity that corresponded to several percent of total protein. Nucleotide sequence analysis of the 5' region of the *pepN* gene allowed a comparison between the deduced and determined amino-terminal primary sequences of aminopeptidase N. The results show that the amino terminus of PepN is not processed and does not possess the characteristics of consensus signal sequences, indicating that aminopeptidase N is probably an intracellular protein. The intracellular location of aminopeptidase N in *L. lactis* was confirmed by immunogold labeling of lactococcal cells.

Lactococci that are used as starter cultures for industrial dairy fermentations contain an efficient but specific proteolytic system for the degradation of caseins, the major milk proteins. The degradation products, small peptides and free amino acids, are essential for the metabolism of the fastidious lactococci and contribute to the development of flavor in fermented-milk products (30). The cascade of proteolytic reactions is initiated by an extracellular cell-envelope-located serine proteinase (6, 8, 15) that produces oligopeptides and larger casein fragments. Successive degradation by both endo- and exopeptidases then generates the amino acids that support the growth of these gram-positive bacteria. The cellular location of these peptidases largely determines their specific contribution to the sequential casein degradation steps and the necessity for particular systems for peptide and amino acid transport (16).

Various peptidases with different substrate specificities have been isolated from lactococci and include metallopeptidases and serine peptidases (for a review, see reference 15). Important biochemical properties of the peptidases, such as location, exact size, and subunit composition, are largely unknown or subject to controversy. In addition, until recently no genetic studies of lactococcal peptidases had been reported. This is in strong contrast with the *Lactococcus lactis* proteinase, which has been analyzed extensively at the genetic level to define its function in the proteolytic system and now serves as a model for protein engineering with the aim of improving the caseinolytic capacity of lactococci (8, 15, 33). Very recently, the lactococcal *pepXP* gene for X-prolyl-dipeptidylaminopeptidase has been cloned and further characterized (19, 20). This aminopeptidase, which belongs to the class of serine peptidases, was first reported to be extracellular but is now assumed to be located

inside the lactococcal cells since sequencing studies of the *pepXP* gene and its product showed that the N-terminal sequence is not processed (19, 20).

A metalloaminopeptidase that shows high activity toward lysyl- and leucyl-*p*-nitroanilide is another peptidase that is presumed to be located extracellularly. Following the initial description of this aminopeptidase in *L. lactis* HP (10), its purification from the cell envelope of *L. lactis* AC1 has been described (12). Recently, an aminopeptidase with similar activity was detected in the crude cell extract and supernatant of *L. lactis* Wg2; it has since been purified and characterized (29). The published subunit molecular masses of the purified AC1 and Wg2 lysyl-aminopeptidases were considerably different (36 and 95 kDa, respectively), despite the similarity in substrate specificity, sensitivity to inhibitors, and presumed location of these enzymes (12, 29). In this report we describe the cloning, characterization, and overexpression in *Escherichia coli* and *L. lactis* of the *L. lactis* gene for lysyl-aminopeptidase. The gene has been designated *pepN* since it complements an *E. coli pepN* mutation. The nucleotide sequence of the 5' region of the *L. lactis pepN* gene has been determined, and the location of aminopeptidase N in lactococci was studied by using immunoelectron microscopy. The results indicate that *L. lactis* PepN is a 95-kDa monomeric, intracellular aminopeptidase.

### MATERIALS AND METHODS

**Bacterial strains, media, and growth.** *E. coli* K803 (36) and Q359 (14) were grown on 1% tryptone (Difco Laboratories, Detroit, Mich.) with 0.5% NaCl, 0.2% maltose, and 10 mM MgSO<sub>4</sub>. *E. coli* MC1061 (3), TG1 (13), 9207 (18),  $\Delta$ H $\Delta$ trp (21), and BL21 (27) were grown on L broth (23), supplemented when appropriate with 10  $\mu$ g of chloramphenicol per ml or 50  $\mu$ g of ampicillin per ml. All *E. coli* strains were grown at 37°C. *L. lactis* subsp. *cremoris* HP (NIZO collec-

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tion) was grown in reconstituted nonfat milk, and the plasmid-free *L. lactis* subsp. *lactis* MG1363 (12) and its derivatives were grown in lactose-M17 broth (E. Merck AG, Darmstadt, Federal Republic of Germany) supplemented when appropriate with 0.5% glucose and/or 10  $\mu$ g of erythromycin per ml. Both strains were grown at 30°C.

**Purification of aminopeptidase N.** *L. lactis* HP cells from an overnight milk culture (10 liters) were harvested, washed, and resuspended to an  $A_{650}$  of 40 in 25 mM imidazole buffer (pH 6.3) containing 100 mM NaCl and 550 mM sucrose. The suspension was supplemented with lysozyme (5 mg/ml) and incubated at 25°C for 90 min. This procedure converted the cells into spheroplasts as revealed by electron microscopy (not shown). The spheroplasts were collected by centrifugation and resuspended in 50 mM imidazole buffer (pH 6.3) containing 100 mM NaCl. Complete lysis was obtained by sonication (five cycles of 30 s) (model W-375; Heat Systems-Ultra Sonics, Inc.), and the debris were removed by centrifugation (20 min at 15,000  $\times$  g). The cell extract, consisting mainly of cytosolic proteins, contained approximately 90% of total PepN aminopeptidase activity. Aminopeptidase N was purified from this extract by ammonium sulfate precipitation (with the PepN aminopeptidase activity appearing in the 28 to 38% [wt/vol] fraction) and Fractogel TSK-butyl-650(s) (E. Merck AG) hydrophobic interaction chromatography (with PepN aminopeptidase activity eluting from the column at an ammonium sulfate concentration of 600 to 500 mM). The active fraction was concentrated, desalted, and transferred to a 0.25 M potassium phosphate buffer (pH 7.0) by using ultrafiltration (50-kDa cutoff; Filtron Technologies). A final purification step included high-performance liquid chromatography (HPLC) gel filtration with an HPLC pump (flow rate, 0.8 ml/min) (model M510; Waters Associates), two coupled columns (Varian TSK3000 and TSK2000, each 300 by 7.5 mm), a variable-wavelength detector used at 280 nm (Lambda Max model 481 LC; Waters), and an injector (model 231/401; Gilson, Middleton, Wis.). The eluant buffer used was 0.25 M potassium phosphate (pH 7.0). The effluent containing aminopeptidase N activity was collected, concentrated by ultrafiltration, and reappplied to the column system to yield a single, symmetrical activity peak.

The relative molecular mass of aminopeptidase N was calculated to be approximately 93 kDa by using a set of calibration proteins with relative molecular masses between 18 and 300 kDa (Boehringer, Mannheim, Federal Republic of Germany). The yield was approximately 4 mg of protein. Further analysis of this preparation showed that aminopeptidase N had an apparent relative molecular mass of 95 kDa on sodium dodecyl sulfate (SDS)-polyacrylamide gels (17) and was contaminated with a protein with a subunit size of 35 kDa (see Fig. 1 and 3). Apparently this component copurified with aminopeptidase N under the conditions used. Part of the purified aminopeptidase N preparation was used to immunize rabbits for antibody production as described previously (9).

**Molecular cloning, expression, and partial nucleotide sequence analysis of the *pepN* gene.** A genomic library of *L. lactis* MG1363 partial *Sau3A* DNA fragments was prepared in *E. coli* K803 by using  $\lambda$ EMBL3 (22) and the EMBL3 Arms Cloning System (Packagene Lambda Packaging System; Pharmacia LKB Biotechnology, Uppsala, Sweden) as described previously (31). The library was screened with the antibodies against aminopeptidase N in the following way. Phages were plated on *E. coli* K803 (10 plates, containing ca. 200 phages each) in top agarose on 1% tryptone agar (Difco Laboratories) with 0.5% NaCl, and after a 3-h incubation at

37°C, a nitrocellulose membrane (BA85; Schleicher & Schuell, Inc., Keene, N.H.) was placed on each plate. This membrane was removed after overnight incubation, treated with aminopeptidase N antiserum, and subjected to incubation with peroxidase-labeled goat anti-rabbit antibodies (Bethesda Research Laboratories). Positive plaques were visualized with 1-chloro-4-naphthol as the substrate and tested for the presence of recombinant phages by propagation on *E. coli* Q359.

Phage DNA from  $\lambda$ NZ118, one of the clones that appeared positive in the immunoscreening and expressed aminopeptidase N activity, was isolated and found to contain an insert of approximately 14 kb of *L. lactis* DNA. A 8.2-kb *PstI* fragment could be isolated from this insert DNA and was used as a source for the *L. lactis pepN* gene in further cloning experiments that were performed by using established procedures (23) and the vector pNZ84. This plasmid is a 2.5-kb derivative of the *E. coli* vector pACYC184 (4), in which the 1.6-kb *HincII-HindIII* fragment, containing part of the tetracycline resistance gene, was replaced by a 37-bp *SmaI-HindIII* fragment from M13mp19 (37) containing the following cloning sites: (*HincII/SmaI-BamHI-XbaI-SalI-PstI-SphI-HindIII*) (5). The unique *SalI* site in pNZ84 is also the unique *HincII* site.

For overproduction of aminopeptidase N, the *E. coli* expression vectors pT7-5 (28) and pNZ28 were used (see Fig. 2). Induction of the  $\phi_{10}$  T7 RNA polymerase promoter in pT7-5 or derivatives was obtained by the addition of 0.4 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) to a growing culture of *E. coli* BL21 harboring the constructs and continued incubation for 1 h. pNZ28 is a 2.8-kb derivative of pPLc28 (21), in which the *EcoRI-HindIII* fragment was replaced by the 51-bp *EcoRI-HindIII* fragment of M13mp18 containing its complete multiple cloning site (34). This plasmid contains the  $\lambda p_L$  promoter in front of the multiple cloning site. *E. coli*  $\Delta$ H $\Delta$ trp harboring pNZ28 derivatives was grown at 28°C and, at an  $A_{600}$  of 0.5, incubated for 1 h at 42°C to inactivate the thermosensitive *cI857* repressor. Cloning in *L. lactis* MG1363 was performed by using the lactococcal vector pIL253 (26), and DNA was introduced by electroporation with a GenePulser (Bio-Rad Laboratories, Richmond, Calif.) as described previously (9, 35).

Sequence analysis by the dideoxy-chain method (24) was performed on single-stranded DNA obtained by cloning in M13mp18 (37), using primers synthesized on a Cyclone DNA synthesizer (Biosearch, San Rafael, Calif.) and Sequenase (U.S. Biochemical Corp., Cleveland, Ohio). All other enzymes were used as specified by the suppliers (Bethesda Research Laboratories, Gaithersburg, Md., and New England BioLabs Inc., Beverly, Mass.).

**Preparation of extracts and enzymatic assays.** *E. coli* or *L. lactis* cells (10 ml) were collected by centrifugation after overnight growth and resuspended in 1 ml of a 50 mM phosphate buffer (pH 7.5). Approximately 1 g of zirconium glass beads (diameter, 0.10 mm; Biospec Products, Bartlesville, Okla.) was added, and cells were disrupted by a treatment of two (*E. coli*) or four (*L. lactis*) cycles of 3 min on a cell disruptor (Beadbeater; Biospec Products). Aminopeptidase N activity was determined at 30°C by monitoring the hydrolysis of the substrate lysyl-*p*-nitroanilide (Fa. Bachem, Bubendorf, Switzerland) as described previously (10). Protein concentrations were determined as described previously (2) by using bovine serum albumin as a standard.

**Western immunoblot analysis.** Samples were prepared for immunoblot analysis as follows. First, phage lysates (200  $\mu$ l) were concentrated by freeze-drying and resuspended in 30  $\mu$ l

of sample buffer (60 mM Tris hydrochloride [pH 6.8], 1% SDS, 1%  $\beta$ -mercaptoethanol, 10% glycerol, 0.01% bromophenol blue). Then lysates of *E. coli* or *L. lactis* were prepared as described above and mixed with equal amounts of sample buffer. After heating at 100°C for 5 min, aliquots of the lysates were applied to a 10% polyacrylamide-SDS gel (17) (Midget System; Pharmacia LKB Biotechnology), which was also used to separate a set of prestained calibration proteins (Bethesda Research Laboratories). Proteins were transferred to a nitrocellulose membrane (BA85; Schleicher & Schuell) as specified by the manufacturer; the membrane was subsequently treated with aminopeptidase N antiserum and then incubated with peroxidase-labeled goat anti-rabbit antibodies.

**Hybridization methods.** A 0.7% agarose gel was blotted for 1 h on a GeneScreen Plus membrane (Dupont, NEN Research Products, Wilmington, Del.), with 0.4 N NaOH-0.6 M NaCl as transfer buffer, by using a blotting device (Vacugene; Pharmacia LKB Biotechnology). A mixed oligonucleotide was synthesized (Cyclone DNA Synthesizer; Biosearch, San Rafael, Calif.) that was based on amino acid residues 11 to 17 of the published amino-terminal sequence of the aminopeptidase of *L. lactis* subsp. *cremoris* Wg2 (29), with the following sequence: 3' GGN CTY TTR ATR TTY TAD AA 5' (N is A, G, C or T; Y is C or T; R is A or G; D is A, G, or T). Hybridization with this probe was performed at 40°C in a 0.5 M sodium phosphate buffer (pH 7.2), and the blot was subsequently washed with  $0.1 \times$  SSC ( $1 \times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (23) at room temperature and finally exposed to X-ray film.

**Immunoelectron microscopy.** Cells of *L. lactis* HP were fixed in phosphate-buffered saline (PBS) (pH 7.4) containing 1% (vol/vol) glutaraldehyde and 1% (vol/vol) *p*-formaldehyde and subsequently embedded in LR White medium (Bio-Rad Laboratories). Immunogold labeling of aminopeptidase N was performed on ultrathin sections with aminopeptidase N antiserum and protein A-gold particles (size, 10 nm; Jansen Lifescience Products, Beerse, Belgium). Sections were examined by using a model JEM 1200 EX electron microscope (JEOL, Tokyo, Japan) at 60 kV.

**Nucleotide sequence accession number.** The GenBank accession number of the reported sequence is M65867.

## RESULTS

**Identification and cloning of the *L. lactis pepN* gene.** A preliminary screening showed the presence of aminopeptidase N activity in all *L. lactis* strains investigated, including the well-studied plasmid-free *L. lactis* MG1363 (1). A library of MG1363 chromosomal DNA in  $\lambda$ EMBL3 was screened twice with aminopeptidase N antiserum. Of approximately 2,000 plaques, 9 reacted positively and contained recombinant phages. Lysates were prepared from six of the best-growing phages and tested for aminopeptidase N activity and for the presence of proteins that reacted with anti-aminopeptidase antibodies on immunoblots. This analysis (Fig. 1) showed that one phage, designated  $\lambda$ NZ118, coded for aminopeptidase N activity and produced a single immunoreactive protein of approximately 95 kDa. The other phages did not show aminopeptidase N activity and appeared to code for immunoreactive proteins that were considerably smaller than that produced by  $\lambda$ NZ118. These results confirm that aminopeptidase N is 95 kDa and indicate that the 35-kDa protein present in the purified preparation used for immunization (Fig. 1, lane 8; also see Materials and Methods) is a contaminant without aminopeptidase N activity.

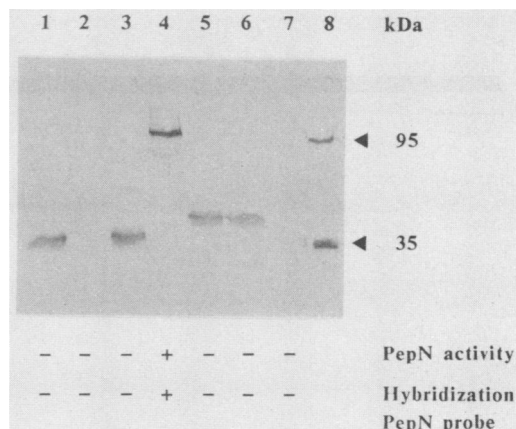


FIG. 1. Immunoblot after SDS-polyacrylamide gel electrophoresis of phage lysates. The presence or absence of aminopeptidase N activity in the phage lysates and the ability or inability of the phage DNA to hybridize with a probe specific for the N-terminal sequence of aminopeptidase N are both indicated by + or -, respectively. Lanes: 1,  $\lambda$ NZ112; 2,  $\lambda$ NZ113; 3,  $\lambda$ NZ115; 4,  $\lambda$ NZ118; 5,  $\lambda$ NZ119; 6,  $\lambda$ NZ129; 7,  $\lambda$ EMBL3; 8, purified aminopeptidase N. The sizes of the immunoreactive proteins (in kilodaltons) present in the purified aminopeptidase N preparation are indicated at the right.

Moreover, the fact that clones were obtained (lanes 1, 3, and 8) that produced only the 35-kDa protein strongly suggests that this protein is not a degradation product of the 95-kDa aminopeptidase N.

For further authentication and localization of the *pepN* gene, phage DNAs were hybridized to an oligonucleotide probe, based on the amino-terminal sequence of the purified aminopeptidase N (29). Only  $\lambda$ NZ118 DNA showed significant hybridization to this PepN-specific probe (Fig. 1), indicating that it contained the *L. lactis pepN* gene.

**Location, orientation of the *pepN* gene, and its overexpression in *E. coli*.** A 5.8-kb *EcoRV-PstI* fragment of  $\lambda$ NZ118 DNA strongly hybridized with the PepN-specific probe and was subcloned into *HincII-PstI*-digested pNZ84, resulting in pNZ1104 (Fig. 2). Cells of *E. coli* MC1061 harboring plasmid pNZ1104 showed a level of aminopeptidase N activity that was significantly higher than the background activity of the same host harboring the vector pNZ84 (Table 1). This correlated well with the presence in those cells of a 95-kDa protein that reacted with the anti-aminopeptidase antibodies (Fig. 3). In addition, the *E. coli pepN* mutant 9207 (18) could be complemented by pNZ1104 (Table 1). These results confirmed that the 5.8-kb *EcoRV-PstI* fragment contained the intact *L. lactis pepN* gene; subsequently, a more detailed restriction fragment map of this DNA was produced (Fig. 2).

To determine the orientation of the *pepN* gene and analyze its overexpression in *E. coli*, the 5.8-kb *EcoRV-PstI* fragment of pNZ1101 containing the *pepN* gene was cloned into the expression vector pNZ28 digested with *SmaI-PstI*, resulting in pNZ1111. If the *pepN* gene in plasmid pNZ1111 is orientated under control of the vector-located  $\lambda p_L$  promoter, it is expected to be overexpressed when pNZ1111 is introduced into *E. coli*  $\Delta H\Delta trp$  and the thermolabile *c1857* repressor of this host is inactivated. The results (Fig. 3) show that aminopeptidase N activity is induced severalfold after induction of the  $\lambda p_L$  promoter at 42°C. These data suggest that the direction of transcription of the *pepN* gene is as shown in Fig. 2.

The degree of overexpression of the *pepN* gene by the  $\lambda p_L$

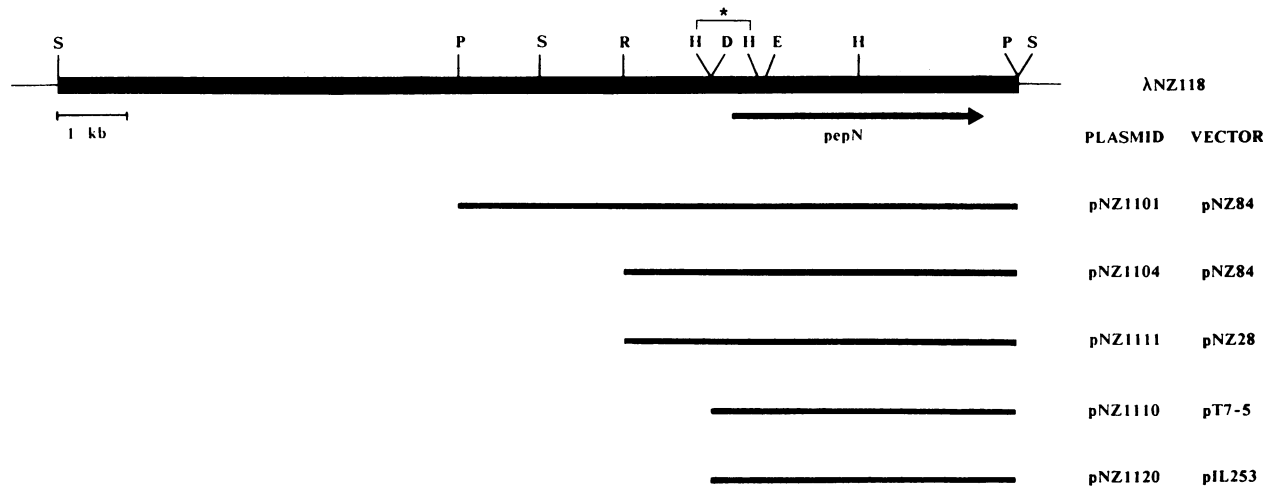


FIG. 2. Restriction map of the *L. lactis* insert DNA in  $\lambda$ NZ118. Fragments that were used for subcloning into *E. coli* or *L. lactis* vectors and the resulting plasmids are indicated. The arrow indicates the approximate location of the *pepN* gene and its orientation. Abbreviations: D, *DraI*; E, *EcoRI* (not all shown); H, *HindIII* (not all shown); P, *PstI*; R, *EcoRV*; S, *SmaI*. The star indicates the 0.7-kb *HindIII* fragment that has been used for sequence analysis.

promoter was limited, and immunoblot experiments showed that the produced aminopeptidase N was subject to proteolysis (Fig. 3). As an alternative method of obtaining high-level production of aminopeptidase N, the T7 expression system in combination with *E. coli* BL21 was used. To this end, a 4.5-kb *DraI-PstI* fragment from pNZ1104 was cloned under control of the T7 RNA polymerase promoter in pT7-5, resulting in plasmid pNZ1110 (Fig. 2). Noninduced and IPTG-induced cells of *E. coli* BL21 harboring pNZ1110 appeared to contain high levels of aminopeptidase N activity (Table 1). In addition, despite some proteolysis, a prominent aminopeptidase N band of approximately 95 kDa was present in the extracts of *E. coli* BL21 harboring pNZ1110 (Fig. 3).

**Cloning and overexpression of the *pepN* gene in *L. lactis*.** Since the 4.5-kb *DraI-PstI* fragment from pNZ1104 allowed high-level expression of the *pepN* gene in *E. coli*, this fragment was also introduced into *L. lactis* MG1363 by

cloning into pIL253 digested with *SmaI* and *PstI*. The resulting plasmid, pNZ1120, had the expected structure and could be stably maintained in *L. lactis* (data not shown). *L. lactis* MG1363 harboring pNZ1120 showed an approximately 20-fold higher specific activity of aminopeptidase N than the untransformed strain or that harboring the vector pIL253 (Table 1). In addition, the 95-kDa band of aminopeptidase N, hardly visible in MG1363 protein extracts, is very pronounced in extracts of *L. lactis* MG1363 harboring pNZ1120 (Fig. 3). These results demonstrate that aminopeptidase N can be overproduced significantly in *L. lactis*.

**Sequence analysis of the 5' region of the *pepN* gene.** Fine restriction site mapping and hybridization analysis of the *pepN* gene allowed the identification and location of a 0.7-kb *HindIII* fragment (Fig. 2) that strongly hybridized with the oligonucleotide probe specific for the N terminus of aminopeptidase N. This fragment was cloned in two orientations into *HindIII*-linearized M13mp18, and both strands were sequenced by using universal and synthetic sequencing primers in the strategy shown in Fig. 4.

The nucleotide sequence of the 0.7-kb *HindIII* fragment (Fig. 4) shows the start of a single open reading frame in the direction expected for that of the *pepN* gene. The first initiation codon of this open reading frame is an ATG at position 324, which is preceded by a consensus lactococcal ribosome-binding site (6). Translation from this site should result in a polypeptide with a deduced amino-terminal sequence that is almost identical to the experimentally determined amino terminus of the purified aminopeptidase N (29) (Fig. 4). From these results, we conclude that the *pepN* gene starts at position 324 and that its expression product, aminopeptidase N, is not processed as would be expected when this protein was to be secreted via a signal peptidase-dependent secretory pathway.

**Immunoelectron microscopic localization of the aminopeptidase N in *L. lactis*.** Aminopeptidase N activity appeared to be detectable in whole cells or cell extracts but not in the supernatant of *L. lactis* cultures (Table 1; results not shown). To further localize aminopeptidase N, we labeled ultrathin sections of whole lactococcal cells by using protein A-gold particles and the antiserum against aminopeptidase N and

TABLE 1. Specific activities of aminopeptidase N produced by *E. coli* and *L. lactis* strains harboring *pepN*-overexpressing plasmids

Strain (plasmid)	IPTG <sup>a</sup>	Mean sp act (nmol/min/mg) ± SD <sup>b</sup>
<i>E. coli</i>		
9207		<0.5
9207(pNZ1104)		332 ± 61
MC1061(pNZ84)		19 ± 3
MC1061(pNZ1104)		184 ± 30
BL21 (pT7-5)		45 ± 3
BL21(pNZ1110)	-	513 ± 103
BL21(pNZ1110)	+	1,083 ± 76
<i>L. lactis</i>		
MG1363(pIL253)		62 ± 3
MG1363(pNZ1120)		1,527 ± 143

<sup>a</sup> The absence (no IPTG addition) or presence (addition of IPTG) of inducing conditions for the *E. coli* T7 promoter is indicated.

<sup>b</sup> Represents the mean and standard deviation of five independent determinations.

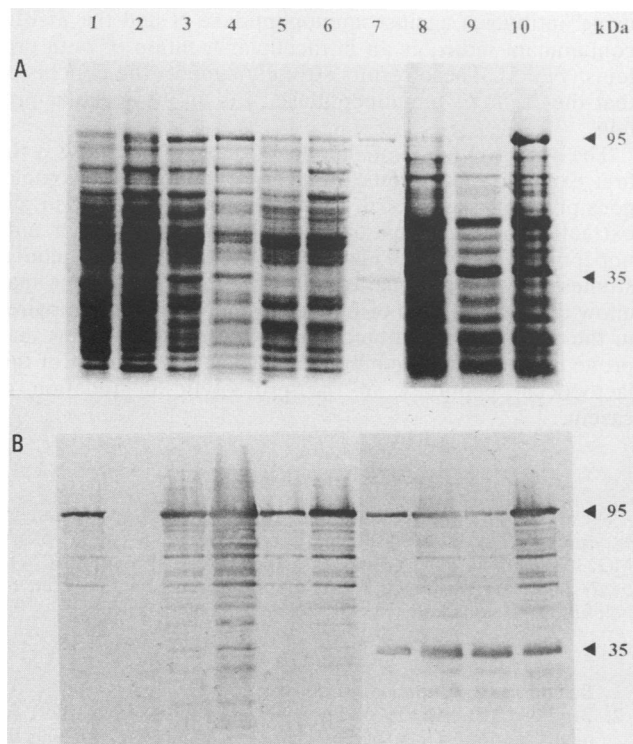


FIG. 3. Coomassie blue-stained gel (A) and immunoblot (B) of extracts of *E. coli* and *L. lactis* producing aminopeptidase N. Lanes: 1, MC1061 harboring pNZ1104; 2, BL21 harboring pT7-5; 3, BL21 harboring pNZ1110; 4, BL21 harboring pNZ1110, induced with IPTG; 5,  $\Delta H\Delta trp$  harboring pNZ1111, grown at 28°C; 6,  $\Delta H\Delta trp$  harboring pNZ1111, induced at 42°C; 7, purified aminopeptidase N; 8, MG1363; 9, MG1363 harboring pIL253; 10, MG1363 harboring pNZ1120. The sizes of the proteins (in kilodaltons) present in the purified aminopeptidase N preparation are indicated at the right.

the 35-kDa contaminant protein (Fig. 5). In all cases, the gold particles were present predominantly inside the cytoplasm, indicating that both aminopeptidase N and its contaminant are intracellular proteins.

## DISCUSSION

We have isolated, cloned, and characterized the chromosomally located *pepN* gene from *L. lactis* MG1363 encoding lysyl-aminopeptidase activity. Sequence analysis of the 5' region of the *pepN* gene has shown that its gene product, aminopeptidase N, is identical to a previously characterized metalloaminopeptidase from *L. lactis* Wg2 with high activity for lysyl- and, to a lesser extent, leucyl-*p*-nitroanilides (29). Similar aminopeptidase activities have been found in many strains of *L. lactis* (1, 10–12, 29). This suggests that aminopeptidase N has an essential role in the metabolism of lactococci and may be involved in nitrogen supply or protein turnover.

The *pepN* gene has been expressed in *E. coli* and *L. lactis* and appeared to encode a 95-kDa protein (Fig. 3). These genetic data are in agreement with our biochemical characterization showing that the aminopeptidase N purified from *L. lactis* HP (Fig. 1 and 3) is a monomer with an apparent molecular mass of 95 kDa (in denaturing gels) or 93 kDa (in gel permeation HPLC). This is in contrast to the subunit size of 36 kDa reported for the lysyl-aminopeptidase isolated

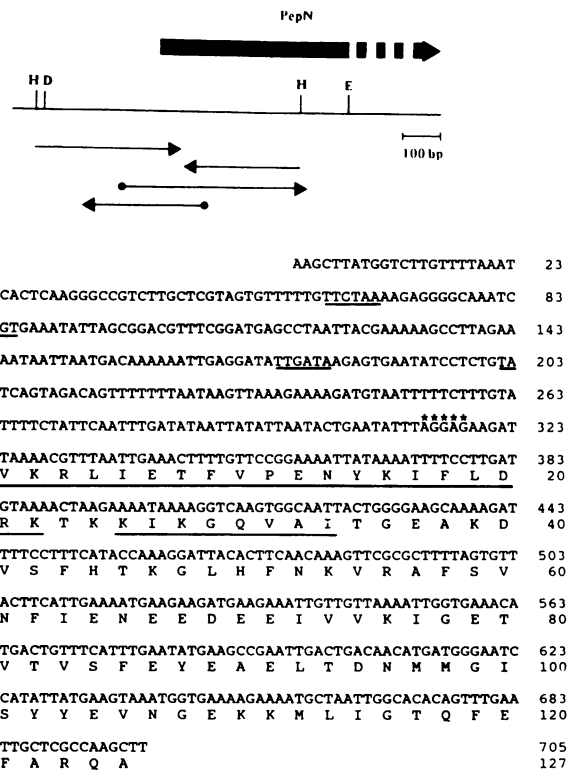


FIG. 4. Sequence strategy of part of the *L. lactis* 1363 *pepN* gene (top) and nucleotide sequence of the 0.7-kb *Hind*III fragment (bottom) with the deduced amino acid sequence of aminopeptidase N. The residues that are identical to the first 34 N-terminal amino acids determined from the purified aminopeptidase N of *L. lactis* Wg2 (29) are underlined in the protein sequence. Abbreviations: H, *Hind*III; D, *Dra*I; E, *Eco*RI. The ribosome-binding site of the *pepN* gene is indicated by the stars, and consensus lactococcal promoter sequences (6) are underlined.

from *L. lactis* AC1 (12). It is remarkable that the reported size of the latter protein corresponds closely to that of the 35-kDa contaminant present in the *L. lactis* HP aminopeptidase N preparation (Fig. 3). Since the latter preparation was used for immunization, the obtained antiserum cross-reacted with the 35-kDa contaminant, explaining why, in the immunological screening of the EMBL3 library, several phages were detected that produced a 35-kDa immunoreactive protein (Fig. 1). The production of an immunoreactive protein of approximately 50 kDa by two of the phages (Fig. 1) may be ascribed to a fusion of part of the gene for either PepN or the 35-kDa protein with a gene of the  $\lambda$ EMBL3 vector, as has been observed previously (9).

Aminopeptidase N is produced at relatively low levels by lactococci (Table 1) (29). To provide an alternative source, we have overexpressed the *pepN* gene in *E. coli* and *L. lactis*. The expression of the *pepN* gene in *E. coli*, using the  $\lambda p_L$  and T7 promoters (in pNZ1110 and pNZ1111, respectively [Fig. 2 and 3]) resulted in a high level of aminopeptidase N production (Table 1; Fig. 3). Unexpectedly, significant overexpression of *pepN* was obtained in *E. coli* BL21 harboring pNZ1110 without IPTG induction (Table 1; Fig. 3). It is unlikely that this is a consequence simply of insufficient repression of the *tac* promoter that controls T7 polymerase gene expression in strain BL21. Alternatively, it is possible that the A+T-rich sequence upstream of the

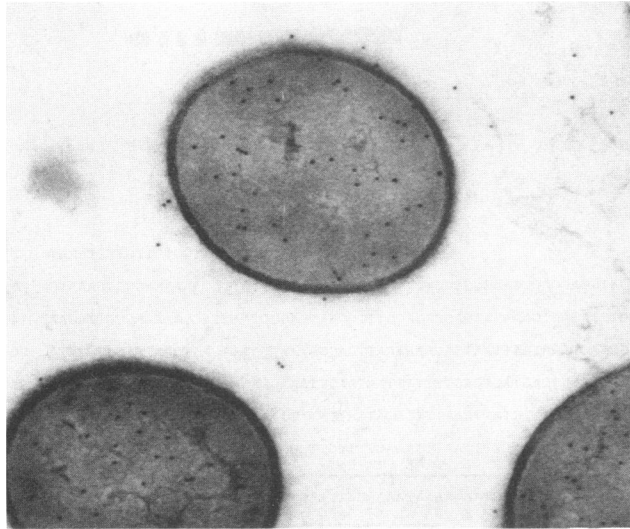


FIG. 5. Immunogold labeling of *L. lactis* HP cells by using the aminopeptidase N antiserum.

*pepN* gene in pNZ1110 contains one or more efficient *E. coli* promoters.

We obtained a degree of overexpression of *pepN* in *L. lactis* MG1363 similar to that in *E. coli*, and we found that the level of aminopeptidase N was increased approximately 20-fold by cloning the *pepN* gene on the vector pIL253 (Table 1). This may well be ascribed to a gene dosage effect since the copy number of pIL253 in *L. lactis* is calculated to be approximately 35 (26). The aminopeptidase N in *L. lactis* MG1363 harboring pNZ1120 amounted to a substantial portion of the total protein (Fig. 3). These results are in agreement with the presence of consensus *L. lactis* promoters (6) present on the A+T-rich fragment preceding the *pepN* gene (Fig. 4). Moreover, pIL253 contains a promoter in the replication region that reads through into the multiple cloning site (7) and may also participate in the overexpression of the *pepN* gene in pNZ1120.

The nucleotide sequence analysis of the 0.7-kb *Hind*III fragment (Fig. 4) allowed for the exact location of the *pepN* gene. In addition, the sequence of the first 127 N-terminal residues of aminopeptidase N could be deduced. Further sequence analysis of the *pepN* gene revealed a continuation of the open reading frame and showed that its deduced amino sequence is homologous to those of eucaryotic and bacterial aminopeptidase N enzymes (25). The amino-terminal sequence of the purified aminopeptidase N (29) shows identity with that deduced from the *pepN* gene sequence (Fig. 4). This allows the exact location of the translational start of the *pepN* gene and the identification of the ribosome-binding site (Fig. 4), which conforms to other lactococcal ribosome-binding sites in showing a high degree of complementarity to the 3' end of the *L. lactis* 16S rRNA (6). In addition, it demonstrates that processing of the amino terminus of the *L. lactis* aminopeptidase N is limited to removal of the initiator methionine, as is common in bacterial systems. The first 35 amino-terminal amino acid residues of aminopeptidase N are highly charged and may form an amphipathic  $\alpha$ -helix, but they do not correspond to a consensus signal peptide (32). These findings suggest that aminopeptidase N is not exported via processing of a signal peptide. Moreover, immunogold labeling of *L. lactis* cells by

using antibodies against aminopeptidase N and the 35-kDa contaminant indicates an intracellular location of both proteins (Fig. 5). These results strongly support the conclusion that the *L. lactis* aminopeptidase N is an intracellular protein.

The overproduction of aminopeptidase N in *L. lactis* is the first example of a significant overproduction of a homologous protein in lactococci. This overproduction allows rapid extraction and purification of milligram amounts of aminopeptidase N (1) that may be used in further biochemical studies. In addition, the availability of the *pepN* gene may allow the construction of *L. lactis* mutants that are impaired in the production of aminopeptidase N. These strains may prove useful in determining the specific contribution of this lactococcal peptidase in the proteolytic degradation of casein.

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