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Genetic Characterization and Physiological Role of Endopeptidase O from *Lactobacillus helveticus* CNRZ32

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A previously identified insert expressing an endopeptidase from a *Lactobacillus helveticus* CNRZ32 genomic library was characterized. Nucleotide sequence analysis revealed an open reading frame of 1,941 bp encoding a putative protein of 71.2 kDa which contained a zinc-protease motif. Protein homology searches revealed that this enzyme has 40% similarity with endopeptidase O (PepO) from *Lactococcus lactis* P8-2-47. Northern hybridization revealed that *pepO* is monocistronic and is expressed throughout the growth phase. CNRZ32 derivatives lacking PepO activity were constructed via gene replacement. Enzyme assays revealed that the PepO mutant had significantly reduced endopeptidase activity when compared to CNRZ32 with two of the three substrates examined. Growth studies indicated that PepO has no detectable effect on growth rate or acid production by *Lactobacillus helveticus* CNRZ32 in amino acid defined or skim milk medium.

Proteolytic enzymes of lactic acid bacteria (LAB) contribute to their ability to obtain essential amino acids from milk and to development of flavor in bacterium-ripened cheese varieties (e.g., Cheddar or Gouda). LAB are multiple-amino-acid auxotrophs and therefore must obtain essential amino acids from the growth medium. The quantities of free amino acids and small peptides present in milk are not sufficient to support the growth of LAB to a high cell density (16). Therefore, LAB require a proteolytic system to obtain essential amino acids from caseins, the primary proteins present in milk. Among LAB, the proteolytic system of *Lactococcus* bacteria and the relationship of specific components to the ability of these organisms to obtain essential amino acids from caseins are the best characterized. Caseins are hydrolyzed by the lactococcal cell envelope-associated proteinase to produce peptides which are transported into the cell by an oligopeptide transport system. Once inside the lactococcal cell, these peptides are hydrolyzed by a variety of endopeptidases, aminopeptidases, and di- and tripeptidases to yield free amino acids (16).

To date, two distinct endopeptidases from lactococci, designated PepO and PepF, have been reported (21, 28, 31). It was found that growth in milk of lactococcal strains lacking either PepO or PepF was indistinguishable from growth of the wild-type strain. However, two highly related enzymes designated PepO2 (12a) and PepF2 (22) have been identified. These results suggest that the milk growth studies may not accurately reflect the importance of PepO and PepF.

While the proteolytic systems of lactobacilli are not as well characterized as those of lactococci, the results obtained to date suggest that their proteolytic systems are similar (14, 25). We are interested in *Lactobacillus helveticus* CNRZ32 due to the demonstrated ability of this strain to accelerate cheese ripening and reduce bitterness when used as an adjunct culture (4, 5). We have focused on proteolytic enzymes from this organism because they are believed to be responsible for its beneficial attributes. Recently, we have focused on endopeptidases from this organism since this class of peptidases is the most poorly characterized in lactobacilli (8). To date, a thiol-dependent endopeptidase from *Lactobacillus helveticus* CNRZ32 has been purified and characterized and the gene encoding this enzyme has been characterized (12). The present report describes the characterization of a metalloendopeptidase gene (*pepO*) of CNRZ32 and the construction and characterization of derivatives lacking PepO. The use of CNRZ32 derivatives deficient in endopeptidase(s) to clarify their role in cheese flavor development is currently under way.

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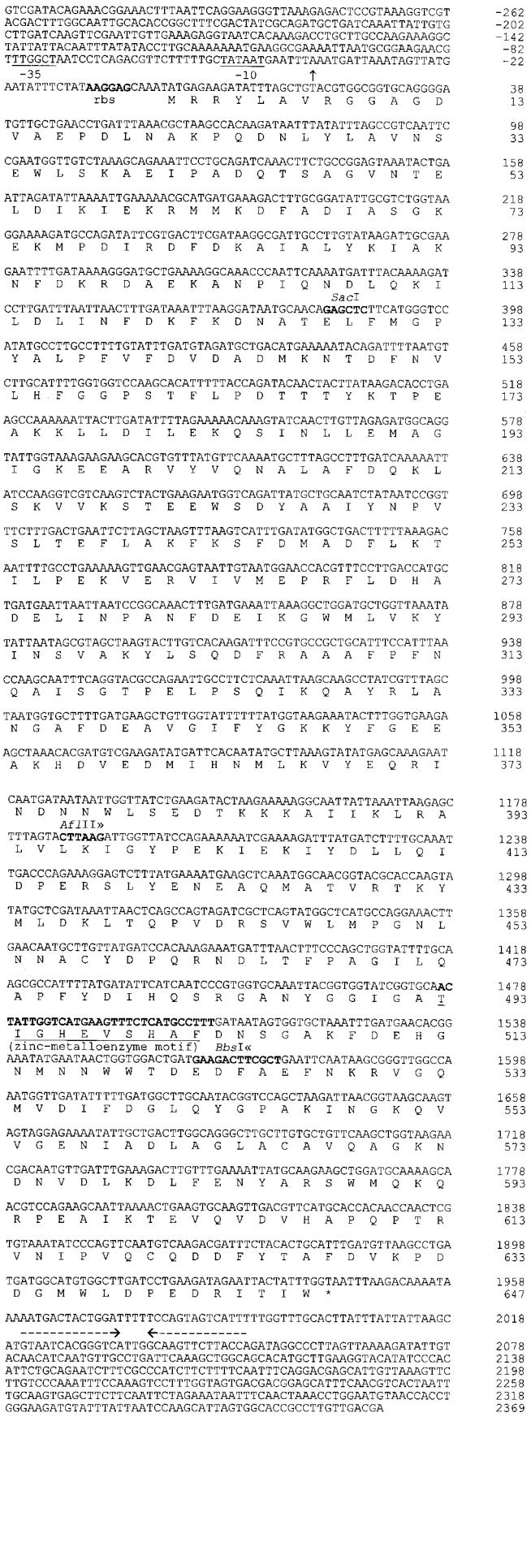
MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *Lactobacillus helveticus* CNRZ32 (15) and JLS200 (CNRZ32 lacking X-prolyl-dipeptidyl aminopeptidase activity, PepX [32]) and their derivatives were grown in MRS broth (Difco Laboratories, Detroit, Mich.) (10) at 37°C. *Lactococcus lactis* LM0230 was obtained from L. L. McKay (University of Minnesota, St. Paul) and propagated at 30°C in M17-glucose broth (Difco Laboratories) (29). *Escherichia coli* DH5α (Gibco-BRL Life Technologies Inc., Gaithersburg, Md.) and DPWC and BW26 (Gold Biotechnology Inc., St. Louis, Mo.) were grown in LB broth (24) at 37°C with aeration. Agar plates were prepared by adding 1.5% (wt/vol) granulated agar (Difco Laboratories) to liquid media. The concentration of antibiotics added to liquid media or agar plates for selection of plasmids in *E. coli* were as follows: pKF1 (12), 1.0 mg of erythromycin per ml; pMOB (Gold Biotechnology), 100 μg of ampicillin or 100 μg of carbenicillin per ml; pSA3 (9), 12.5 mg of tetracycline or 100 μg of chloramphenicol per ml. To select for pSA3 or pTRKL2 (23) in *Lactobacillus helveticus* or *Lactococcus lactis*, 5 μg of erythromycin per ml was used. All antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Molecular cloning. Plasmid isolation from *E. coli* and chromosomal DNA isolation from *Lactobacillus helveticus* were performed as described by Sambrook et al. (24). Plasmid isolation from lactococci was conducted as described by Anderson and McKay (2). Restriction enzymes and T4 DNA ligase were purchased from Gibco-BRL Life Technologies Inc. and were used as recommended by the manufacturer. Electroporations were conducted with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.). Electroporation of *E. coli* was performed as recommended by the manufacturer (Bio-Rad). Electroporations of *Lactobacillus helveticus* CNRZ32 and JLS200 were performed essentially as described by Bhowmik and Steele (7). The only differences were the following: (i) instead of electroporation buffer, cells were washed with ice-cold, sterile, double-distilled water; and (ii) 50 mM proline was added to the electroporation buffer. Electroporation of *Lactococcus lactis* LM0230 was performed as described by Holo and Nes (13). Subcloning of pKF1 (12)-derived fragments into pMOB was conducted essentially as described by Sambrook et al. (24).

DNA sequencing and sequence analysis. PCR and DNA sequencing reactions were performed in a Perkin-Elmer (Norwalk, Conn.) model 480 thermal cycler. DNA sequencing reactions were conducted with the Prism Ready Reaction DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.). DNA templates were purified by using the modified alkaline lysis-polyethylene glycol precipitation procedure recommended by Applied Biosystems, Inc. Additional primers were designed by using the Affinity program supplied by Ransom Hill Bioscience, Inc. (Ramona, Calif.) and were synthesized by using GIBCO-BRL (Grand Island, N.Y.) Custom Primers. Nested sets of Tn*1000* insertions were generated by using the Tn*1000* kit (Gold Biotechnology,



Inc.). Vector- and transposon-specific primers supplied with the Tn*1000* kit were used for mapping of the Tn*1000* insertion sites by PCR. DNA sequencing was conducted with the primers supplied with the Tn*1000* kit and with synthesized primers. DNA sequence determination was conducted by the Nucleic Acid and Protein Facility of the University of Wisconsin—Madison Biotechnology Center, by using an ABI model 370/3 automated sequencer. Sequences were analyzed by use of the Genetics Computer Group (Madison, Wis.) sequence analysis package. Protein homology searches were performed by using the BLAST network service (1).

Construction of LAB derivatives. A fragment of pKF1 insert was cloned into pSA3. A 381-bp internal in-frame deletion was introduced by digestion with restriction enzymes *Afl*II and *Bbs*I (see Fig. 1), filling-in with Klenow fragment, and ligation to yield pSUW50. The deletion was confirmed by restriction endonuclease digestions and DNA sequencing. Construction of *Lactobacillus helveticus* CNRZ32 PepO-deficient derivatives was conducted via gene replacement with pSUW50 (6). Identification of the mutants was accomplished by performing PCR with *pepO* internal primers and Southern hybridization with digoxigenin-labeled DNA probes generated by PCR from the same set of primers (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The nucleotide sequences of the primers were 5′CCGAATGGTTGTCTAAAGCA3′ (YC-1) and 5′CCAGC ATCCAGCCTTTAATTTC3′ (YC-2).

A *Sma*I-*Sal*I fragment containing a complete copy of *pepO* from pKF1 was subcloned into pTRKL2 to yield pSUW51. A *Lactococcus lactis* LM0230 derivative containing pSUW51 was constructed by electroporation and confirmed by plasmid analysis.

Enzyme assays. Overnight cultures were harvested by centrifugation at 3,840 × *g*, and the pellet was washed and suspended in 50 mM Na_2HPO_4 (pH 8.0; Sigma). Cell extracts from *Lactobacillus helveticus* and *Lactococcus lactis* were obtained by alternately vortexing the samples with glass beads and then cooling them on ice, for 1 min each, with six repetitions. The protein content of cell extracts was determined by the method of Lowry et al. (18) with the Sigma Total Protein Kit by using bovine serum albumin (Sigma) as the standard. Substrates previously used to screen for endopeptidase activity (12), *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide (*p*NA), *N*-benzoyl-Pro-Phe-Arg-*p*NA, and *N*-benzoyl-Val-Gly-Arg-*p*NA (Sigma), were employed at final concentrations of 0.1, 0.5, and 0.5 mM, respectively. Enzyme assays were conducted with cell extracts normalized to 30 μg of protein/ml in 50 mM Na_2HPO_4 (pH 8.0) and preequilibrated at 37°C. Reactions were initiated by the addition of substrate. Reactions were stopped by the addition of 200 μl of 30% acetic acid to 800-μl reaction mixtures. Absorbance at 410 nm was determined by using a Beckman (Fullerton, Calif.) DU-65 spectrophotometer. Reaction rates were verified to be linear under these conditions and were quantified on the basis of release of *p*NA (extinction coefficient of 8.8 mM^{-1} cm^{-1} at 410 nm) (11). Enzyme assays were performed in duplicate on two independently prepared cell extracts. Endopeptidase activity was expressed as micromoles of *p*NA released per minute per milligram of protein. The endopeptidase activities of *Lactobacillus helveticus* CNRZ32 and *Lactococcus lactis* LM0230 were normalized to 100%. The relative endopeptidase activities of CNRZ32 PepO$^-$ and LM0230(pSUW51) were calculated relative to those of their parental strains.

Growth studies. Growth studies were performed in double-steamed, pasteurized skim milk medium (pasteurized skim milk was steamed for 20 min, held at 42°C for 1 h, and then steamed for another 20 min) and amino acid defined medium (salts were prepared according to the ingredients of MRS broth, with the supplement of complete amino acids and glucose as the carbon source) (8a). Cultures propagated in MRS broth at 42°C to exponential phase were washed and resuspended in 0.85% NaCl to the original volume. A 0.1% inoculation (initial cell density, approximately 1.0×10^6 cells/ml) was made into both media, and the cultures were incubated at 42°C. Samples for pH and absorbance determinations were taken at 1-h intervals. The pH was determined with a pH meter (model 410A; Orion Research, Boston, Mass.) with an Ingold puncture-type pH probe (LoT406-M6-DXK-S7/25; Mettler-Toledo, Urdorf, Switzerland). The cell density in amino acid defined medium was determined by monitoring absorbance at 600 nm. The cell density in skim milk medium was determined by monitoring the absorbance at 600 nm of clarified samples (8a). Briefly, 0.5 ml of skim milk culture was mixed with 0.5 ml of 2 M borate–200 mM EDTA (pH 8.0) and incubated at 55°C for 10 min. The cells were then harvested by centrifugation and washed once with 1.0 ml of 2 M borate–200 mM EDTA (pH 8.0). The cell pellet was washed twice with 100 mM BisTris buffer (bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane) (pH 6.5), and the absorbance at 600 nm was determined from dilutions (if necessary) in the 0.03 to 0.30 linear range. All sampling was performed in triplicate from duplicate growth curves.

FIG. 1. Nucleotide sequence and deduced amino acid sequence of *pepO* from *Lactobacillus helveticus* CNRZ32. A putative Shine-Dalgarno sequence is shown in boldface type and labeled rbs. The −10 and −35 regions are indicated as well as the zinc-metalloprotease motif. The two horizontal arrows indicate the putative transcriptional terminator. The 5′ end of the *pepO* mRNA is marked with a vertical arrow. Relevant restriction endonuclease sites are shown in boldface type and labeled. The nucleotides between the ≫≪ symbols were deleted in the PepO$^-$ derivatives.

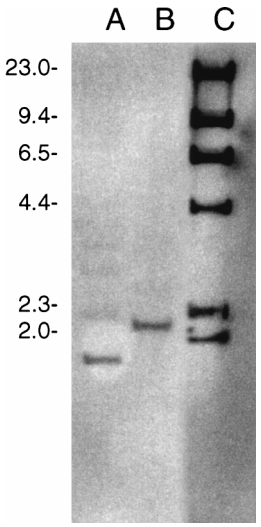


FIG. 2. Detection of *Lactobacillus helveticus* CNRZ32 *pepO* mutants by Southern hybridization. Lanes: A, CNRZ32 *pepO*; B, CNRZ32 wild type; C, digoxigenin-labeled λ DNA/*Hin*dIII molecular weight markers (sizes of markers, in thousands, are shown on the left).

Southern hybridization. A 774-bp internal *pepO* fragment (nucleotides 98 to 871) was used to synthesize a digoxigenin-labeled probe by PCR with the YC-1 and YC-2 primers and the Genius system (Boehringer Mannheim). Southern hybridizations were performed by the procedure supplied by the manufacturer.

RNA methods. Total RNA was isolated by using the RNeasy kit (Qiagen). A 1,548-bp internal *pepO* fragment (nucleotides 387 to 1934) was amplified and used to synthesize a digoxigenin-labeled probe with the Genius system (Boehringer Mannheim) for Northern hybridization. The nucleotide sequences of the two primers were 5′CTTCATGGGTCCATATGCC3′ and 5′GTAATTCTATC TTCAGGATC3′. RNA molecular weight markers, solutions, and reagents used in Northern hybridization and chemiluminescent detection were purchased from Boehringer Mannheim. Northern hybridizations were performed by the procedure supplied by the manufacturer. Mapping of the 5′ end of the *pepO* transcript was conducted by using the 5′ rapid amplification of cDNA (5′ RACE) kit (version 2.0; Gibco-BRL). The nucleotide sequences of the three primers used for 5′ RACE were 5′CTGTATTTTTCATGTCAGCATC3′ (YC-3), 5′CTCCGGC AGAAGTTTG3′ (YC-4), and 5′TTTGATCTGCAGG3′ (YC-5). First-strand cDNA synthesis was performed with primer YC-3. Nested amplification of first-strand cDNA was carried out with primer YC-4 and the anchor primer supplied by the kit. Sequencing reactions were conducted with primer YC-5 by using the nested amplification product as the template.

Nucleotide sequence accession number. The sequence for *pepO* has been submitted to GenBank and assigned accession no. AF019410.

RESULTS

Subcloning of pKF1. Previously, an endopeptidase-positive clone, designated pKF1, was identified in a *Lactobacillus helveticus* CNRZ32 genomic library constructed in *E. coli* DH5α (12). A restriction endonuclease map revealed an insert size of 5.7 kb (data not shown). All attempts to subclone the entire insert into pMOB were unsuccessful. Three pKF1 fragments were subcloned separately into pMOB. Enzyme assays indicated that none of the subcloned fragments expressed endopeptidase activity (data not shown).

Tn*1000* mutagenesis and DNA sequence analysis. Sequence analysis was employed to identify the pKF1 region which confers endopeptidase activity. Insertion sites of Tn*1000* within the three subcloned fragments were determined by PCR. The junctions of the subcloned fragments were sequenced by using synthesized primers and pKF1 as the template. The nucleic acid sequence of *pepO* is shown in Fig. 1. An open reading frame (ORF) of 1,941 bp which encodes a putative protein of 647 amino acids was identified. This protein has 40% sequence similarity to PepO from *Lactococcus lactis* P8-2-47 (20). There is a Shine-Dalgarno sequence (AAGGAG; $\Delta G = -12.8$ kcal) (26) 6 bases upstream from the putative start codon AUG; in addition, a putative transcriptional terminator ($\Delta G = -22.4$ kcal) (30) was identified 16 bases downstream of the putative stop codon UAA. Additionally, a search of the PROSITE Dictionary of Protein Sites and Patterns (3) with the deduced amino acid sequence identified a zinc-protease motif (His-Glu-Xxx-Xxx-His). No signal sequence was detected from the hydrophilicity plot (17).

CNRZ32 *pepO*-negative derivatives. Two CNRZ32PepO-negative derivatives were constructed: a CNRZ32 *pepO* single mutant and a CNRZ32 *pepX pepO* double mutant. The CNRZ32 *pepX pepO* mutant was constructed from JLS200. Results from both PCR (data not shown) and Southern hybridization (Fig. 2) confirmed that an approximately 400-bp deletion had been introduced into the chromosomal *pepO* gene. Southern hybridization (Fig. 2) with total chromosomal DNA digested with *Pst*I detected single bands which hybridized with the *pepO* probe in both CNRZ32 (2.2 kb) and its *pepO* mutant (1.8 kb).

Growth characteristics. The growth and acidification rates for the CNRZ32 wild type, five *pepO* mutants, two *pepO*$^+$ revertants, two *pepX pepO* mutants, and two *pepX pepO*$^+$ revertants in both amino acid defined medium and skim milk medium were compared (data not shown). No differences among the CNRZ32 wild type, *pepO* mutants, or the *pepO*$^+$ revertants were observed. Similarly, no differences among the *pepX*, *pepX pepO* mutants, or *pepX pepO*$^+$ revertants were observed.

Enzyme assay. The *pepO* mutant examined had 79 and >94% lower activities than that of CNRZ32 with *N*-benzoyl-Phe-Val-Arg-*p*NA and *N*-benzoyl-Val-Gly-Arg-*p*NA, respectively (Table 1). The introduction of the CNRZ32 *pepO* into *Lactococcus lactis* LM0230 on the low-copy-number vector pTRKL2 (6 to 9 copies/cell) did not result in a significant increase in endopeptidase activity (data not shown).

mRNA analysis. Two transcripts were detected in CNRZ32 throughout the growth phase (Fig. 3). One transcript had a calculated size of 2.2 kb, which corresponds to the size of the ORF of *pepO*. The other transcript had a calculated size of 1.5

TABLE 1. Endopeptidase activities of *Lactobacillus helveticus* CNRZ32 and a CNRZ32 *pepO* mutant

Strain	Endopeptidase activity on substrate of:					
	N-benzoyl-Phe-Val-Arg-*p*NA		*N*-benzoyl-Pro-Phe-Arg-*p*NA		*N*-benzoyl-Val-Gly-Arg-*p*NA	
	Means ± SD[a]	RA (%)[b]	Mean ± SD	RA (%)	Mean ± SD	RA (%)
CNRZ32	0.39 ± 0.01	100	0.081 ± 0.001	100	0.085 ± 0.004	100
CNRZ32 *pepO*	0.083 ± 0.001	21	0.081 ± 0.003	100	BQL[c]	<6

[a] Mean of specific activity (micromoles of *p*NA minute^{-1} milligram of protein^{-1}) ± standard deviation.
[b] RA, relative activity.
[c] BQL, below quantifiable level (0.01 μmol of *p*NA min^{-1} mg of protein^{-1}).

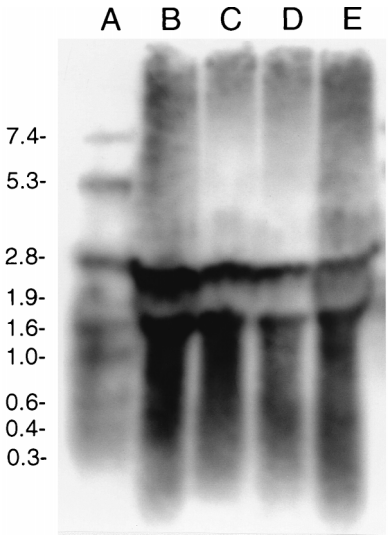


FIG. 3. Detection of the *Lactobacillus helveticus* CNRZ32 *pepO* transcript during growth in MRS broth by using Northern hybridization. Lanes: A, digoxigenin-labeled RNA molecular weight markers; B to E, CNRZ32 total RNA isolated from early, mid-log, late log, and stationary phases (optical densities at 600 nm of 0.6, 1.2, 2.0, and 3.5), respectively. Sizes of molecular weight markers, in thousands, are shown on the left.

kb. Similar analysis with a CNRZ32 *pepO* mutant detected transcripts of 1.8 and 1.5 kb (data not shown).

Sequencing of the nested amplification product from 5′ RACE revealed that the transcription start site was located 41 nucleotides upstream of the start codon (Fig. 1).

DISCUSSION

In this study, a previously identified endopeptidase-expressing clone (12) was characterized and determined to contain a 5.7-kb insert. Nucleic acid sequencing identified a 1,941-bp *pepO* ORF with an upstream AT-rich sequence which might serve as the putative −10 and −35 promoter regions. Data which suggest that the CNRZ32 *pepO* gene is monocistronic include (i) the putative promoter region and the putative terminator, and (ii) results from Northern hybridization. The high level of protein sequence homology of the CNRZ32 PepO to the PepO from *Lactococcus lactis* P8-2-47 suggests an ancestral association between these two enzymes. In addition, a metalloprotease motif (His-Glu-Xxx-Xxx-His) identified from the deduced PepO sequence was also present in strain P8-2-47 (20). The lack of a signal sequence suggests an intracellular location for PepO. The lactococcal PepO is also believed to be located intracellularly (27, 31).

In contrast to the lactococcal *pepO* (20, 28, 31), nucleic acid sequence analysis of 2.82 kb upstream and 0.55 kb downstream of the CNRZ32 *pepO* gene suggests that this gene is not associated with oligopeptide transport genes. A second copy of *pepO* in lactococcal strains, designated *pepO2*, has been reported recently (12a). The presence of single bands in Southern hybridizations with *pepO* as the probe in both CNRZ32 and its *pepO* derivatives indicates that there is only one copy of *pepO* in CNRZ32. Additionally, the >94% reduction in hydrolysis of *N*-benzoyl-Val-Gly-Arg-*p*NA by the *pepO* mutant suggests that there is a single copy of *pepO* in CNRZ32 and that this substrate could function to selectively quantify PepO activity.

To evaluate the physiological role of PepO, studies were conducted to compare the growth and acidification rates of the CNRZ32 wild type and *pepO*, *pepO pepX*, and *pepX* mutants in both amino acid defined medium and skim milk medium. The results revealed that the *pepO* and *pepO pepX* strains did not differ significantly in their growth or acidification rates from those of the wild type and the *pepX* mutant, respectively. This is similar to the results reported by Mierau et al. (19) for PepO in lactococci. These results suggest one or more of the following: (i) that PepO is not involved in the hydrolysis of milk-derived peptides, (ii) that other peptidases possess overlapping specificities with PepO, and (iii) that alternative milk-derived peptides can be utilized to obtain essential amino acids. Further investigation is required to determine what role, if any, PepO has in the development of cheese flavor.

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