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# Cloning and DNA Sequence Analysis of an X-Prolyl Dipeptidyl Aminopeptidase Gene from *Lactococcus lactis* subsp. *lactis* NCDO 763

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***Lactococcus lactis* subsp. *lactis* NCDO 763 (also designated ML3) possesses an X-prolyl dipeptidyl aminopeptidase (X-PDAP; EC 3.4.14.5). X-PDAP mutants were selected by an enzymatic plate assay on the basis of their inability to hydrolyze an L-phenylalanyl-L-proline-β-naphthylamide substrate. A DNA bank from *L. lactis* subsp. *lactis* NCDO 763 was constructed in one of these X-PDAP mutants, and one clone in which the original X-PDAP phenotype was restored was detected by the enzymatic plate assay. The X-PDAP gene, designated *pepXP*, was further subcloned and sequenced. It codes for a protein containing 763 residues. Comparison of the amino-terminal sequence of the X-PDAP enzyme with the amino acid sequence deduced from the *pepXP* gene indicated that the enzyme is not subjected to posttranslational modification or exported via processing of a signal peptide. The *pepXP* gene from *L. lactis* subsp. *lactis* NCDO 763 is more than 99% homologous to the *pepXP* gene from *L. lactis* subsp. *cremoris* P8-2-47 described elsewhere (B. Mayo, J. Kok, K. Venema, W. Bockelmann, M. Teuber, H. Reinke, and G. Venema, Appl. Environ. Microbiol. 57:38–44, 1991) and is also conserved in other lactococcal strains.**

Lactic acid bacteria used by the dairy industry are fastidious microorganisms. To grow, they need a number of essential growth factors, including amino acids and small peptides. However, these compounds are available in limited amounts in milk. To grow to high cell densities, the lactic acid bacteria depend on their proteolytic enzymes, which hydrolyze casein to peptides and free amino acids (26). Caseins have a high proline content, and their hydrolysis by the cell wall proteinase from *Lactococcus lactis* subsp. *lactis* (formerly *Streptococcus lactis*) (27, 28, 41) leads to proline-rich oligopeptides. Since peptide bonds involving a proline residue need specific enzymes to be hydrolyzed, such enzymes may be an essential component in the process of casein degradation.

An X-prolyl dipeptidyl aminopeptidase (X-PDAP; capable of releasing dipeptides of the sequence X-Pro from the N-terminal end of peptide chains; EC 3.4.14.5) has been demonstrated in several genera of lactic acid bacteria, including *Lactobacillus*, *Streptococcus*, and *Lactococcus* (5). X-PDAPs have recently been purified from *L. lactis* subsp. *cremoris* (15) and *L. lactis* subsp. *lactis* NCDO 763 (42) and characterized. These enzymes share a common substrate specificity, are inhibited by diisopropylfluorophosphate, and have a dimeric structure, with subunits of identical molecular mass in the vicinity of 80 kDa.

We describe here the cloning and characterization at the nucleotide level of the DNA region carrying the gene coding for an X-PDAP from *L. lactis* subsp. *lactis* NCDO 763. A similar X-PDAP was found in *L. lactis* subsp. *cremoris* P8-2-47 (23).

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## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The bacterial strains and plasmids used are listed in Table 1. *L. lactis* subsp. *lactis* strains were grown in M17 medium (38) in which lactose had been replaced by glucose or in Elliker medium (10) at 30°C. *Bacillus subtilis* and *Escherichia coli* were grown in Luria-Bertani medium (22) at 37°C. When needed, erythromycin (5 μg/ml for *L. lactis* subsp. *lactis* and 0.3 μg/ml for *B. subtilis*) or ampicillin (50 μg/ml for *E. coli*) was added to the culture medium. Plasmid pBluescript was obtained from Stratagene (La Jolla, Calif.).

**Mutagenesis.** *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis was performed on a mid-log-phase culture in Elliker broth as described previously (25) with 100 mM citrate buffer (pH 5.5) containing 250 μg of NTG per ml. After 60 min of incubation at 30°C, mutagenized cells were collected by centrifugation and washed twice in 100 mM phosphate buffer (pH 7.0). A 4-h expression culture was performed after resuspension of the cells in Elliker broth. Survival after mutagenesis was estimated by plating aliquots of the suspension before and after NTG treatment.

**Enzymatic plate assay for the selection of X-PDAP mutants.** Mutagenized colonies were screened by a plate staining procedure adapted from that used by Miller and Mackinnon (24) and more recently by Atlan et al. (2) for the isolation of aminopeptidase mutants of *Salmonella typhimurium* or *Lactobacillus delbrueckii* subsp. *bulgaricus*. Both of these procedures make use of amino-acyl β-naphthylamide which, following hydrolysis by aminopeptidase, yields β-naphthylamine. Reaction of β-naphthylamine with fast garnet produces a dark red color. To select X-PDAP mutants, we used L-phenylalanyl-L-proline-β-naphthylamide. Cells were grown on Elliker medium for 24 h at 30°C. The colonies were screened for enzymatic activity by pouring the plates with a mixture of 0.2 ml of L-phenylalanyl-L-proline-β-naphthylamide (Bachem, Budendorf, Switzerland) solution (10 mg/ml

TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Characteristics | Reference or source |
|---|---|---|
| *L. lactis* subsp. *lactis* | | |
| NCDO 763 | First described as ML3, contains a spontaneous plasmid complement | 8 |
| TIL1 | Mutant of NCDO 763 lacking X-PDAP activity | This work |
| IL1403 | Plasmid free, $R^-/M^-$ | 7 |
| *B. subtilis* 1012 | *leuA8 metB5* $R^-/M^+$ | 31 |
| pIL252 | $Em^r$, 4.6 kb | 33 |
| pBluescript | $Ap^r$, M13 *ori*, pBR322 *ori* | Stratagene |

in dimethylformamide), 1 ml of fast garnet GBC (Sigma, St. Louis, Mo.) (10 mg/ml in water), and 5 ml of 50 mM phosphate buffer (pH 7.0). After a few minutes of incubation at an ambient temperature, the mutants clones appeared as white colonies among dark red wild-type colonies.

**Molecular cloning.** Plasmid and chromosomal DNAs were prepared as previously described (21, 34). Restriction endonucleases and T4 DNA ligase were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used as recommended by the supplier. Procedures for DNA manipulation, transformation of *E. coli* cells, and cloning were essentially as described by Maniatis et al. (22). Transformation of *L. lactis* subsp. *lactis* was done by electroporation as previously described (19). Competent cells of *B. subtilis* were transformed as described by Anagnostopoulos and Spizizen (1).

**DNA sequence analysis.** *E. coli* clones for sequencing were obtained by subcloning specific DNA fragments in the pBluescript plasmid and by using exonuclease III and mung bean nuclease (Stratagene) to generate a series of overlapping clones. Single-stranded DNA was prepared as described by Vieira and Messing (40) and sequenced essentially in accordance with the protocol accompanying the 370A DNA sequencer (Applied Biosystems, San Jose, Calif.). In brief, the DNA was used in dideoxynucleotide chain termination sequencing reactions with *Taq* DNA polymerase and fluorescent dye-coupled primers (Promega). In some clones, DNA structural instability was observed during single-stranded DNA production. The DNA sequence of the insert was determined on the double-stranded DNA template by the standard manual dideoxynucleotide sequencing method (32) with synthetic primers (obtained from Appligène, Illkirch, France) designed by use of DNA sequences that had already been determined. The reported sequence was determined on both strands. The DNA and protein sequences were analyzed with BISANCE and University of Wisconsin GCG software packages, implemented at the Centre InterUniversitaire d'Informatique à Orientation Biomédicale (Paris, France).

**Southern hybridizations.** Southern hybridizations were done as described by Maniatis et al. (22) with DNA probes prepared with a nick translation kit (Amersham Corp., Arlington Heights, Ill.) and [$\alpha$-$^{32}$P]dCTP (Amersham).

**Crude extract and enzyme assay.** Cell extracts were prepared from 150-ml aliquots taken from cultures in M17 broth at different times. Cells were harvested by centrifugation and washed twice in 50 mM $\beta$-glycerophosphate buffer (pH 7.0) containing 20 mM $CaCl_2$. They were resuspended in 5 ml of 50 mM triethanolamine buffer (pH 7.0) containing 10 mM $MgCl_2$ and 1 mg of lysozyme per ml. After incubation for 2 h at 37°C, the suspension was centrifuged at 20,000 × *g* for 15 min to separate the crude extract from membrane and cell debris. X-PDAP activity was determined in the supernatant with L-alanyl-L-proline-*p*-nitroanilide as the substrate as described by Zevaco et al. (42).

**Determination of the N-terminal sequence.** X-PDAP was purified from an intracellular crude extract of *L. lactis* subsp. *lactis* NCDO 763 cells grown on M17 medium. After elimination of nucleic acids with DNase and RNase, the extract was submitted to ion-exchange chromatography on a MonoQ HR 10/10 column (Pharmacia, Uppsala, Sweden) with 50 mM Tris hydrochloride buffer (pH 7.5). Gel filtration of the concentrated active fractions was performed on an Ultrogel ACA 34 column (IBF, Villeneuve la Garenne, France) with 50 mM Tris hydrochloride buffer (pH 7.5). A third chromatography step on a MonoQ HR 10/10 column with 50 mM Tris hydrochloride buffer (pH 8.5) led to an electrophoretically pure protein (42). The purified sample was electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore) as described by Moos et al. (29). Following staining with Coomassie blue, the band was excised and placed in the cartridge block of an Applied Biosystems model 477A liquid pulsed-phase sequencer coupled with an Applied Biosystems 120A high-pressure liquid chromatography system. Sequencing was performed in accordance with Applied Biosystems standard procedures.

**Nucleotide sequence accession number.** The GenBank, EMBL, and DDBJ nucleotide sequence accession number is M35865.

## RESULTS

**Mutagenesis and characterization of mutants.** Exposure of *L. lactis* subsp. *lactis* NCDO 763 to NTG (250 $\mu$g/ml) for 60 min resulted in 55% survival. Three mutants producing white colonies were obtained from 15,000 colonies. Comparison of the X-PDAP activity in the crude extracts of the parental strain and the mutants confirmed that the latter did not have this activity. One of these, designated TIL1, was further used. The growth curves of strain NCDO 763 and mutant TIL1 in milk at 30°C were compared, and respective maximum specific growth rates ($\mu_{max}$) of 1.6 and 1.0 $h^{-1}$ were measured.

**Cloning of the X-PDAP determinant.** Total DNA prepared from *L. lactis* subsp. *lactis* NCDO 763 was partially digested with *Sau*3A endonuclease. DNA fragments (12 $\mu$g) larger than 10 kb and purified by centrifugation through a sucrose gradient were ligated with 2 $\mu$g of *Bam*HI-cleaved pIL252 DNA at a concentration of 100 $\mu$g/ml. The ligation mixture was used to transform *L. lactis* subsp. *lactis* TIL1 by electroporation. From 48,000 $Em^r$ transformants obtained and screened for X-PDAP activity by the enzymatic plate assay, one colony had recovered the parental phenotype. This transformant contained, in addition to its original plasmid complement, a 17.8-kb plasmid, designated pTIL1, which was purified by transforming plasmid-free *L. lactis* subsp. *lactis* IL1403 by electroporation. A transformant carrying pTIL1 alone was further used as a source of pTIL1 DNA. Further transfer of pTIL1 DNA to *L. lactis* subsp. *lactis* TIL1 and recovery of the parental X-PDAP phenotype in the $Em^r$ transformants ruled out the possibility that the recovery of the parental phenotype was due to reversion.

A restriction map of pTIL1 was constructed by single and

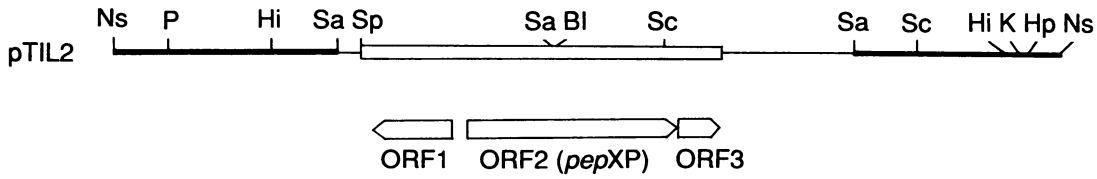


FIG. 1. Composite showing the restriction map of plasmid pTIL2, the sequenced region, and the deduced ORFs. The thick and thin lines represent pIL252 DNA and the cloned insert, respectively. The open box corresponds to the sequenced region. The abbreviations of restriction enzymes are as following: Bl, *Bgl*I; Hi, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; Nd, *Nde*I; Ns, *Nsi*I; P, *Pvu*II; Sa, *Sac*I; Sc, *Sca*I; Sp, *Spe*I. For *Hin*dIII, *Pvu*II, and *Nsi*I, not all restriction sites are marked.

double digestions with several restriction endonucleases, and subcloning experiments were done with *Bgl*I, *Eco*RV, *Sac*I, or *Sca*I. The smallest recombinant plasmids still conferring the X-PDAP phenotype were made from two adjacent *Sac*I fragments from pTIL1. In one of these plasmids, designated pTIL2, the *Sac*I fragments were inserted in the reverse orientation relative to pTIL1 (Fig. 1).

Southern blot hybridization of undigested or *Sac*I-cleaved NCDO 763 DNA probed with pTIL2 DNA demonstrated that the cloned DNA originated from the NCDO 763 chromosome (data not shown).

Specific X-PDAP activity conferred by plasmid pTIL1 or pTIL2 to mutant TIL1 was compared with that of the parental strain NCDO 763. Activity measured during growth of TIL1 was not detectable. In contrast, the specific activities in NCDO 763 and TIL1 containing either pTIL1 or pTIL2 were not significantly different, being 0.169, 0.174, and 0.150 optical density units per mg of protein per min, respectively, at the end of the late-log phase of growth.

pTIL2 DNA was transformed into competent cells of *B. subtilis* 1012, a restriction-deficient derivative of *B. subtilis* Marburg, which is described as a preferable host for the cloning of heterologous DNA (13) and does not express X-PDAP activity. All $Em^r$ transformants acquired X-PDAP activity, demonstrating that the determinant was expressed in *B. subtilis*.

**DNA sequence analysis.** The nucleotide sequence of a 3,963-bp region has been determined. The location of this region in the pTIL2 DNA insert is presented in Fig. 1. Figure 2 displays the complete nucleotide sequence. Codon preference analysis (12) of the sequence revealed three open reading frames (ORFs), designated ORF1 (289 codons), ORF2 (763 codons), and ORF3 (166 codons) (Fig. 1). ORF1 is oriented in the opposite direction as compared with ORF2 and ORF3. Putative ribosome-binding sites complementary to the 3′ end of the *L. lactis* subsp. *lactis* 16S rRNA (9) were found 11 and 6 bp upstream of ORF1 and ORF2, respectively, but no ribosome-binding site-like structure was found upstream of ORF3. ORF1 and ORF2 are separated by a 172-nucleotide region which contains four candidate sequences that can fit the consensus for lactococcal promoters (9), with −35 regions starting at position 1010 (putative ORF1 promoter) and positions 954, 1041, and 1052 (putative ORF2 promoters). A putative transcription termination signal consisting of a stem loop structure followed by seven thymidine residues was found immediately downstream of ORF1. No typical transcription termination structure was detected in the 70-bp sequence downstream of ORF3.

**ORF2 is the structural gene for X-PDAP.** To identify the gene coding for X-PDAP, we determined the sequence of the first 10 N-terminal amino acids of the purified X-PDAP enzyme. The sequence Met-Arg-Phe-Asn-His-Phe-Ser-Ile-Val-Asp was found; this sequence matched the sequence deduced from the nucleotide sequence at the beginning of ORF2. This analysis ascertained the start of the X-PDAP gene and established that the X-PDAP protein was not subjected to posttranslational modification. The X-PDAP gene was designated *pepXP*. It encodes a 763-residue polypeptide with a calculated molecular mass of 87,696 Da.

**Homology search.** The GENPRO (release 63, March 1990) and the NBRF (release 23, January 1990) protein data bases were searched for proteins homologous to those deduced from ORF1, the *pepXP* gene, and ORF3. The protein specified by ORF1 was found to share significant homology with the *E. coli* glycerol facilitator protein (30) and the homologous enzyme from *Streptomyces coelicolor* (37). Like the glycerol facilitator protein, the protein specified by ORF1 is an integral transmembrane protein and was predicted to possess six transmembrane α-helical domains. No significant homology was detected between the protein specified by *pepXP* or ORF3 and the GENPRO or NBRF protein data bases.

The hydropathy profile (17) of the X-PDAP protein did not reveal any hydrophobic segment likely to be a signal peptide sequence, a transmembrane α-helical domain, or a membrane anchor.

## DISCUSSION

We isolated the X-PDAP determinant from the *L. lactis* subsp. *lactis* NCDO 763 chromosome by direct shotgun cloning into an isogenic X-PDAP mutant. Three ORFs were detected in the cloned fragment by nucleotide sequence analysis. Determination of the amino-terminal sequence of the X-PDAP protein allowed the identification of ORF2 as the *pepXP* gene. It encodes a 763-residue protein with a calculated molecular mass of 87,696 Da, a value in good agreement with the molecular mass of 85 kDa reported for the X-PDAP protein (42). The possible role of the flanking ORFs in X-PDAP activity remains to be examined.

The fact that the amino-terminal sequence of the protein matched the sequence deduced from the beginning of *pepXP* indicated that the X-PDAP protein is not subjected to posttranslational modification of its amino terminus and, in particular, is not exported through processing of a signal peptide. This fact is in agreement with the results of a previous study demonstrating that almost all enzyme activity was detected in the *L. lactis* subsp. *lactis* NCDO 763 cytoplasmic fraction (42) and indicates that the enzyme is probably intracellularly located. This probable localization raises the question of the role of the X-PDAP enzyme in the cascade of events participating in casein breakdown.

As no significant uptake of free proline has been detected in *L. lactis* subsp. *lactis* ML3 (36), this amino acid must be supplied in the form of peptides. The proline-containing peptides obtained after casein hydrolysis by the cell wall

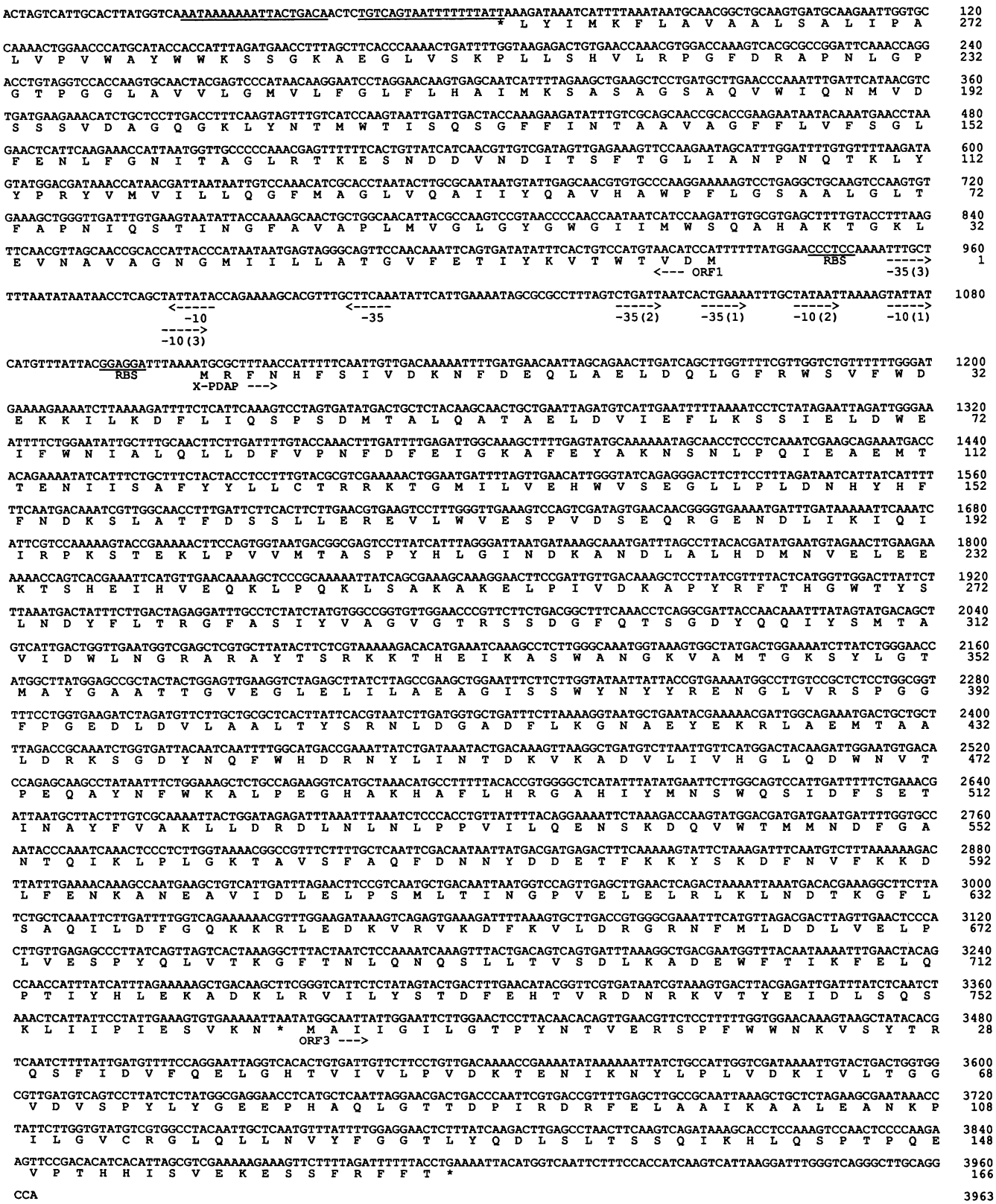


FIG. 2. Nucleotide and deduced amino acid sequences of ORF1, the X-PDAP gene, ORF3, and the flanking regions. The numbers to the right refer to nucleotides (top lines) and amino acid residues (bottom lines). Potential ribosome-binding sites (RBS) are indicated by underlining of the relevant sequences. Potential promoter −35 and −10 boxes are underlined by arrows oriented in the direction of transcription. The putative terminator at the end of ORF1 is underlined. Stop codons are indicated by asterisks.

proteinase are more than five amino acids long (27, 28, 41) and are thus too large to be translocated across the cell membrane (20). These facts imply the existence of an exocellular peptidase(s) able to hydrolyze oligopeptides by the scheme proposed by Laan et al. (18) and Thomas and Pritchard (39). The small proline peptides produced in this way could be further translocated through the membrane and hydrolyzed by X-PDAP. Ultimately, the X-Pro dipeptides released by X-PDAP could be hydrolyzed by a prolidase that has been detected in lactococci (14; 28a).

The fact that X-PDAP mutants are still able to grow on milk, albeit at a reduced specific growth rate, can be explained in two ways. Either *L. lactis* subsp. *lactis* is auxotrophic for proline or X-PDAP is not the only intracellular enzyme able to hydrolyze prolyl peptides. An iminopeptidase (proline aminopeptidase) detected in *L. lactis* subsp. *cremoris* (11) could offer an alternative pathway for the degradation of prolyl peptides.

A comparison of the nucleotide sequence of *pepXP* and its flanking regions from the *L. lactis* subsp. *lactis* NCDO 763 chromosome with the corresponding sequence from the *L. lactis* subsp. *cremoris* P8-2-47 chromosome (23) indicates a very high homology. Both enzymes are 763-residue proteins, and only 7 residues are different from each other because of single nucleotide substitutions. Five substitutions lead to nonhomologous amino acid replacements (Ala-50 → Asp-50; Thr-130 → Asn-130; Asn-573 → His-573; Val-649 → Ala-649; and Ser-692 → Asn-692), suggesting that this region is highly conserved in lactococci. Southern hybridization experiments with chromosomal DNA from different *Lactococcus* strains and with a *pepXP* DNA fragment as a probe (data not shown) revealed that the homologous region was indeed present in all six strains tested.

The *L. lactis* subsp. *lactis* NCDO 763 X-PDAP is inhibited by diisopropylfluorophosphate (42), a fact which is regarded as presumptive evidence for the involvement of a seryl residue in the catalytic mechanism (3). Many such procaryotic serine proteolytic enzymes, including four proteinases from *B. subtilis* (35), a proteinase from *L. lactis* subsp. *cremoris* (16), and a proteinase from *Streptococcus pyogenes* (6), show conserved amino acid sequences around their active sites. We thus expected some homology of X-PDAP with these enzymes. However, the X-PDAP protein does not share significant homology with any protein in the protein data bases. Despite careful comparison of the complete amino acid sequence of X-PDAP with the sequences around the active sites of the serine proteinases, no convincing homology was found and the active serine of X-PDAP could not be identified. It is noteworthy that another serine proteinase that has been found in *Staphylococcus aureus* V8 (4) shows no significant homology to the proteinases of this group. The fact that the X-PDAP from *Lactococcus* species shares no homology with other serine proteinases suggests a different evolutionary origin for the X-PDAP gene.

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