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Cloning, heterologous expression, and sequencing of a novel proline iminopeptidase gene, *pepI*, from *Lactobacillus delbrueckii* subsp. *lactis* DSM 7290

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The gene for proline iminopeptidase from *Lactobacillus delbrueckii* subsp. *lactis* DSM 7290 coding for an enzyme that hydrolyses the synthetic substrate L-prolyl-β-naphthylamide (Pro-βNA) was cloned in *Escherichia coli*. An enzymic plate assay was used to screen for positive clones. The gene, designated *pepI*, was subcloned into vector pUC18 and sequenced. The nucleotide sequence revealed an 882 bp open reading frame encoding 294 amino acids, coding for an enzyme with a calculated molecular mass of 32883 Da. By cloning under control of the *lac* promoter the peptidase was highly expressed. Sequence analysis showed that *pepI* is of a new sequence type, distinct from all peptidases so far sequenced. Amino acid homology to the active site of a *Pseudomonas putida* esterase and inhibitor studies of the enzyme imply involvement of a serine residue in catalysis.

Keywords: iminopeptidase, serine protease, *Lactobacillus delbrueckii* subsp. *lactis*, nucleotide sequence analysis

INTRODUCTION

Lactobacilli growing in milk need to possess an efficient enzymic system to utilize the abundant nutrients present. Using a coordinated combination of proteinases, peptidases, and amino acid and peptide transport systems, they are able to use milk protein, especially casein, as a source of amino acids essential for growth. The proteolytic system of lactobacilli used as starter cultures in the industrial dairy fermentations is an important factor influencing dairy product quality (e.g. organoleptic properties, or taste, and texture). This has resulted in increased fundamental research concerning the genes and corresponding enzymes involved, which may contribute to the improvement of industrial strains. Additionally the genes of individual proteinases or peptidases may serve as food-grade markers in plasmids suitable for genetic engineering.

The large peptides generated by proteinases during casein degradation are further hydrolysed into smaller peptides and amino acids by the action of peptidases. The catalytic activity of the proline iminopeptidase (EC 3.4.11.5) is to cleave N-terminal proline residues from peptides. This bond is less susceptible to the action of aminopeptidases of broad specificity because proline residues confer structural constraints on the peptide (Yaron & Naider, 1993). Casein has an extraordinarily high content of proline residues (16·7% for β-casein; Casey & Meyer, 1985) and the iminopeptidase is able to remove the blocking proline residues, thus making the peptides again accessible for other enzymes. Proline iminopeptidase activity has been reported for a variety of organisms, but as far as we know only a *Bacillus* gene has been cloned and sequenced (Kitazono *et al.*, 1992). This paper deals with the cloning, sequencing and expression of the proline iminopeptidase from *Lactobacillus delbrueckii* subsp. *lactis* DSM 7290 in *Escherichia coli*.

Abbreviations: 3,4-DCI, 3,4-dichloroisocoumarin; E-64; L-*trans*-epoxysuccinylleucylamide(4-guanidino)-butane; *p*-NA, *p*-nitroanilide; Pro-βNA, L-prolyl-β-naphthylamide.

The GenBank/EMBL/DDBJ accession numbers for the novel nucleotide sequence data reported in this paper are Z26948 and Z26951.

METHODS

Bacterial strains, plasmids, and growth conditions. *Escherichia coli* K12 strain ER1562 (Raleigh *et al.*, 1988) was used for molecular cloning and strain CM89 (Miller & Schwartz, 1978) for preparation of cell extracts. The low-copy-number vector pLG339 (Stoker *et al.*, 1982) was from N. Stoker and pUC18

(Yanisch-Perron *et al.*, 1985) was obtained from Pharmacia. *E. coli* was grown at 37 °C in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) at 37 °C. Ampicillin or kanamycin were added to concentrations of 200 μg ml^{-1} and 40 μg ml^{-1}, respectively.

Transformations. *E. coli* was transformed by electroporation using a Bio-Rad Gene Pulser as described by Dower *et al.* (1988).

Recombinant DNA techniques. Restriction enzymes and other nucleic-acid-modifying enzymes were used as recommended by the manufacturers. Isolation of plasmid DNA from *E. coli* was performed as described by Sambrook *et al.* (1989).

Isolation of chromosomal DNA from *L. delbrueckii* subsp. *lactis* DSM 7290 and preparation of a plasmid library in the low copy number vector pLG339 (Stoker *et al.*, 1982) was performed as described previously (Klein *et al.*, 1993).

Molecular cloning of the *L. delbrueckii* subsp. *lactis pepI* gene. A gene bank of size-fractionated partial *Sau*3A fragments of total *L. delbrueckii* subsp. *lactis* DNA constructed with vector pLG339 (Stoker *et al.*, 1982) was used to transform *E. coli* strain ER1562 as described previously (Klein *et al.*, 1993). Colonies with proline iminopeptidase activity were identified by a plate staining method originally described by Miller & Mackinnon (1974). If the chromogenic substrate Pro-βNA is cleaved, the reaction of the β-naphthylamine with fast garnet GBC (Sigma) can be monitored by the formation of a red, non-diffusible azo dye. During subcloning the transformants, selected on ampicillin- or kanamycin-containing LB-plates, were screened for those showing proline iminopeptidase enzyme activity with Pro-βNA as a substrate.

DNA sequence analysis. DNA sequencing of the chromosomal insert of pJK505 was carried out with a pair of universal sequencing primers adjacent to the multiple cloning site of pUC18. Synthetic oligonucleotide primers, deduced from the investigated sequence, were synthesized (Applied Biosystems model 392), and allowed direct sequencing of double-stranded plasmid DNA purified by NUCLEOBOND AX100 columns (Machery-Nagel). The DNA sequence of each strand was determined using the T7 DNA polymerase sequencing kit (Pharmacia), which is based on the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977), in the presence of [^{35}S]dATPαS (Amersham). For computer-assisted sequence analysis the Microgenie (Beckman), PC-Gene (IntelliGenetics), and HUSAR (GENIUSnet) software were used.

Preparation of cell extracts. Cell pellets of *E. coli* from a 50 ml overnight culture were washed with 50 mM Tris/HCl pH 8·0, pelleted by centrifugation and resuspended in 500 μl of the same buffer. The bacteria were sonicated on ice (Bandelin sonifier; Sonopuls HD60) until more than 90 % of the cells were broken. Cell debris was removed by centrifugation at 51000 ***g*** and 4 °C for 60 min in a Hereus Biofuge RS28.

The supernatant of the preparation contained approximately 50 mg protein ml^{-1} as determined by the Lowry method and was used for enzyme assays and SDS-PAGE (Laemmli, 1970). The content of PepI protein in cell extracts was determined videodensitometrically (Cybertech CS1, Image Documentation System) with Coomassie-stained SDS-gels, calibrated with bovine serum albumin standards.

Enzyme assay and effects of various chemical reagents. For characterization of the enzyme, *p*-nitroanilide (*p*-NA) substrates (Bachem) were dissolved in water and added to the reaction mixture [10 mM Tris/HCl, pH 8·0, and varying amounts of cell extracts (10–2000 ng)] to a concentration of 1 mM in a volume of 250 μl. Release of *p*-nitroaniline was measured after a 10 min incubation at 37 °C at 405 mm in a LKB Ultrospec Plus spectrophotometer.

To study the mechanism of enzyme action, the inhibitors 3,4-dichloroisocoumarin (3,4-DCI) at a concentration of 0·1 mM, pepstatin A at 1 μg ml^{-1}, L-*trans*-epoxysuccinylleucylamide(4-guanidino)-butane (E-64) at 0·1 mM, and 1,10-phenanthroline or EDTA at 1 mM were added to the extracts and incubated for 30 min at 37 °C. The substrate Pro-*p*-NA was added and activity was measured spectrophotometrically by the release of *p*-nitroaniline.

RESULTS AND DISCUSSION

Cloning of the *L. delbrueckii* subsp. *lactis* DSM7290 *pepI* gene

E. coli ER1562 was transformed with the plasmid library of DSM 7290 chromosomal DNA in pLG339 and the *pepI* gene could be isolated from colonies showing peptolytic activity on the substrate Pro-βNA. In the plate assay this substrate was not cleaved by the *E. coli* host strain, nor was it cleaved by *E. coli* harbouring the recently cloned genes for X-prolyl-dipeptidyl aminopeptidase (Meyer-Barton *et al.*, 1993) and aminopeptidase N (Klein *et al.*, 1993) from *L. delbrueckii* subsp. *lactis* DSM 7290. From approximately 6500 transformants screened, four Pro-βNA-cleaving colonies could be detected. Restriction

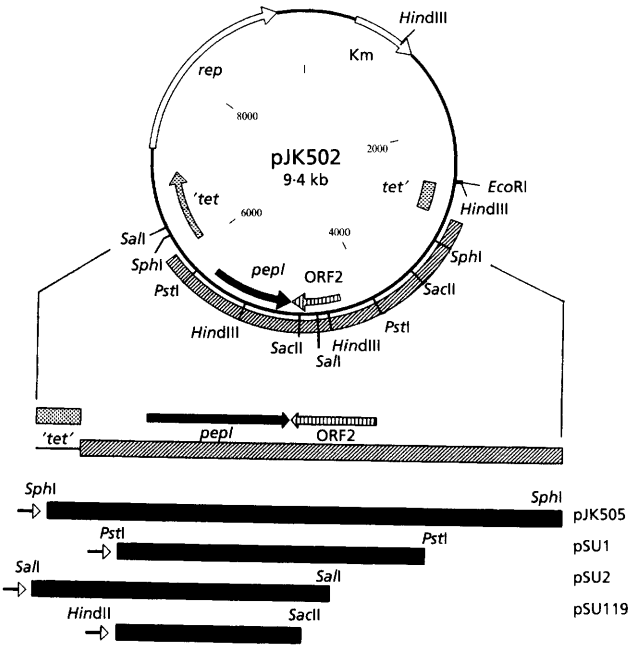

Fig. 1. Genetic map of pJK502. The 3·2 kb insert of chromosomal DNA is indicated as a hatched segment, with the *pepI* gene located along the black arrow. The linear expansion of the insert illustrates the subcloning steps. The different restriction fragments used for subcloning into vector pUC18, all spanning the *pepI* gene, are indicated as shaded bars.

analysis revealed that all plasmids isolated had inserts ranging in size from 3·2 to 8·0 kb, with a conserved identical core region. Plasmid pJK502, with the smallest insert size of 3·2 kb, was subjected to further analysis (Fig. 1).

Subcloning and nucleotide sequence analysis

Subcloning of a 3·2 kb *Sph*I fragment from pJK502 in pUC18 resulted in plasmid pJK505 (Fig. 1) expressing the PepI activity coded on a high-copy-number plasmid. The complete nucleotide sequence of both strands of this *Sph*I fragment was determined using synthetic oligonucleotide primers deduced from the investigated sequences. Analysis of the nucleotide sequence determined (2958 bp, 51·4 mol % C+G) uncovered the putative open reading frame for *pepI* (Fig. 2) extending from an ATG codon at nucleotide 410 to a TAA stop codon at position 1292, which is sufficient to encode a protein of 294 amino acids corresponding to a protein with a molecular mass of 32883 Da. A second open reading frame (ORF2) located on the opposite DNA strand, ranging from ATG at position 1812 to the TAG stop codon at position 1291, is capable of coding a protein of 173 amino acids with a molecular mass of 19822 Da.

Overexpression of PepI protein in *E. coli*

Crude cell extracts of *E. coli* CM89 harbouring plasmids pJK502 or pJK505, both coding for *pepI*, were subjected to SDS-PAGE (Fig. 3). A 34 kDa protein could be detected; this value corresponds well with the molecular mass of the enzyme predicted from nucleotide sequence analysis.

The subcloning experiments, which were performed in order to minimize the *pepI* coding region, are outlined in Fig. 1. Cloning of the 3·2 kb *Sph*I fragment from the low-copy-number plasmid pJK502 into high-copy-number vector pUC18, resulting in pJK505, increased gene dosage, but this had only a minor effect on protein overexpression. The knowledge of the complete pJK505 nucleotide sequence allowed cloning of defined DNA fragments. The *Pst*I fragment from pJK505 was subcloned into pUC18, both possible insert orientations were obtained and both constructs expressed PepI protein, thus indicating that the *pepI* promoter sequence is functional in *E. coli*. But only the plasmid (pSU1) with the *lac* promoter upstream of *pepI* showed a drastic increase of PepI expression (Fig. 3). Plasmid pSU2, with *pepI* on a 1·8 kb *Sal*I fragment, has a spacing region of 1 kb between the *lac* promoter and the structural gene and PepI is less efficiently expressed than in pSU1. We further succeeded in cloning the small *pepI*-expressing 1140 bp *Hin*cII/*Sac*II fragment from pSU1 into pUC18, resulting in pSU119.

The amount of PepI protein expressed was correlated with the copy number of the vectors used, but a considerable increase could be observed after cloning under control of the *lac* promoter. In *E. coli* CM89(pSU119) the *pepI* product constituted about 50 % of the cytoplasmic proteins (Fig. 3). This remarkable high-level expression of the *pepI* product appeared not to be lethal for the *E. coli* host, but the high-copy-number plasmids were structurally unstable and PepI activity was lost after approximately 80 generations with antibiotic selection. In earlier experiments with *pepX* (encoding X-prolyl-dipeptidyl aminopeptidase; Meyer-Barton *et al.*, 1993), we were not successful in cloning the peptidase gene into pUC18, and with *pepN* (aminopeptidase N; Klein *et al.*, 1993) subcloning into high-copy-number vectors resulted in extremely unstable plasmids. A reason for this was probably the strong overexpression in *E. coli* under control of the original promoter even if cloned in low-copy-number plasmids.

A protein which could be associated with ORF2 gene product (19·8 kDa) was not detectable by SDS-PAGE.

Homologies of the *L. delbrueckii* subsp. *lactis* sequence

Searching the EMBL database with the investigated nucleotide sequence resulted in no significant homologies; only after translation of the two proposed open reading frames were sequences with homologies detected in the protein databases.

The ORF2 gene product is similar to a repressor protein of *Bacillus subtilis pai*1 (Honjo *et al.*, 1990) (Fig. 4). In *Bacillus* this repressor is involved in reduction of extracellular and cell-associated protease levels, when present in high copy number, and it is considered to function as a transcriptional regulator. The physiological function of the lactobacillal ORF2 gene product remains to be investigated.

For *pepI* the most significant homology is observed in a small, but functionally important segment of a 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase (Menn *et al.*, 1991), an enzyme involved in degradation of toluene, which contains the serine catalytic site (Fig. 5). Comparison of both protein sequences over their entire length results only in a non-significant homology of 23·8 % (70 in 294 amino acids). This result shows that *pepI* is of new sequence type, distinct from prolyl-aminopeptidase, the only one having so far been cloned and sequenced being from *Bacillus coagulans* (Kitazono *et al.*, 1992), also distinct from the Pro-β-naphthylamidase from porcine liver (Matsushima *et al.*, 1991), which turned out to be a carboxylesterase, and from all other aminopeptidases hitherto sequenced.

The absence of a signal peptide sequence and the hydrophilicity plot of the amino acid sequence according to Kyte & Doolittle (1982), which does not show transmembrane domains, indicate that PepI of *L. delbrueckii* subsp. *lactis* might be an intercellularly located enzyme. No secreted peptidase has yet been found in lactic acid bacteria. However, an extracellular location of peptidases is discussed since most peptides released from casein by the proteinase are too large to be taken up by the

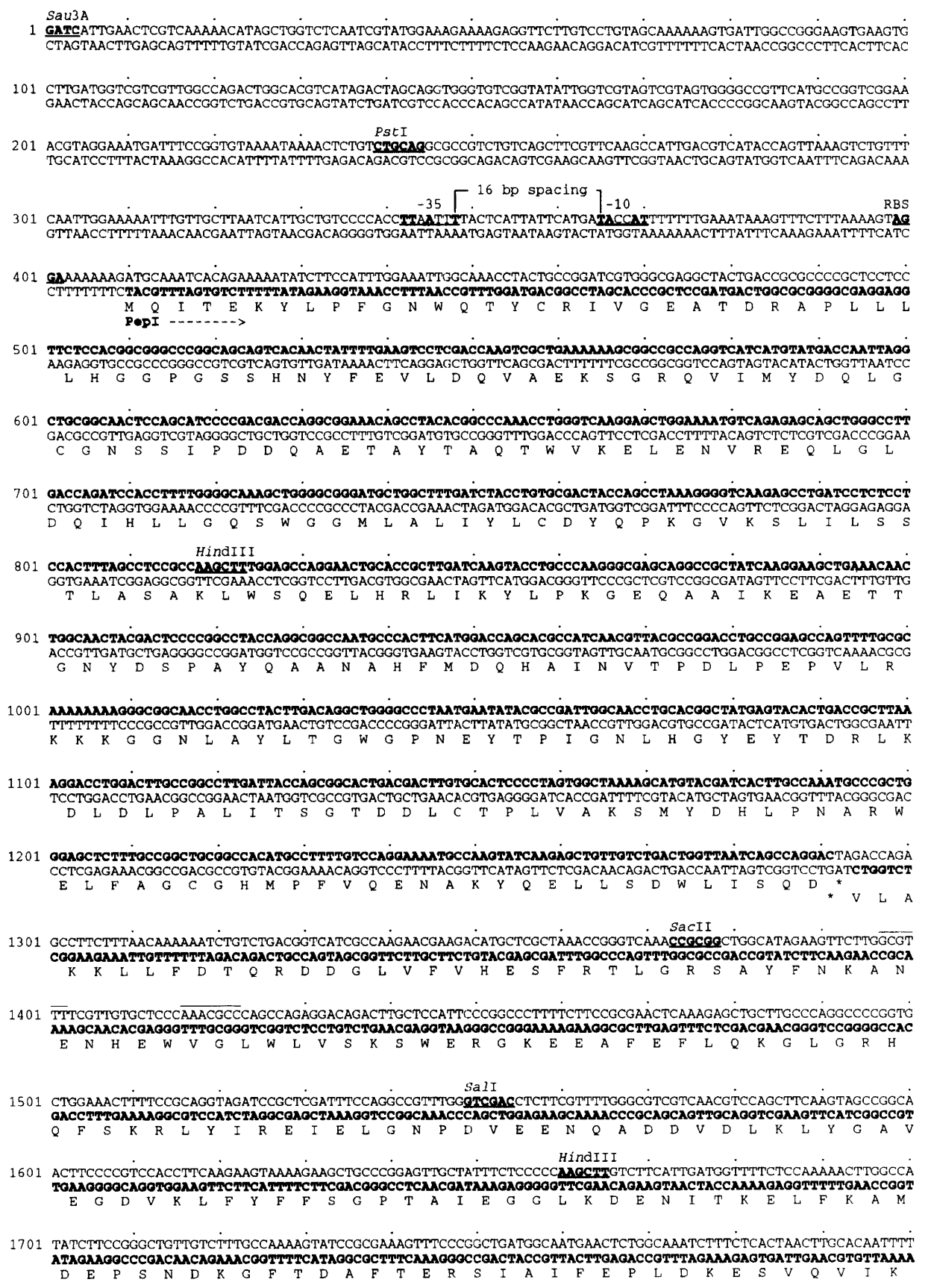

Fig. 2. For legend see facing page.

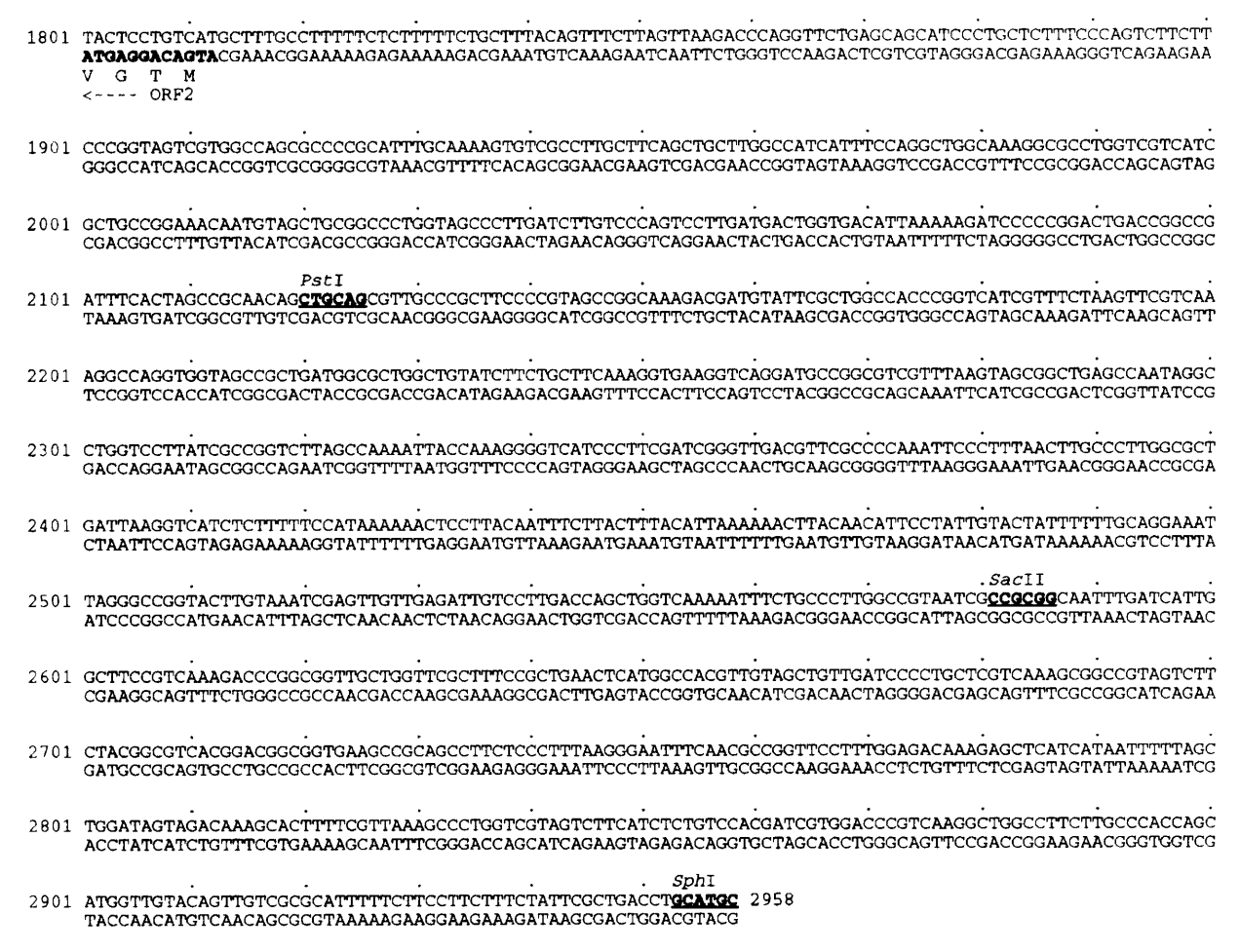

Fig. 2. Nucleotide sequence of the chromosomal insert of pJK505. The upper strand codes for *pepI* (nt 410–1291) and the lower strand for a gene (nt 1812–1294) with high homology to the *pai*1 repressor of *B. subtilis*. The deduced amino acid sequences are given below the nucleotide sequence. Five base pairs upstream of the putative *pepI* ATG start codon is a ribosome-binding site with a tetranucleotide stretch (AGGA) complementary to the *E. coli* 16S rRNA (Shine & Dalgarno, 1974); sequences that resemble promoter −10 and −35 elements (Van de Guchte *et al.*, 1992) are underlined (conserved nucleotides in bold type). An inverted repeat (overlined at bp 1396 to 1422) with a ΔG value of −18 kcal mol^{-1} (−75·3 kJ mol^{-1}) might function as transcription terminator. Some pertinent restriction enzyme sites are also indicated.

cell (Tan *et al.*, 1993). Possibly a yet unknown mechanism might be responsible for translocation of peptidases to the cell surface.

Partial characterization of the overproduced peptidase I

Since PepI protein was highly overproduced in *E. coli* harbouring the gene coded on plasmid pSU119, cell extracts were prepared from strain CM89(pSU119). This strain, lacking the peptidase genes *pepA*, *pepB*, *pepD*, *pepN* and *pepQ*, was chosen to exclude as much background peptidase activity as possible.

To determine the mechanism of enzyme action, we treated cell extracts of CM89(pSU119) with various protease inhibitors. 3,4-DCI (0·1 mM) inhibited the enzyme activity to a final relative activity of 8·9 %, whereas pepstatin A (inhibition to 89 %), E-64 (inhibition to 86·5 %), 1,10-phenanthroline or EDTA (inhibition to 84 %) had no significant effect on enzyme activity. These results suggest that the enzyme is classifiable as serine protease, which is consistent with its homology to the *P. putida* serine esterase active site (Fig. 5), implying that PepI has a catalytic centre with a serine residue.

To determine the substrate specificity of PepI a variety of *p*-NA substrates (Pro-, Ala-, Arg-, Asp-, Glu, Gly-, His-, Leu-, Lys-, Phe-, Ala-Pro-, Gly-Ala-, Gly-Phe-, Gly-Pro-, Gly-Trp-*p*-NA) were incubated with cytoplasmic extracts of CM89(pSU119), but among these only Pro-*p*-NA was effectively cleaved. This rapid test is only an indication of enzyme specificity, valuable if compared with the specificities of other cloned peptidases, and of course a more detailed characterization will be performed with purified enzyme. The purification and characterization of PepI will be described in the near future, as well as its subcellular location in *Lactobacillus* and determination of the N-terminal sequence, in order to confirm the coding region of the nucleotide sequence.

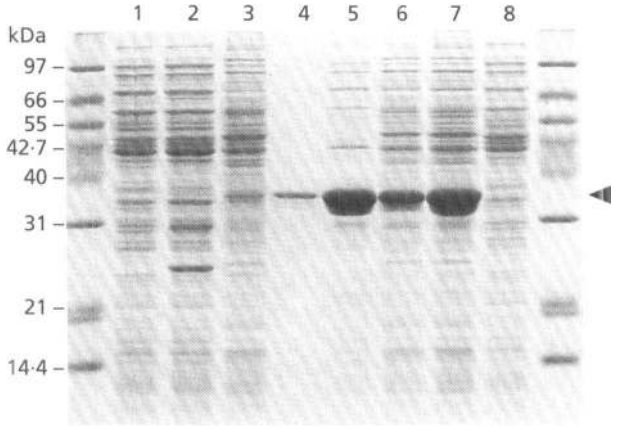

Fig. 3. Expression of PepI protein in *E. coli* strain CM89. Separation of crude cell extracts by SDS-PAGE (12%, w/v, acrylamide). Proteins were visualized by Coomassie staining. The gel contained molecular mass marker proteins (both flanking lanes) and cell extracts of *E. coli* CM89(pLG339) (lane 1), CM89(pJK502) (lane 2), CM89(pJK505) (lane 3), CM89(pSU1) (lanes 4 and 5; 1/10 of cell extract in lane 4), CM89(pSU2) (lane 6), CM89(pSU119) (lane 7) and CM89(pUC18) (lane 8). The position of PepI is marked by an arrowhead.

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                    10        20        30        40        50        60
pai1_ba     MS-VKMKKCSREDLQTLQQLSIETFNDIFKEQNSPENMKAYLESAFNTEQLEKELSNMSS
ORF2        MTGVKIVQVSEKDLPEFIAISRETFADTFGKDNSPEDMAKFLEKTINEDKLGGEIATPGS
            *. **. . * .**... ..* ***.*.* ..****.*  .**...*...*. *... .*

pai1_ba     QFFFIYFDHEIAGYVKVNIDDAQSEEMGAESLEIERIYIKNSFQKHGLGKHLLNKAIEIA
ORF2        FFYFLKVDGEVAGYLKLDVDDAQNEEVDPNGLEIERIYLRKSFQHRGLGKQLFEFAEEKG
             *.*.  * *.***.*...****.**.....********...***..****.*.. * * .

pai1_ba     LERNKKNIWLGVWEKNENAIAFYKKMGFVQTGAHSFYMGDEEQTDLIMAKTLI
ORF2        REWSKSVLWLGVWEHNENAKNFYASRGLTRFSEHVFVLGDDRQTDFLLKKALV
             *..*. .******.**** .** ..*... ..* * .**. ***... *.*.
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Fig. 4. Alignment resulting from CLUSTAL analysis of the ORF2 gene product (173 amino acids) as predicted from pJK505 nucleotide sequence analysis, with the *B. subtilis pai*1 repressor protein (172 amino acids), the translation product of EMBL entry M36471. Values of 45% identity and 84% similarity were calculated. Identical (*) and similar (.) amino acids are marked.

```
                nt
PEPI           644 YTAQTWVKELENVREQLGLDQIHLLGQ S WGGMLALIY
                    *  ..**  *  . . * **.. *.*  *  **.* .
TODF_PSEPU      78 YGVESWVAHLAGILDALELDRVDLVGN S FGGALSLAF
                aa
                                                105  ↑ active site
```

Fig. 5. Homology of the *pepI* gene product with 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase of *P. putida* (Swissprot database entry: TODF_PSEPU) resulting from BLASTX analysis. The PepI amino acid sequence starting at nucleotide 644 is numbered as in Fig. 2. Identities, 14/37 (37%); positives, 23/37 (62%).

ADDENDUM

After the completion of this work and acceptance of our manuscript, we became aware that cloning and sequencing, as well as purification and characterization, of a proline iminopeptidase from a closely related subspecies, *Lactobacillus delbrueckii* subsp. *bulgaricus*, was about to be published (Atlan *et al.*, 1994; Gilbert *et al.*, 1994). Comparison of the sequences revealed a very high homology not only of the deduced PepI amino acid sequence (identities: 287 of 295 amino acids) but also for the up- and downstream regions of the reported nucleotide sequence. As could be expected from this, the enzymic properties of the enzymes are very similar (manuscript in preparation).

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