Cloning and nucleotide sequence analysis of pepV, a carnosinase gene from Lactobacillus delbrueckii subsp. lactis DSM 7290, and partial characterization of the enzyme

Klaus F. Vongerichten, Jürgen R. Klein, Hugo Matern and Roland Plapp

Cell extracts of Lactobacillus delbrueckii subsp. lactis DSM 7290 were found to exhibit unique peptolytic ability against unusual β-alanyl-dipeptides. In order to clone the gene encoding this activity, designated pepV, a gene library of strain DSM 7290 genomic DNA, prepared in the low-copy-number plasmid pLG339, was screened for heterologous expression in Escherichia coli. Recombinant clones harbouring pepV were identified by their ability to allow the utilization of carnosine (β-alanyl-histidine) as a source of histidine by the E. coli mutant strain UK197 (pepD, hisG). Complementation was observed in a colony harbouring a recombinant plasmid (pKV101), carrying pepV. A 2.4 kb fragment containing pepV was subcloned and its nucleotide sequence revealed an open reading frame (ORF) of 1413 nucleotides, corresponding to a protein with predicted molecular mass of 51998 Da. A single transcription initiation site 71 bp upstream of the ATG translational start codon was identified by primer extension. No significant homology was detected between pepV or its deduced amino acid sequence with any entry in the databases. The only similarity was found in a region conserved in the ArgE/DapE/CPG2/YscS family of proteins. This observation, and protease inhibitor studies, indicated that pepV is of the metalloprotease type. A second ORF present in the sequenced fragment showed extensive homology to a variety of amino acid permeases from E. coli and Saccharomyces cerevisiae.

Keywords: β-alanyl-dipeptides, peptidase V, pepV, carnosinase, Lactobacillus delbrueckii subsp. lactis

INTRODUCTION

Lactobacilli used as starter cultures in dairy fermentations require exogenously supplied amino acids for growth. Due to the rather low content of free essential amino acids in milk they are dependent on their proteolytic abilities to degrade milk casein. Lactobacilli possess a complex proteolytic system composed of proteinases and a variety of peptidases (Abo-Elnaga & Plapp, 1987; Law & Kolstadt, 1983; Thomas & Pritchard, 1987), which act in a cascade to hydrolyse casein to small transportable peptides and amino acids. The quality of dairy products depends on the property of the proteolytic system since it affects rapidity of growth and concomitant acid production. Moreover, the taste of the fermented food relies on the production of flavourous peptides and amino acids resulting from the degradation of milk casein. In view of the economic importance of lactobacilli, intensive research is currently being carried out to investigate the proteolytic enzymes and the corresponding genes. This could lead to the development of starter strains with improved properties that may be used to increase the efficiency of milk fermentations. Furthermore, genes of individual proteinases and peptidases may serve as markers for the construction of food-grade vectors suitable for genetic engineering.

Only a few Lactobacillus genes coding for proteolytic enzymes have so far been cloned and sequenced, including three genes from Lactobacillus delbrueckii subsp. lactis DSM
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Replicon, relevant feature</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lb. delbrueckii</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>subsp. lactis DSM 7290</td>
<td>Source of plasmid library, pepV&lt;sup&gt;+&lt;/sup&gt;</td>
<td>ColE1, high copy number</td>
<td>Klein et al. (1993)</td>
</tr>
<tr>
<td>E. coli K12, TA3472</td>
<td>ΔhisOGDCBHAFAF1E</td>
<td>ColE1, high copy number</td>
<td>Ames &amp; Adeshir (1980)</td>
</tr>
<tr>
<td>E. coli K12, CM17</td>
<td>F&lt;sup&gt;+&lt;/sup&gt; leu-9 Δ(pro-lac) met thyA</td>
<td>ColE1, medium copy number</td>
<td>Miller &amp; Schwartz (1978)</td>
</tr>
<tr>
<td>E. coli K12, UK20</td>
<td>CM17, glyA</td>
<td>pSC105, low copy number</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>E. coli K12, UK197</td>
<td>TA3472, Δ(pepD-proBA)</td>
<td>pSC105, from pLG339</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC18</td>
<td></td>
<td>pSC105, from pLG339</td>
<td>Yanisch-Peron et al. (1985)</td>
</tr>
<tr>
<td>pBR322</td>
<td></td>
<td>pSC105, from pLG339</td>
<td>Sutcliffe (1979)</td>
</tr>
<tr>
<td>pJK13</td>
<td>pepD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>pSC105, from pLG339</td>
<td>Klein et al. (1986)</td>
</tr>
<tr>
<td>pKV101</td>
<td>pepV&lt;sup&gt;+&lt;/sup&gt;</td>
<td>pSC105, from pLG339</td>
<td>This study</td>
</tr>
<tr>
<td>pKV102</td>
<td>pepV&lt;sup&gt;+&lt;/sup&gt;</td>
<td>pSC105, from pLG339</td>
<td>This study</td>
</tr>
<tr>
<td>pKV103</td>
<td>pepV&lt;sup&gt;+&lt;/sup&gt;</td>
<td>pSC105, from pLG339</td>
<td>This study</td>
</tr>
<tr>
<td>pKV104</td>
<td>pepV&lt;sup&gt;+&lt;/sup&gt;</td>
<td>pSC105, from pLG339</td>
<td>This study</td>
</tr>
</tbody>
</table>

7290: pepX encoding the X-prolyl-dipeptidyl-aminopeptidase (Meyer-Barton et al., 1993), pepN encoding a lysyl-aminopeptidase (Klein et al., 1993) and pepL coding for proline iminopeptidase (Klein et al., 1994). In addition, sequences of pepP, the proline iminopeptidase gene from *Lb. delbrueckii* subsp. *bulgaricus* (Atlan et al., 1994), and of a proteinase gene (Holck & Naes, 1992) from *Lactobacillus paracasei* have been published. The peptidease V from *Lb. delbrueckii* subsp. *lactis* DSM 7290, described in this report, is capable of hydrolysing the peptide bond of the unusual dipeptide β-alanyl-L-histidine (carnosine) and other β-alanyl-dipeptides. Carnosinase activities have been previously reported for *Salmonella typhimurium* (Kirsh et al., 1978), *Escherichia coli* (Klein et al., 1986) and mammals (Kunze et al., 1986) but, as far as we know, only the gene from *E. coli* has been cloned and sequenced (Henrich et al., 1990). In this communication we describe the cloning, sequencing and heterologous expression of pepV from *Lb. delbrueckii* subsp. *lactis* DSM 7290 in *E. coli*. The gene has been designated pepV in correspondence with a dipeptidase from *Lactococcus lactis* NCDO 712 (P. Stroman, personal communication), to which it shows significant amino acid homology. Although pepV complements a mutation of pepD in *E. coli*, the present report indicates that pepV and pepD do not belong to the same enzyme family.

**METHODS**

**Bacterial strains, plasmids and media.** The strains and plasmids used are summarized in Table 1. *E. coli* was grown at 37 °C either in Luria-Bertani medium (Sambrook et al., 1989) or in Davis minimal medium (Davis & Mingioli, 1950), supplemented with the required amino acids at a concentration of 50 μg ml<sup>-1</sup>, 0.5% glucose and thiamin (5 μg ml<sup>-1</sup>, final concentration). For selection of pepV transformants, minimal medium contained 100 μg β-alanyl-L-histidine ml<sup>-1</sup> (Bachem). Kanamycin was added to the culture medium at a concentration of 40 μg ml<sup>-1</sup> when appropriate. Cultures of *Lb. delbrueckii* subsp. *lactis* DSM 7290 were incubated at 42 °C in MRS medium (De Man et al., 1960).

**Transformations.** *E. coli* was transformed by electroporation using a Bio-Rad Gene Pulser as described by Dower et al. (1988). Restriction enzymes and other nucleic-acid-modifying enzymes were obtained from Boehringer Mannheim, United States Biochemical, New England Biolabs, or Pharmacia and were used as recommended by the manufacturers. Isolation of plasmid DNA from *E. coli* was performed as described by Birnboim & Doly (1979).

**Molecular cloning of pepV from *Lb. delbrueckii* subsp. *lactis*.** The method used to clone pepV relied on complementation of UK197, an *E. coli* pepD mutant (Table 1). In this strain, both relevant markers pepD and hisG were deleted. A genomic library of size-fractionated Sau3A fragments of total DNA from *Lb. delbrueckii* subsp. *lactis* DSM 7290 constructed in the low-copy-number vector pLG339 (Stoker et al., 1982), as described by Klein et al. (1994), was used to transform UK197. pepV<sup>+</sup>-Positive transformants were identified by their ability to grow on minimal agar plates supplemented with carnosine and kanamycin.

**DNA sequence analysis.** Nucleotide sequencing of pKV104 was initiated with M13 sequencing primers adjacent to the multiple cloning site, and subsequently oligonucleotide primers (Applied Biosystems, model 392) deduced from the investigated sequences were applied. Double-stranded plasmid DNA, purified on NUCLEOBOND AX100 columns (Macherey-Nagel), was used as a template. The sequence of each strand was determined using the Applied Biosystems model 373A DNA Sequencing System and the required PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit, which is based on the dideoxynucleotide chain termination method (Sanger et al., 1977). All chemicals were obtained from Applied Biosystems.
and used as recommended. For computer-assisted DNA and amino acid sequence analyses the Microgenie (Beckman), PC/GENE (IntelliGenetics) and HUSBAN (GENUSnet) software were used.

Isolation of RNA. Total RNA isolation from Lb. delbrueckii subsp. lactis DSM 7290 was based on the method of Ikemura & Dahlberg (1973) and was modified as follows. An inoculum of 5 ml overnight (16 h) culture of Lb. delbrueckii subsp. lactis DSM 7290 was mixed with 5 ml prewarmed MRS medium and incubated for 2 h at 37 °C. Cells were pelleted by centrifugation, washed once with water, resuspended in 1:14 ml 6.5% (w/v) sucrose in 50 mM Tris/HCl (pH 8), 1 mM EDTA plus 6.75 mg lysozyme and 50 U mutanolysin (Sigma). After incubation for 1 h at 37 °C, 0.15 ml 0.25 M EDTA in 50 mM Tris/HCl (pH 8) and 84 μl 20% SDS were added. The lysed cells were subjected to proteinase K digestion at a concentration of 50 μg ml⁻¹ for 30 min at 55 °C. The solution was extracted twice with Tris-buffered phenol (pH 7), once with phenol/chloroform and twice with chloroform/isomyl alcohol (24:1, v/v). The supernatant was added to 1 vol. 4 M LiCl and RNA was allowed to precipitate overnight at 4 °C. After centrifugation (14 000 × g, 20 min, 4 °C), the RNA pellet was dissolved in 1 ml H₂O, followed by a second LiCl precipitation for 4 h at 4 °C. The pellet was washed in 80% (v/v) ethanol, and dissolved in 20 μl H₂O. After a final ethanol precipitation the RNA was stored as an ethanolic suspension at -70 °C. The RNA concentration was determined by measuring the A₂₆₀ after centrifugation of an aliquot of this suspension and dissolution of the sediment in distilled water (25 A₂₆₀ units = 1 mg RNA ml⁻¹). RNA preparations were analysed on 1% (w/v) agarose gels before use.

Primer extension analysis. To determine the 5' end of pepV mRNA, primer extension analysis was performed as described by Henrich et al. (1993), using a synthetic oligonucleotide (27-mer, see Fig. 4), which was hybridized to RNA from Lb. delbrueckii subsp. lactis DSM 7290. The size of the 32P-labelled cDNA was determined by a parallel DNA sequencing reaction of a pepV coding plasmid, initiated by the same primer.

Preparation of cell-free extracts. Cell pellets of E. coli from 100 ml of an overnight culture were washed with 20 ml 50 mM Tris/HCl (pH 8), pelleted by centrifugation at 7500 g for 10 min and resuspended in 1 ml of the same buffer. The bacteria were disrupted by ultrasonication on ice (Bandelin sonifier, Sonopuls HD60) and cell debris was removed by centrifugation at 51 000 g, 50 mg protein ml⁻¹ as determined by the Lowry method. The supernatants by an L-alanine dehydrogenase-NAD coupled reaction, exactly as described by Williamson (1985). One unit of pepstatine V activity was defined as the amount of enzyme that liberates 1 nmol l-alanine min⁻¹ under the conditions described.

To study the mechanism of enzyme action, the inhibitors 3,4-dichloroisoucamin (3,4-DCI) at a concentration of 0.1 mM, pepstatine A at 1 μg ml⁻¹, L-trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64) at 10 μM, and 1,10-phenanthroline or EDTA at 1 mM were added to the extracts and incubated for 30 min at 37 °C. The substrate β-alanyl-l-alanine, was added and activity was measured spectrophotometrically as described above.

Non-denaturing PAGE. Crude cell extracts were subjected to disc gel electrophoresis on polyacrylamide gels (7%, pH 8) according to Davis (1964). After electrophoresis, peptidase activity was detected by incubation of the gels with the reaction mixture in the dark at 37 °C (Sugiura et al., 1977). The reaction mixture was prepared immediately before use, as follows: 1.3 ml of a 2% (w/v) agar solution (45 °C) in 0.1 mM CoCl₂, 0.1 mM MnCl₂, supplemented with 2.5 mg peptide as a substrate, was added to 1.3 ml of a solution containing 5 mg 2-(p-iodophenyl)-3-p-nitrophenyl-5-phenyltetrazolium hydrochloride (INT), 1 mg of phenazine methosulfate (PMS) in 10 ml 50 mM Tris/HCl (pH 8) and 12 μl L-amino acid oxidase (1 mg ml⁻¹) from Crotalus adamanteus (Boehringer Mannheim). L-Amino acid oxidase efficiently catalyses the deamination of phenylalanine, tyrosine, tryptophan, leucine, isoleucine and methionine, while histidine, arginine and valine are rather poor substrates. All other amino acids are not detectable by this reaction. When using peptides containing L-alanine as substrate, L-amino acid oxidase was replaced by 10 μl L-alanine dehydrogenase (5 mg ml⁻¹) and 100 μl NAD solution (6 mg ml⁻¹).

RESULTS AND DISCUSSION

Cloning of pepV from Lb. delbrueckii subsp. lactis DSM 7290

Since cloning and transformation procedures are still only moderately successful in lactobacilli we decided to screen the plasmid library of Lb. delbrueckii subsp. lactis DSM 7290 prepared in the low-copy-number vector pLG339 for heterologous expression in E. coli. Recombinant plasmids carrying pepV were identified in E. coli UK197 transformants, which were capable of utilizing β-alanyl-l-histidine as a source of histidine. The only positive clone which exhibited pepV activity harboured a recombinant derivative of pLG339, carrying a chromosomal Sau3A fragment 4 kb in size. This plasmid, designated pKV101, was the subject of further analyses.

Subcloning of pepV and expression in E. coli

In order to shorten the pepV-containing insert, a 1.6 kb SauA fragment was deleted from pKV101 (Fig. 1), thus generating pKV102. This deletion reduced the insert size to 2.4 kb without affecting the expression of pepV (Fig. 2). Subcloning of the 2.4 kb BamHI–SauA fragment into the vectors pWSK129 and pWSK130 (Wang & Kushner, 1991) resulted in plasmids pKV103 and pKV104, which carry the inserted DNA in opposite orientations with
Fig. 1. Genetic map of plasmid pKV101. (a) The chromosomal DNA insert of 4 kb is indicated as a shaded segment with pepV and ORF2' located along the solid arrows. (b) The 2.4 kb BamHI-SalI fragment subcloned into pLG339, pWSK129 and pWKS130 is shown as a shaded bar.

Fig. 2. Expression of pepV in E. coli strain UK197. Crude cell extracts were separated by SDS-PAGE as indicated in Methods. Lanes: 1, molecular mass marker proteins; 2, UK197(pLG339); 3, UK197(pKV101); 4, UK197(pKV102); 5, UK197(pKV103); 6, UK197(pKV104). The position of PepV is marked by an arrowhead.

K. F. VONGERICHEN and OTHERS

I

Sall

BamHI

Fig. 7. Genetic map of plasmid pKV101. (a) The chromosomal DNA insert of 4 kb is indicated as a shaded segment with pepV and ORF2' located along the solid arrows. (b) The 2.4 kb BamHI-SalI fragment subcloned into pLG339, pWSK129 and pWKS130 is shown as a shaded bar.

123456
97 -

66.2 -

55 -

42.7 -

40 -

31 -

21.5 -

14.4 -

respect to the vectorial lac promoters. Peptidase V was expressed from pKV103 as well as from pKV104 and complemented E. coli UK197 in its peptidase D mutation without induction of the lac promoter, indicating that the original Lactobacillus pepV promoter is likely to be present on the cloned fragment and functional in E. coli. Attempts to clone the 2.4 kb BamHI-SalI section into the high-copy-number vector pUC18 (Raleigh et al., 1988), with pepI/ in opposition to the lac promoter, as well as into the medium-copy-number vector pBR322 (Sutcliffe, 1979), were not successful.

Cell extracts of E. coli UK197 complemented by the four different pepV-containing plasmids (pKV101, pKV102, pKV103 and pKV104) were subjected to SDS-PAGE. As shown in Fig. 2, one additional protein band of 52 kDa appeared specifically in extracts of all the complemented clones, but not in transformants harbouring vector pLG339. These results indicate that the 52 kDa band is likely to represent peptidase V. Expression of pepV in E. coli is high since about 25% of the cytoplasmic proteins are constituted by PepV protein (Fig. 2). The high level of expression under the control of the original promoter might result in lethal over-expression if pepV is cloned in high-copy-number vectors. This might explain the failure of subcloning pepV in plasmids like pUC18 or pBR322 (Meyer-Barton et al., 1993). Comparable observations were made for peptidases X and N (Meyer-Barton et al., 1993; Klein et al., 1993).

Substrate specificity of peptidase V

To compare the substrate specificity of the Lactobacillus peptidase V with peptidase D of E. coli, crude extracts of E. coli strain UK20 [pepD], UK20(pKV102) [pepV+] or UK20(pJK13) [pepD·], were subjected to native disc gel electrophoresis. The subsequent histochemical staining with a variety of different substrates permitted the detection of the two cloned peptidases in the presence of the host background. Since different peptidases could be easily distinguished by their characteristic Rf values, this method allowed the determination and comparison of substrate specificities without the necessity for protein purification. As visible in Fig. 3, lane 2, a band of Rf 0.9, indicating peptidase V activity, could be observed for cell-free extracts of UK20 incubated with trimethionine. This band could not be detected for cell-free extracts of UK20 incubated with the same substrate (Fig. 3, lane 1). Peptidase D activity of UK20(pJK13) incubated with β-alanyl-l-tyrosine as the specific substrate is indicated by a band of Rf 0.6 (Fig. 3, lane 3). In Table 2 the substrate specificities of the two peptidases are compared. Both enzymes cleave a variety of dipeptides, especially those with the unusual β-alanyl residue. While peptidase V was capable of hydrolysing some tripeptides, peptidase D acts solely as a dipeptidase. The activity of another di-/tripeptidase (DTP) from Lactobacillus helveticus CNRZ 32 was described by Nowakowski et al. (1993). Since DTP was not further characterized or sequenced, we were only able to compare the two enzymes at the level of substrate specificities. DTP and PepV have some overlapping substrate specificities but we also found substrates which were hydrolysed specifically by only one of the two peptidases. Accordingly, PepV cleaves L-phenylalanyl-L-leucine, but was not active on trileucine, which is a
peptides from *Lactobacillus lactis*

Fig. 3. Activity staining of crude cell extracts separated by native disc electrophoresis after incubation with two different peptides. Lanes: 1, UK20 with trimethionine; 2, UK20(pKV102) with trimethionine; 3, UK20(pJK13) with β-Ala-1-Tyr. The peptidase V activity from *Lb. delbrueckii* subsp. *lactis* (marked by an asterisk) is visible in lane 2 beside the *E. coli* peptidases A, B, B′ and N. Activity bands with *R* values characteristic of PepD and PepV were observed exclusively in extracts of *E. coli* harbouring the respective plasmids. The results obtained with a wide variety of peptides are summarized in Table 2.

Specific substrate of DTP. On the other hand the PepV substrate 1-phenylalaninyl-1-leucine was reported not to be hydrolysed by DTP. This comparison shows that PepV and DTP are different peptidases. However, since both enzymes were isolated from closely related *Lactobacillus* strains, significant homologies might be expected at the level of nucleotide or amino acid sequences.

In order to determine the mechanism of enzyme action we incubated cell extracts from UK20(pKV102) with different types of protease inhibitors (3,4-DCI for serine proteases, pepstatin A for proteases with aspartate-active sites, E-64 for cysteine proteases and 1,10-phenanthroline or EDTA for metalloproteases). The results indicated that the enzyme is a metalloprotease since full inhibition with 1,10-phenanthroline or EDTA could be detected, whereas the other inhibitors used had no significant effect on enzyme activity. Further characterization of the enzyme will be performed after purification.

**DNA sequence analysis**

The nucleotide sequence of the BamHI–SalI fragment in pKV104 was determined for both DNA strands (Fig. 4). Analysis of the sequence, consisting of 2403 bp, revealed the presence of two ORFs separated by 248 bp. ORF1 (1413 bp, 54.1 mol % G+C), starting with an ATG codon at position 221 and extending to the TAA stop codon at

**Table 2. Comparison of substrate specificities of *Lactobacillus* peptidase V and *E. coli* peptidase D**

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Peptidase V</th>
<th>Peptidase D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracts of UK20, UK20(pJK13) and UK20(pKV102) were analysed by activity staining after native PAGE. Extracts of UK20 served as a negative control containing only endogenous <em>E. coli</em> peptidases. The amino acids underlined are those with the potential to react either with the amino acid oxidase or, in the case of alanine, with L-alanine dehydrogenase after release by peptidases. This specificity for distinct amino acids allowed the prediction that Pep V cleaves tripeptides from the N-terminus but not from the carboxy-terminus (e.g. release of phenylalanine from L-Phe-Gly-Gly but not from Gly-Gly-L-Phe).</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 4. For legend see facing page.
nucleotide 1630, is sufficient to encode a protein of 470 amino acids with a calculated molecular mass of 51998 Da. This value agrees closely with the 52 kDa molecular mass of peptidase V as estimated by SDS-PAGE. A potential ribosome-binding site (AGGAG) is present 7 bp upstream of the predicted ATG start codon, which is similar to those reported for E. coli (Shine & Dalgarno, 1974) and Lactococcus lactis (van de Gucht, 1992).

The hydrophilicity plot of the deduced amino acid sequence according to Rao & Argos (1986) did not show any obvious membrane-spanning domains (data not shown) and searching the N-terminus did not reveal any putative signal peptide sequence. This suggests that PepV might be an intracellularly located enzyme. However, the cellular distribution of PepV in Lb. delbrueckii subsp. lactis DSM 7290 needs to be further investigated. ORF2, located on the same DNA strand downstream of pepV, runs from the unusual TTG start codon at position 1878 to the end of the cloned insert at position 2403. Since no translation stop codon could be detected, ORF2 does not encode a complete gene.

**Determination of the transcription initiation site**

To identify the precise location of the 5' end of the pepV mRNA, we performed primer extension analysis. A synthetic oligonucleotide primer of 27 nt complementary to nucleotides 242-268 of the antisense strand (Fig. 4) was hybridized to mRNA isolated from Lb. delbrueckii subsp. lactis DSM 7290. The extended cDNA indicated that the start point of pepV transcription is the A residue at position 149 (Fig. 5). Analysis of the nucleotide sequence in the close range upstream of the +1 region revealed a putative promoter area with considerable homology to the E. coli consensus promoter (Harley & Reynolds, 1987). The two detected conserved sequences TTGcCA and TAGAAT closely resemble the −35 and −10 boxes of the E. coli consensus promoter, respectively. Moreover the two boxes are separated by 17 bp, which complies with the canonical spacing in E. coli. Taken together, the similarity of the Lactobacillus transcription and translation signals to those observed in E. coli is consistent with the observation that heterologous expression of pepV was very efficient in E. coli. As in the case of this Lactobacillus gene, considerable homologies were found between the expression signals of Lc. lactis and E. coli (van de Gucht et al., 1992).

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

When searching the EMBL database with the pepV nucleotide or amino acid sequence, no significant homology to any sequences could be detected. No similarities were found with any peptidase or protease sequenced previously. Although complementation of E. coli pepD by the Lactobacillus pepV gene was used for screening, the sequences of the two genes are distinct. Comparison with an unpublished dipeptidase from Lc. lactis NCDO 712 (P. Stromman, personal communication) revealed considerable homology (47% identity over their entire length of 470 amino acids). Because of this striking homology, indicating a close phylogenetic relation between the

---

**Fig. 4.** Nucleotide sequence of the chromosomal insert of plasmid pKV104. The coding region for pepV (nt 221–1630) and the partial ORF for the predicted amino acid permease (nt 1878–2403) are translated into the amino acid sequence given below the nucleotide sequence. Seven nucleotides upstream of the pepV ATG start codon, and six nucleotides upstream of the ORF2 TTG start codon, well-conserved ribosome-binding sites (RBS) are present. Primer extension, using a 27-mer oligonucleotide complementary to mRNA (nt 242–268), identified nt 149 as the transcriptional start (A-residue shown above the nucleotide sequence of pepV). Searching the upstream region of the mRNA-starting point revealed the potential promoter region indicated. The potential ORF2 promoter is predicted by sequence analysis. The underlined region of dyad symmetry displaying typical features of a rho-independent transcription terminator is centred around nucleotide 1733 (Brendel & Trifonov, 1984). It is capable of forming a G+C-rich stem–loop structure with a ΔG value of −146 kJ mol⁻¹ (−11.1 kcal mol⁻¹), and is followed by a stretch of 8 T-residues. The two flanking restriction sites used for subcloning are indicated.
K. F. Vongerichten and Others

have been designated respective peptidases from pattern matching and thus allowing detection of domains et

We searched PepV with BLIMPS (BLOCKS IMPROVED Searcher), a searching tool that scores a protein sequence 'signatures' specific to a family or group of proteins (Boyen et al., 1992; Meinnel et al., 1992; Spormann et al., 1991). The enzymes of this family, acetylornithine deacetylase (coded by gene argE from E. coli), succinyldiaminopimelate desuccinylase (dapE from E. coli), carboxypeptidase G2 (cpg2 from Pseudomonas), vacuolar carboxypeptidase S (yscS from yeast), have been shown to be evolutionarily and functionally related. They share the common characteristics of hydrolysis of amide bonds in substrates with similar structure, dependence on cobalt or zinc for activity, and a size of 40 to 60 kDa, and they also show a number of regions of sequence similarity. Two conserved regions were selected as prosite signature patterns, one located in the N-terminal section of the enzymes and the second located in the central part. Each pattern contains one of the two conserved histidine residues in these enzymes, which are thought to be involved in the binding of the metal ions.

We searched PepV with BLIMPS (BLOCKS IMPROVED Searcher), a searching tool that scores a protein sequence against the BLOCKS database. This program is capable of detecting and evaluating distant relationships. We further searched PepV with prosite, a similar program, using pattern matching and thus allowing detection of domains with biological functions, active sites of enzymes and sequence 'signatures' specific to a family or group of proteins (Bairroch, 1992, 1993). Both analyses revealed homology to the signature sequence of the ArgE/DapE/CPG2/YscS family of proteins (Boyen et al., 1992; Meinnel et al., 1992; Spormann et al., 1991). The enzymes of this family, acetylornithine deacetylase (coded by gene argE from E. coli), succinyldiaminopimelate desuccinylase (dapE from E. coli), carboxypeptidase G2 (cpg2 from Pseudomonas), vacuolar carboxypeptidase S (yscS from yeast), have been shown to be evolutionarily and functionally related. They share the common characteristics of hydrolysis of amide bonds in substrates with similar structure, dependence on cobalt or zinc for activity, and a size of 40 to 60 kDa, and they also show a number of regions of sequence similarity. Two conserved regions were selected as prosite signature patterns, one located in the N-terminal section of the enzymes and the second located in the central part. Each pattern contains one of the two conserved histidine residues in these enzymes, which are thought to be involved in the binding of the metal ions. 

**Fig. 6.** Progressive alignment of the PepV protein sequence with proteins of the ArgE/DapE/CPG2/YscS family, using the program treed (Feng & Doolittle, 1987). The PepV sequence displays only weak homology over its entire length (ArgE 20%, DapE 17.8%; CPG2 18.7%; CPSI 17.9%) but the consensus regions Mla and Mlb are well conserved. Strictly conserved amino acids in all five sequences are marked by an asterisk. The ArgE/DapE/CPG2/YscS family signatures are boxed and labelled Mla, Mlb and MII. The prosite pattern Mib was recently replaced by Mla, containing one of the two histidine residues demanded for binding of the metal ions. Consensus pattern Mla (last update October 1993): [FYI-G-x-H-x-

L. lactis DSM 7290 and L. lactis NCDO 712, both these genes have been designated pepV.

![Image](image-url)
alignment revealed conservation of two adjacent glutamic acid residues (Fig. 6), which are likely to participate in chelating of the presumed metal ion as found in a number of Zn-metalloproteases.

This finding might indicate a new family of peptidases with homology to the ArgE/DapE/CPG2/YscS proteins but, due to the lack of the second consensus pattern, having a different catalytic site. The inhibition of PepV by metal ion chelating agents confirms our classification as a metalloenzyme, but the physiological activity of PepV, as an exclusively peptide-degrading enzyme, is not certain. The cleavage of unusual P-alanyl peptides and the homology to the ArgE/DapE/CPG2/YscS family might suggest participation in metabolic pathways having nothing in common with casein degradation.

The putative ORF2 protein showed significant homologies to the N-termini of a variety of amino acid permeases from different species (Table 3). The size of the most homologous permeases from E. coli ranges from 456 to 489 amino acids, so we calculated that approximately 40% of the presumed Lactobacillus gene is present on the cloned fragment. Since permeases are involved in the overall process of casein utilization, detailed knowledge of the transport systems specific for the products of degradation is needed. We are therefore currently screening the plasmid library for the missing part of the gene using colony blot hybridization with a labelled DNA probe.

### ACKNOWLEDGEMENTS

This work was supported by the BRIDGE T-Project 'Biotechnology of Lactic Acid Bacteria' of the EC and by the Bundesministerium für Forschung und Technologie grant 0319281B. The authors are responsible for the content of this publication. We acknowledge the excellent technical assistance of Bernd Winkelmann.

### REFERENCES


### Table 3. Amino acid (aa) homologies of the putative amino acid permease from Lb. delbrueckii subsp. lactis DSM 7290 (BlastX-Analysis*)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Organism</th>
<th>Percentage identity (aa residues)</th>
<th>Percentage similarity (aa residues)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aromatic amino acid transport protein (aroP)</td>
<td>E. coli</td>
<td>45 (72/159)</td>
<td>73 (112/159)</td>
</tr>
<tr>
<td>Phenylalanine specific permease (pheP)</td>
<td>E. coli</td>
<td>45 (71/156)</td>
<td>69 (109/156)</td>
</tr>
<tr>
<td>γ-Aminobutyric acid permease (gabP)</td>
<td>E. coli</td>
<td>34 (53/155)</td>
<td>60 (94/155)</td>
</tr>
<tr>
<td>Lysine specific permease (lysP)</td>
<td>E. coli</td>
<td>29 (40/137)</td>
<td>56 (77/137)</td>
</tr>
<tr>
<td>Proline specific permease</td>
<td>E. coli</td>
<td>37 (33/89)</td>
<td>61 (55/89)</td>
</tr>
<tr>
<td>Arginine permease</td>
<td>S. cerevisiae</td>
<td>32 (38/118)</td>
<td>55 (65/118)</td>
</tr>
<tr>
<td>General amino acid permease</td>
<td>S. cerevisiae</td>
<td>37 (40/107)</td>
<td>63 (63/107)</td>
</tr>
<tr>
<td>Histidine permease</td>
<td>Saccharomyces cerevisiae</td>
<td>45 (17/37)</td>
<td>81 (30/37)</td>
</tr>
<tr>
<td>Proline specific permease</td>
<td>Aspergillus nidulans</td>
<td>53 (23/43)</td>
<td>86 (37/43)</td>
</tr>
</tbody>
</table>

*Altschul et al. (1990).


Received 21 March 1994; revised 2 June 1994; accepted 5 June 1994.