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

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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 51 998 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

Abbreviations: 3,4-DCI, 3,4-dichloroisocoumarin; E-64, L-trans-epoxy-succinyl-leucylamide-(4-guanidino)-butane; DTP, di-/tri-peptidase.

The GenBank/EMBL/DBJ accession number for the nucleotide sequence reported in this paper is Z31377.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Replicon, relevant feature	Reference or source
Strains			
<i>Lb. delbrueckii</i> subsp. <i>lactis</i> DSM 7290	Source of plasmid library, <i>pepV</i> ⁺		Klein <i>et al.</i> (1993)
<i>E. coli</i> K12, TA3472	$\Delta(hisOGDCBHAFIE)$		Ames & Adeshir (1980)
<i>E. coli</i> K12, CM17	F ⁻ <i>leu-9</i> $\Delta(pro-lac)$ <i>met thy.A</i>		Miller & Schwartz (1978)
<i>E. coli</i> K12, UK20	CM17, <i>gly.A</i>		Laboratory collection
<i>E. coli</i> K12, UK197	TA3472, $\Delta(pepD-proB.A)$		Laboratory collection
Plasmids			
pUC18		ColE1, high copy number	Yanisch-Peron <i>et al.</i> (1985)
pBR322		ColE1, medium copy number	Sutcliffe (1979)
pJK13	<i>pepD</i> ⁺	ColE1, high copy number	Klein <i>et al.</i> (1986)
pLG339		pSC105, low copy number	Stoker <i>et al.</i> (1982)
pWSK129, pWSK130		pSC105, from pLG339	Wang & Kushner (1991)
pKV101	<i>pepV</i> ⁺	pSC105, from pLG339	This study
pKV102	<i>pepV</i> ⁺	pSC105, from pLG339	This study
pKV103	<i>pepV</i> ⁺	pSC105, from pLG339	This study
pKV104	<i>pepV</i> ⁺	pSC105, from pLG339	This study

7290: *pepX* encoding the X-prolyl-dipeptidyl-aminopeptidase (Meyer-Barton *et al.*, 1993), *pepN* encoding a lysyl-aminopeptidase (Klein *et al.*, 1993) and *pepI* coding for proline iminopeptidase (Klein *et al.*, 1994). In addition, sequences of *pepIP*, 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 peptidase 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. Ströman, personal communication), to which it shows significant amino acid homology. Although *pepV* complemented 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 μ g ml⁻¹, 0.5% glucose and thiamin (5 μ g ml⁻¹, final concentration). For selection of *pepV* transformants, minimal

medium contained 100 μ g β -alanyl-L-histidine ml⁻¹ (Bachem). Kanamycin was added to the culture medium at a concentration of 40 μ g ml⁻¹ 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*⁺-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 HUSAR (GENIUSnet) 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/isoamyl 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 (14000 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 A_{260} after centrifugation of an aliquot of this suspension and dissolution of the sediment in distilled water (25 A_{260} 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 ³⁵S-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 7000 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 51000 g and 4 °C for 60 min. Cell extracts from *Lactobacillus* were prepared from 50 ml cultures as described by Klein *et al.* (1993). The supernatant of both preparations contained approximately 50 mg protein ml⁻¹ as determined by the Lowry method. The content of PepV protein in cell extracts was determined videodensitometrically (Cybertech CS1, Image Documentation System) with Coomassie-stained SDS gels, calibrated with serum albumin standards.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Crude extracts were analysed by SDS-PAGE (12.5%, w/v, acrylamide) using the discontinuous buffer system of Laemmli (1970). Gels were stained with Coomassie blue according to Weber & Osborn (1969).

Assay of peptidase V activity and effects of various chemical reagents. Peptidase V was assayed in 0.66 ml reaction volumes by incubating 350 µl of crude extracts (*E. coli* or *Lb. delbrueckii* subsp. *lactis* DSM 7290) at 37 °C in the presence of 0.1 M Tris/HCl (pH 9), 0.1 M CoCl₂, and 7.27 mg β-alanyl-L-alanine ml⁻¹ as a specific substrate (Kirsh *et al.*, 1978). After 0, 10, 25 and 40 min, 0.2 ml samples were removed, heated at 90 °C for 5 min,

and centrifuged for 15 min at 12000 g and 4 °C. L-Alanine, the product of the reaction, was determined in 180 µl aliquots of the supernatants by an L-alanine dehydrogenase-NAD coupled reaction, exactly as described by Williamson (1985). One unit of peptidase 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-dichloroisocoumarin (3,4-DCI) at a concentration of 0.1 mM, pepstatin 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 *SaI* 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 *Bam*HI-*SaI* 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 *Bam*HI–*Sal*I fragment subcloned into pLG339, pWSK129 and pWKS130 is shown as a shaded bar.

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 *Bam*HI–*Sal*I section into the high-copy-number vector pUC18 (Raleigh *et al.*, 1988), with *pepV* 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. 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

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.

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 *R_F* 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 *R_F* 0.9, indicating peptidase V activity, could be observed for cell-free extracts of UK20(pKV102) 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 *R_F* 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

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-L-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_f 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 L-phenylalaninyl-L-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 *Bam*HI–*Sa*I 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

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 *E. coli* 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).

Peptides	Peptidase V UK20(pKV102)	Peptidase D UK20(pJK13)
β -Ala-L-Ala	+	+
β -Ala-L-His	+	+
β -Ala-L-Ile	+	–
β -Ala-L-Leu	+	+
β -Ala-L-Met	+	+
β -Ala-L-Phe	+	+
β -Ala-L-Trp	+	+
β -Ala-L-Tyr	+	+
β -Ala-L-Val	+	–
L-Ala- β -Ala	–	–
L-Ile- β -Ala	+	–
L-Leu- β -Ala	+	–
L-Met- β -Ala	+	–
L-Phe- β -Ala	+	–
L-Val- β -Ala	–	–
D-Ala-L-Leu	+	+
L-Ala-L-Ala	+	+
Gly-L-Phe	+	+
L-Leu-Gly	+	–
L-Leu-L-Leu	+	–
L-Met-L-Met	+	+
L-Phe-L-Phe	+	+
L-Phe-L-Leu	+	–
L-Pro-L-Leu	+	+
L-Ala-L-Ala-L-Ala	–	–
L-Ala-Gly-Gly	–	–
L-Ala-L-Val-L-Leu	–	–
L-Ala-L-Val-L-Leu	–	–
Gly-Gly-L-His	–	–
Gly-Gly-L-Phe	–	–
Gly-Gly-L-Met	–	–
Gly-L-Phe-L-Ala	–	–
L-His-Gly-Gly	–	–
L-His-L-Pro-L-Leu	–	–
L-His-L-Pro-L-Val	–	–
L-Leu-Gly-Gly	–	–
L-Leu-L-Leu-L-Leu	–	–
L-Met-L-Ala-L-Ser	+	–
L-Met-L-Ala-L-Ser	–	–
L-Met-L-Met-L-Met	+	–
L-Phe-Gly-Gly	+	–
L-Phe-L-Phe-L-Phe	+	–
Gly-Gly-Gly-L-Ala	–	–
L-Phe-Gly-Gly-L-Phe	–	–
L-Phe-L-Phe-L-Phe-L-Phe	–	–

Sali 10 20 30 40 50 60 70 80 90 100
 GTCGACCCCCAGTCGATACCGGTGGTCTTTACTGAAAGAGTGATTGATATCGCGGCAACTAAATACCGCCGCTAAAGCTGCGGGAAGCGGGCTTTTTCG

110 120 130 140 150 160 170 180 190 200
 TTGGCGTCTTGT -35 | 17 nt spacing | -10 TTTATTTAGGTGGTGTAGTCTTTTGTAACTAATTTCAAAAAACAGCAGGCATTGAA
 TTGCCA potential promoter TAGAAT

210 220 230 240 250 260 270 280 290 300
 ATAAACATTAGGAGAAAAAGATGGACTTAACTTTAAAGAACTGGCGGAAGCAAGGACGCGATCTTAAAGACTTAGAGGAATTGATCGCCATTGA
 M D L N F K E L A E A K K D A I L K D L E E L I A I D

310 320 330 340 350 360 370 380 390 400
 CTCTTCAGAGACCTGGAAAACGCCACGGAAGATACCCAGTTGCCAAGGTCCTGGTTGACGCCATGACCAAGTTCCTCAGCTTTGCCAAGCGGGACGGC
 S S E D L E N A T E E Y P V G K G P V D A M T K F L S F A K R D G

410 420 430 440 450 460 470 480 490 500
 TTTGACACGAAAACCTTCGCCAACTACGCCGGCCGGTCAACTTCGGTGCAGGCGACAAGCGCCTGGGCATCATCGGCCACATGGACGTAGTGCCAGCTG
 F D T E N F A N Y A G R V N F G A G D K R L G I I G H M D V V P A

510 520 530 540 550 560 570 580 590 600
 GTGAAGGCTGGACCCGGGACCCCTTCAAGATGGAAATCGACGAAGAAGGCCGGATCTACGGCCGGGCGAGCGCCGACGACAAGGGGGCCAGCTTAACTGC
 G E G W T R D P F K M E I D E E G R I Y G R G S A D D K G P S L T A

610 620 630 640 650 660 670 680 690 700
 CTACTACGGGATGCTTTTGTCTCAAGAAGCTGGCTTTAAGCCAAAGAAGATCGACTTCGTCTTAGGGACCAACGAAGAAACCACTGGGTGGGATC
 Y Y G M L L L K E A G F K P K K K I D F V L G T N E E T N W V G I

710 720 730 740 750 760 770 780 790 800
 GACTACTACTTGAAGACGAAACGACTCCAGACATCGTCTTCTCACCAGCGCTGAATACCCGATCATCAATGGTGAACAAGGGATCTTACCCTGGAAT
 D Y Y L K H E P T P D I V F S P D A E Y P I I N G E Q G I F T L E

810 820 830 840 850 860 870 880 890 900
 TCAGCTTCAAGAACGATGATACTAAGGCGGACTATGTTTAGACAAGTTCAAGGCCGGGATGCCACTAACGTGACTCCGCGAGTTACCCGGGCTACTAT
 F S F K N D D T K G D Y V L D K F K A G I A T N V T P Q V T R A T I

910 920 930 940 950 960 970 980 990 1000
 CTCTGGACCTGACCTGGAAGCTGTTAAATTGGCTTACGAAGCTTTTGGCAGACAAGGAATTTGGACGGATCATTTGAAATTAATGACGAAAGCGCCGAT
 S G P D L E A V K L A Y E S F L A D K E L D G S F E I N D E S A D

1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
 ATCGTCTTGATCGGCCAAGGGGCCACGCTTCAGCTCCGCAAGTCGCAAGAACTCAGCAACCTTCTGGCCCTCTTCTGGACCAATACGCTTTTGCCG
 I V L I G Q G A H A S A P Q V G K N S A T F L A L F L D Q Y A F A

1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
 GCCGGGCAAGAAGCTTCTCCACTTCTGGCTGAAGTGAACACGAAGACTTCTATGGCAAGAAGCTGGGCATCTTCCACCAGTATGATCTGATGGGCGA
 G R D K N F L H F L A E V E H E D F Y G K K L G I F H H D D L M G D

1210 1220 1230 1240 1250 1260 1270 1280 1290 1300
 TCTGGCCAGCAGCCGCTCCATGTTGACTACGAACACGCCGCAAGGCCCTCTTGAACAATGTCCGCTACCCGCAAGGGACTGACCCGGACACCATG
 L A S S P S M F D Y E H A G K A S L L N N V R Y P Q G T D P D T M

1310 1320 1330 1340 1350 1360 1370 1380 1390 1400
 ATCAAGCAGGTTCTGGCAAGTTTCAGCGGCATCTGGATGTCACTTACAACGGCTTTGAAGAACCAGCACTACGTGCCAGGCAGCCCAATGGTGAGA
 I K Q V L D K F S G I L D V T Y N G F E E P H Y V P G S D P M V Q

1410 1420 1430 1440 1450 1460 1470 1480 1490 1500
 CTTTGTCTAAGGTTTACGAAAAACAAACCGGCAAGCCGGGCCAGAGTCGTAATCGGCGGCGGACTTACGGCCGCTCTTTGAGCGCGGGTTCCTT
 T L L K V Y E K Q T G K P G H E V V I G G G T Y G R L F E R G V A F

1510 1520 1530 1540 1550 1560 1570 1580 1590 1600
 CGGGGCGCAGCCGGAAGCGCCCAATGGTTATGCACGCGCCCAACGAATTCATGATGCTGGACGATTGATCCTGTCCATCGCTATCTACGCTGAAGCC
 G A Q P E N G P M V M H A A N E F M M L D D L I L S I A I Y A E A

1610 1620 1630 1640 1650 1660 1670 1680 1690 1700
 ATCTACGAATTGACCAAGGACGAGAGCTGTAAGAAAAAGCCAAAAGGTAATTACTCAGGTGACTTAATTTTCTTTAGTCTAACCCGGTCAAAACAA
 I Y E L T K D E E L

1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
 GCATTTTGTAGCTTTAGCAGGTTGTCAATTAATTTTATTTTGCAGCGCTGCTTTTGTGCCCTTCTTAAAGGATTTTATC TTTACAA AAAAGACTGAAT
 potential terminator

1810 1820 1830 1840 1850 1860 1870 1880 1890 1900
 CC TATATT ATACAACCATAAATCTTATTTTATTTTATTTTACTTTTATTTTCTAGTTGCTTTAGT AGGA AGGACTTGCTTTATGGAGACCGCAGCAT
 | -10 RBS L L Y G D R S 1
 ORF2---->

1910 1920 1930 1940 1950 1960 1970 1980 1990 2000
 TGAAACACGGACGGGACTATCCGCTCCTTGTCCAACCGGCACGTGCGATGATTGCCATTGGCGGCACGATCGGTACTGGCCTCTTCTGGGGGCGGG
 E N T D G T I R S L S N R H V Q M I A I G G T I G T G L F L G A G

2010 2020 2030 2040 2050 2060 2070 2080 2090 2100
 ACCACGATTTCCGCCACTGGTCCCTCAGTTATTTTATCTACGCCATCATGGGCTATTTTCTTCTTCTTCTTGGCGGCTTAGGAGAGATGTTCTATT
 T T I S A T G P S V I F I Y A I M G L F F F F L L R A L G E M F Y

2110 2120 2130 2140 2150 2160 2170 2180 2190 2200
 TTGACTCCAACAGCCACCTTCGCTCCTTCATCAGATACCTGGGTGAGGCGGCTGGCGGCTGGACCTACTGGATCGGCATCGCTCTT
 F D S N S H T F V S F I T R Y L G E A A G R F A G W T Y W I G I L F

2210 2220 2230 2240 2250 2260 2270 2280 2290 2300
 TGCCTGCATGGCGGAGCTGACGCGCTTCAACCTATGTACAGTACTGGCTGCCCGGCTGCCCGCTGGCTGATTGAAGTTAGCGTCTAGGCTCTTGT
 A C M A E L T A V S T Y V Q Y W L P G L P A W L I E V S V L G L L

2310 2320 2330 2340 2350 2360 2370 2380 2390 2400
 ACCCTGCTCAACTTGACGCGCGGCAAGCTTTTGGCGAACTGAATCTGGTTTGCCATGATCAAGATCATCGCCATCATCTCTTGGTCGTAACCGGGA
 T L L N L T A A K L F G E T E F W F A M I K I I A I I S L V V T G

BamHI
 TCC
 I

Fig. 4. For legend see facing page.

Fig. 5. Mapping of the 5' end of the *pepV* transcript by primer extension. The 27-mer oligonucleotide (Fig. 4) complementary to the sequence near the 5' end of the *pepV* coding region was used to prime cDNA synthesis of RNA from *Lb. delbrueckii* subsp. *lactis* DSM 7290 (lane 1). Reference sequencing reactions (lanes A, C, G and T) were performed with the same primer on double-stranded plasmid pKV104 as the template. The sequence of the antisense DNA strand in the region of transcription is shown to the right of the panel. The 3' terminus of the extended product complementary to the 5' end of the *pepV* RNA assigned to an A-residue is marked by an asterisk.

nucleotide 1630, is sufficient to encode a protein of 470 amino acids with a calculated molecular mass of 51 998 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 Guchte, 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 Guchte *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. Ströman, 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 $-14.6 \text{ kcal mol}^{-1}$ (61.1 kJ mol^{-1}), and is followed by a stretch of 6 T-residues. The two flanking restriction sites used for subcloning are indicated.

Lb_PepV	MDLNF	KELAEAKKDAIL	KDLEELI	AID	SSED
Ec_DapE	MS	CPVIE	LT	SLS	PDD
Ec_ArgE	MKNKL	PPFIE	IY	SIS	ATE
Ps_CpG2	MRPSIHRTAIAAVLATAFVAGTALAQKRDNVLFQAATDEQPAVAK				TLEKLVNIETG
Sc_CpS1	MIALPVEKAPRKSLSWQRHRAFIISGIVALIIIGTFFLTSLGLHPAPPHEAKRPHHGKGMHSPKCEKIEPLSPSFKHSVDITLHDPAPFRNSSIEKLSNAVRI				

Lb_PepV	ENATEEYPVGKGPVDAMTKFL	* AKRDGFDTENFANYA	GRVNFAGAGDKR	* LGIIGHMDVVPAGE	GWTRDPF	KMEIDEEGR I	* YG	
Ec_DapE	AGCQ	ALLIERLQAI	GFTVERMDFADTQ N F	WAWRQGQ ET	LAFAGHTDVPVPGDA	DRWINPFF	EPTI RDGM L	FG
Ec_ArgE	EALDQSNADLITLLADWFKDL	GFNVEVQVPVGTNR KFN	MLASIGQGAGG	LLLAGHTDTPVPDD	GRWTRDPF	TLTE HDGK L	YG	
Ps_CpG2	TGDAEGIAAAGNFLEAELKNL	GFTVTRSKSAGLVGDN I	VGKIKGRGGKN	LLLMHMDTVYLKG	ILAKAPF	RV	EGDKA	YG
Sc_CpS1	PTVVQDKNPNPADDPDFYKHFYELHDYFEKTFPNIHKHLKLEKVNELGLLYTWEGSDPDLKP			LLMAHQDVVPVNNETLSSWKFPFF		SGHYDPETDFV	WG	

Mia

Lb_PepV	RGSADDKGPS	LTAYYGMILLKEAGFKPKKIDFVLGTN	EE	TNWVGIDYLLKHEPTPDIVFSPDAEYPIINGEQGIFTLEFSFKNDTCKGDYVLDKFKAGI				
Ec_DapE	RGADDMKGS	AAMVVAERFVAQHPNHTGRFLAFLITS	EE	ASAHNGTVKV	VEALMARNERLDYCLVGEPSI		EVVGDVVKNGRRGSL	
Ec_ArgE	LGTADMKGFF	AFILDALR DVDVTKLKPILATAD	EE	TSMAGARYFA	ETAL	RPDCAIIGEPSL	QPV	RAHKGHI
Ps_CpG2	PGIADDKGN	AVILHTLKLKEYGVRDYGTITVLFNTD	EE	KGSFGSRDLI	QEEAKL	ADYVLSFEPTS		AGDEKLSLGTSGIA
Sc_CpS1	RGSNDCKNLL	IAFEAIEQLLDGFKPNRTIVMSLGF	EE	ASGTLGAASL	ASFLHRYGDDGIYSIIDEGEGIMEVD		KDVFVATPINAERGVY	

Mib

Lb_PepV	ATNVTQVTRATIS	* GPDLEAVKLAYE	SFLADKELDGSFEINDESADIVLIGQGAHASAPQVGKN	SATFLALFLDQYAF	GRDKN				
Ec_DapE	TCNLT	IHGVOGHEVA YPHLA	DNPVHRAAPFNLN	AI	E	WDQ	NEF	FP	ATSMQ
Ec_ArgE	SNAIR	IQQQSGHSS DPARG	VNAIELMHDAIGHIL	QLRDNLKE		RYHY	EAFVTP	YPTLN	
Ps_CpG2	YVQVN	ITGKASHAGAAPELG	VNALVEASDLVLRM	NIDDKAKN		LRF	NWTIA	KAGN	
Sc_CpS1	DFEVS	ILGHGGHSSVPPDHT	TIGIASLITEFEANPFDEYEFNDNPIYGLLTCAAEHSKSLSKDVKKITLGAFFCPRRKDKLVEYISNQSHLRLTIRTO						

	↑	↑	↑	↑
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MII

Lb_PepV	FLHFLAEVEHEDFY	GKKLGIFHHDDLMGDLASSPSMFDYEHAGKASLLNNVRYPGQTDPTMIKQVLDKFSGLDVTYNGFEFHYVPGS	DPMVQ					
Ec_DapE	IANIQAGTGSNN	VIP GELFVQF	NFRFSTELTD	EMIKA	QVLALLEKHQLRYTVDDWNLGQPFLLTARG	K	LVD	
Ec_ArgE	LGHIVGGDASNR	ICACCELHMDI	RPLPGMTLNLNGLND		ALAPVSEWRPGLRTVDELHPPIPGYECP	N	H	
Ps_CpG2	VSNIIIPASA	TLNADV	RYARNEDFDAAMKTL	EE	RAQQKLP	PEADV	KVIVTRGRPAFNAGEGG	KKLVD
Sc_CpS1	AVDIINGGVKANALPETTRFLINHRINLHSSV	AEVFERNIEYAKKIAEKYGYGLSKNGDDYIIPETELGHIDITLLRELEPAPLSPSSGSPVWDILAG						

Lb_PepV	TLLKVYEKQTKPGHE	VVIG	GGTYGRLFER	GVAF	GAQPENG	PMVMHAANEFMMLDDLILSIAIYAEAIYELTKDEEL
Ec_DapE	AVVNAVEHYNEIKPQL	LTTG	GTSDGRFIARMGAQV	VELGPVN		ATIHKINECVNAADLQLLARMYQRIEQLVA
Ec_ArgE	QLVEVVEKLLGAKTEV	VNY	CTEAPFIQTLCPTL	V LGPGS		INQAHPDEYLETRFKPTRELITQVIHHFCWH
Ps_CpG2	KAVAYYKAGGTLGVE	ERTG	GGTDAAYALSGKPVIESLGLPG			FGYHSDKAEYVDISAIPRLRYMAARLIMDLGAGK
Sc_CpS1	TIQDVVFENGVLQNNEEFYVTTLGFSGMTDTKYYNLSKNIYRFVGS	I	IDIDLKTLHVSNEHVDVPGHLSAIAFVVEYIVNVNEYA			

Fig. 6. Progressive alignment of the PepV protein sequence with proteins of the ArgE/DapE/CPG2/YscS family, using the program TREE (Feng & Doolittle, 1987). The PepV sequence displays only weak homology over its entire length (ArgE 20%; DapE 17.8%; CPG2 18.7%; CPS1 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 Mlb 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): [FY]-G-x-G-x-A-D-x-K-G-x(2)-A. Consensus pattern Mlb: [LIVMF]-x-[GSA]-H-x-D-x-V-x(9,13)-P-F. Consensus pattern MII: I-x-G-x(2)-[SAG]-H-x-[SAG]-x(1,2)-P-x(2)-[SAGT]; the strictly conserved amino acids in this pattern are labelled by an arrow.

respective peptidases from *Lb. delbrueckii* subsp. *lactis* DSM 7290 and *Lc. lactis* NCDO 712, both these genes have been designated *pepV*.

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 (Henikoff & Henikoff, 1991; Wallace & Henikoff, 1992). 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 (Bairoch, 1992, 1993).

Both analyses revealed homology to the signature sequence of the ArgE/DapE/CPG2/YscS family of proteins (Boyen *et al.*, 1992; Meinel *et al.*, 1992; Spormann *et al.*, 1991). The enzymes of this family, acetylornithine deacetylase (coded by gene *argE* from *E. coli*), succinyl-diaminopimelate desuccinylase (*dapE* from *E. coli*), car-

boxypeptidase 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.

Peptidase V from *Lactobacillus* appeared to contain only the first motif, and not the second histidine-containing pattern (Fig. 6). The same was observed for the lactococcal enzyme (P. Strøman, personal communication; data not shown). PepV, being a metallohydrolase, might have a different histidine residue involved in catalysis. The

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

Enzyme	Organism	Percentage identity (aa residues)	Percentage similarity (aa residues)
Aromatic amino acid transport protein (<i>aroP</i>), 456 aa	<i>E. coli</i>	45 (72/159)	73 (112/159)
Phenylalanine specific permease (<i>pheP</i>), 458 aa	<i>E. coli</i>	45 (71/156)	69 (109/156)
γ -Aminobutyric acid permease (<i>gabP</i>), 466 aa	<i>E. coli</i>	34 (53/155)	60 (94/155)
Lysine specific permease (<i>lysP</i>), 489 aa	<i>E. coli</i>	29 (40/137)	56 (77/137)
Proline specific permease 570 aa	<i>S. cerevisiae</i>	37 (33/89)	61 (55/89)
Arginine permease, 590 aa	<i>S. cerevisiae</i>	32 (38/118)	55 (65/118)
General amino acid permease, 601 aa	<i>S. cerevisiae</i>	37 (40/107)	63 (63/107)
Histidine permease, 532 aa	<i>Saccharomyces cerevisiae</i>	45 (17/37)	81 (30/37)
Proline specific permease, 570 aa	<i>Aspergillus nidulans</i>	53 (23/43)	86 (37/43)

* Altschul *et al.* (1990).

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 β -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.

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