A non-essential glutamyl aminopeptidase is required for optimal growth of *Lactococcus lactis* MG1363 in milk

K. J. A. I'Anson,¹ S. Movahedi,³ H. G. Griffin,¹ M. J. Gasson¹
and F. Mulholland²

Author for correspondence: K. I'Anson. Tel: +44 1603 255000. Fax: +44 1603 507723.

¹ Institute of Food Research,
Norwich Laboratory,
Norwich Research Park,
Colney, Norwich NR4 7UA,
UK

² Institute of Food Research,
Reading Laboratory, Earley
Gate, Whiteknights Road,
Reading RG6 6BZ, UK

³ Biochemistry and
Molecular Biology
Department, Leeds
University, Leeds LS2 9JT,
UK

Degenerate PCR primers were designed from the N-terminal amino acid sequence of a glutamyl aminopeptidase (PepA) from *Lactococcus lactis*. These primers were used to screen a lambda library for clones containing the gene (*pepA*) encoding PepA. The DNA sequence of a 2.1 kb fragment containing *pepA* was determined. The sequence revealed the presence of one complete and two incomplete open reading frames (ORFs). The complete ORF encodes a putative protein of 353 amino acids with a predicted N-terminal sequence identical to that determined for purified PepA. The *pepA* gene was subcloned on an *Escherichia coli* plasmid vector and production of active PepA was confirmed by means of a zymogram. Mutants of *L. lactis* in which the *pepA* gene was inactivated grew to normal cell densities in milk but exhibited a reduced growth rate during the exponential phase. Thus whilst PepA is required for optimal growth it is not essential.

Keywords: aminopeptidase, PCR screening, *Lactococcus lactis* subsp. *lactis*, nucleotide sequence analysis

INTRODUCTION

The growth of lactic acid bacteria (LAB) in milk is limited by the initial concentration of free amino acids and small peptides. For rapid multiplication, LAB rely on their ability to break down the milk protein, casein, to provide essential amino acids for cell protein synthesis. As a consequence of this, LAB have a specialized proteolytic system to allow the efficient degradation and utilization of casein. The proteolytic system consists of a membrane-bound proteinase (Laan *et al.*, 1989) which has been extensively studied (for reviews see Kok, 1990; Visser, 1993; Pritchard & Coolbear, 1993); transport systems to allow uptake of the resultant amino acids and peptides (Poolman, 1993; Hagting *et al.*, 1994; Kunji *et al.*, 1995); and several intracellular peptidases for further degradation of the generated peptides. In an attempt to fully understand this system, several of the peptidase genes of *Lactococcus lactis* have been isolated, cloned and sequenced (Mayo *et al.*, 1991; Ströman, 1992; Chapot-Chartier *et al.*,

1993; Mierau *et al.*, 1993b, 1994; van Alen-Boerrigter *et al.*, 1991; Monnet *et al.*, 1994).

Glutamic acid is one of the essential amino acids required by *L. lactis* in order to sustain growth (Mills & Thomas, 1981). Its concentration in milk is below the minimum required for cell protein synthesis (Thomas & Mills, 1981) and the involvement of the LAB proteolytic system is necessary to provide this essential nutrient from the milk proteins. LAB peptidases capable of releasing glutamate from milk proteins and peptides are of potential significance in this process.

Glutamate is an important flavour-enhancing component in many foods. It is also one of the most prevalent amino acids in cheese, and cheese flavour development coincides with the increasing content of glutamic acid during ripening (Puchades *et al.*, 1989). The peptidases of LAB are considered to play an important role in cheese ripening processes (Law, 1987). Of these peptidases, glutamyl aminopeptidase (PepA), which has activity against N-terminal aspartyl- and glutamyl-containing peptides (Exterkate & de Veer, 1987; Niven, 1991), is potentially involved both in the release of glutamate for growth of LAB in milk, and in the accumulation of glutamate in cheese.

Abbreviations: LAB, lactic acid bacteria; PepA, glutamyl aminopeptidase; pNA, p-nitroanilide.

The EMBL accession number for the sequence reported in this paper is X81089.

In this paper we describe the cloning and sequencing of the *pepA* gene, encoding PepA, from *L. lactis* subsp. *cremoris* MG1363 (Gasson, 1983) and its expression in *Escherichia coli*. To investigate the role of PepA in intracellular proteolysis we constructed *pepA* mutants and examined their ability to grow on milk.

METHODS

Bacterial strains, plasmids and growth conditions. *Escherichia coli* MC1022 (Casadaban & Cohen, 1980) was used as recipient in molecular cloning. *E. coli* LE392 (Sambrook *et al.*, 1989) was used to propagate lambda phage. *E. coli* was grown in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) at 37 °C. When appropriate, ampicillin was added to a concentration of 200 mg ml⁻¹. The vector pUC18 (Yanisch-Perron *et al.*, 1985) was from Pharmacia. Plasmid pFI872 is pUC18 containing a 4.2 kb *EcoRI* insert from *L. lactis* carrying the *pepA* gene. FI7894 is MC1022 containing pFI872. Plasmid pFI950 is pCRII (Invitrogen Corporation) with a 0.76 kb PCR product (truncated *pepA* gene) and erythromycin resistance gene cloned into the multiple cloning site. The *L. lactis* strains used were MG1363 (Gasson, 1983), FI5876 (Dodd *et al.*, 1990) and MG4695 (MG1363 with the pLP712: Gasson, 1983). *L. lactis* strains FI8362, FI8364 and FI8365 are *pepA* mutants with the pFI950 integrated into the chromosome. *L. lactis* was grown in M17 (Terzaghi & Sandine, 1975) supplemented with 0.5% (w/v) glucose or lactose and when appropriate with erythromycin (5 µg ml⁻¹) at 30 °C without agitation. Growth analysis of *pepA* mutants was carried out in 10% (w/v) low-temperature reconstituted skimmed milk (kindly supplied by G. Fitzgerald, University of Cork) steamed for 15 min on two successive days.

Growth analysis of *pepA* mutants. The OD₆₀₀ of exponentially growing cultures (in broth) was determined and cultures diluted with broth to give the same optical density in each culture. Ten microlitres of the diluted culture was used to inoculate 10 ml 10% reconstituted skimmed milk and incubation performed at 30 °C. Samples were taken at hourly intervals. The pH was measured and the OD₆₀₀ determined using a UVikon 860 spectrophotometer (Kontron Instruments) by the method of Kanasaki *et al.* (1975): 100 µl milk culture was mixed with 900 µl 0.5 M sodium borate (pH 8.0) containing 30 mM EDTA. The actual values of OD₆₀₀ and pH for each time point were calculated as the mean of three individual readings from three separate cultures (i.e. nine separate readings) for each strain. These experiments were repeated several times to confirm that readings were accurate. Statistical significance of the results was determined by Student's *t*-test.

DNA manipulations. Transformation of, and plasmid isolation from, *E. coli* were carried out as described by Sambrook *et al.* (1989). Chromosomal DNA was isolated from *L. lactis* by the method of Lewington *et al.* (1987). Lambda DNA was isolated using a Qiagen lambda DNA kit (Diagen) according to the manufacturer's instructions. Restriction endonucleases and T4 ligase were purchased from Gibco-BRL (Life Technologies) and used as recommended by the supplier. PCR products were cloned in pCRII (Invitrogen Corporation) following the manufacturer's instructions for use of the TA cloning kit. Transformation of *L. lactis* was performed by electroporation as described by Holo & Nes (1989). Southern hybridizations were performed using an ECL direct labelling and detection kit (Amersham) according to the manufacturer's instructions.

Determination of N-terminal sequence. PepA was purified according to the procedure of Niven (1991). The resulting

purified enzyme was blotted onto polyvinylidene difluoride membranes (Problott, Applied Biosystems) as described by Gooderham (1984). The blotted protein was visualized by staining with Coomassie Blue G-250 and the bands excised. N-terminal protein sequencing was performed on these bands, placed directly into the sequencer reaction cartridge, using a 470A gas-phase protein sequencer equipped with a 120A on-line phenylthiohydantoin analyser (Applied Biosystems), according to the procedure of Hunkapillar *et al.* (1983).

Primers used for library screening. Based on the codon usage in *L. lactis* (van de Guchte *et al.*, 1992), pools of degenerate primers were designed from the N-terminal sequence of PepA. Primer 1C (designed from the first 11 residues of the N-terminal sequence) consisted of a pool of 256 oligonucleotides with the sequence 5'-ATGGAA(T/C)TATTTGAT(G/A)A(A/T)-GT(A/T)(G/A)(T/A)TGC(A/T)(T/C)TAAC; primer 5C (designed from the last 10 residues of the N-terminal sequence) was a pool of 128 oligonucleotides with the sequence 5'-ATC(A/T)AC(A/T)AC(A/T)GG(A/T)CGTTCAAA(A/T)-CC(A/T)GT(A/T)GT. These primers generated a product of about 75 bp from lactococcal chromosomal DNA.

Construction of the lambda-ZAP library. The lambda-ZAP library was custom-made by Stratagene using *L. lactis* FI5876 (Dodd *et al.*, 1990) genomic DNA.

Direct PCR screening of the lambda-ZAP library. Screening was carried out by a modification of the method of Griffin *et al.* (1993). The lambda-ZAP library was plated out onto ten plates to give a plaque density of about 200 p.f.u. per plate (Sambrook *et al.*, 1989). A plaque lift was performed on each plate using nylon membranes (Amersham). Each filter was placed plaque side up in a sterile Petri dish and washed in 2 ml SM buffer (Sambrook *et al.*, 1989). A 10 µl aliquot was taken from each sample for analysis by PCR with primers 1C and 5C. The products were analysed by PAGE. If a plate contained one or more phage plaques that had the target sequence, a band of approximately 75 bp could be seen in the gel. In this way plates containing positive plaques were identified. A further plaque lift was carried out from each positive plate and the filter divided into ten segments (with the position of each segment marked on the positive plate). Each filter segment was placed in a 1.5 ml Eppendorf tube containing 200 µl SM buffer, vortexed, and 10 µl aliquots were subjected to PCR with primers 1C and 5C. In this way, separate positive segments were identified. The individual plaques (10–20 per segment) from those segments were 'picked' using a sterile glass Pasteur pipette into 200 µl SM buffer. Again 10 µl aliquots were analysed by PCR with primers 1C and 5C and any positive plaques were plaque-purified (Sambrook *et al.*, 1989).

Isolation of lambda DNA and subcloning of the lactococcal fragment. DNA was isolated from the plaque-purified positive plaque using a Qiagen Lambda isolation kit (Diagen). The lambda DNA was digested with *EcoRI* (lactococcal fragments were introduced into the lambda-ZAP library on *EcoRI* fragments). A 4.2 kb *EcoRI* fragment was cloned into pUC18 and plasmids containing the appropriate fragment were identified by performing PCR on the white colonies (Gussow & Clackson, 1989).

DNA sequence analysis. Sequencing reactions were carried out using the Applied Biosystems 'Prism' ready reaction DyeDeoxy terminator cycle sequencing kit and an Applied Biosystems 373A DNA sequencer according to the manufacturer's directions. Part of the 4.2 kb chromosomal insert of pFI872 was sequenced using Universal and Reverse primers. Following this, synthetic primers were designed from the deduced sequence and synthesized (Applied Biosystems model 394).

These were then used to sequence both strands of the DNA insert.

The Wisconsin Genetics Computer Group sequence analysis software package, version 7 (University of Wisconsin Biotechnology Center, Madison) was used to perform sequence comparisons, analysis of non-coding regions and to deduce protein sequences. Analysis of the DNA coding regions was done using the DNA strider program (Marck, 1988) on an Apple Macintosh LCIII computer.

Preparation of cell extracts. Overnight cultures (500 ml) of either *E. coli* or *L. lactis* were pelleted by centrifugation and washed with 50 mM Tris/HCl pH 7.5. The pellet was resuspended in 40 ml of the same buffer and subjected to French Pressing at a constant pressure of 1000 p.s.i. (6.9 MPa). Cell debris was removed by centrifugation at 42000 *g*, 4 °C for 60 min in a Sorvall RC5C centrifuge.

Detection of PepA activity. To show expression of PepA in *E. coli* FI7894, crude cell extracts were applied to non-denaturing PAGE gels (8%, w/v, acrylamide, pH 8.0) according to the method of Davis (1964), and the protein visualized by staining with Coomassie Blue. PepA activity in these gels was localized by a modification of the method of Hermsdorf (1978). The gels were overlaid with 1% (w/v) agar containing 130 mM MnSO₄, 40 mM EDTA, 50 mM Tris pH 8.0, 2 mM Glu-Trp, 0.1 mg dianisidine ml⁻¹, 0.1 mg amino acid oxidase ml⁻¹ and 0.2 mg peroxidase ml⁻¹. Oxidation of dianisidine produces a dark orange zone indicating glutamyl aminopeptidase activity. PepA activity of *pepA* mutant strains was determined by the method of Niven (1991): 50 µl cell extract was added to 450 µl 0.1 M Tris/HCl, pH 7.5, and 500 µl 2 mM Glu-pNA. This was incubated for 1 h at 50 °C and the reaction stopped by the addition of 500 µl 30% (v/v) acetic acid. The sample was then centrifuged at 10000 *g* for 5 min and the absorbance determined at 410 nm.

Determination of protein concentration. Protein concentration in cell extracts was determined using the Bio-Rad DC Protein assay according to the manufacturer's instructions, using bovine serum albumin standards.

RESULTS AND DISCUSSION

Library screening and sub-cloning

PepA has been purified from *L. lactis* NCDO 712 (Niven, 1991). The sequence of 25 residues at the N-terminus of the purified enzyme was determined by automated gas-phase sequencing as: Met-Glu-Leu-Phe-Asp-Lys-Val-Lys-Ala-Leu-Thr-Glu-Ile-Gln-Ala-Thr-Ser-Gly-Phe-Glu-Gly-Pro-Val-Arg-Asp. This sequence was used to design the degenerate primers subsequently used. PCR amplification of *L. lactis* chromosomal DNA with oligonucleotide primers 1C and 5C resulted in production of a 75 bp fragment. The FI5876 lambda ZAP library was screened with these primers (as described in Methods) and positive plaques were identified by the generation of a 75 bp product. Approximately 3000 plaques were screened in this way and four gave a positive result. DNA from one of the four plaques was isolated and a 4.2 kb *EcoRI* fragment was subcloned into pUC18 to create pFI872, which was then introduced into *E. coli* MC1022 to generate strain FI7894.

A strong PepA activity was found in the crude extract of *E. coli* FI7894 when assayed with Glu-pNA (data not

Fig. 1. (a) Coomassie Blue staining of non-denaturing PAGE to show expression of PepA in *E. coli* FI7894. Lanes: A, extract from *E. coli* MC1022 (not diluted); B, cell extract from *E. coli* FI7894 (diluted 50× with 50 mM Tris/HCl, pH 7.5); C, purified PepA from *L. lactis* MG1363 (10 µg). (b) Non-denaturing PAGE-zymogram to show activity of PepA in *E. coli* FI7894. Lanes: A, cell extract from *E. coli* FI7894 (diluted 50× with 50 mM Tris/HCl, pH 7.5); B, purified PepA from *L. lactis* MG1363 (10 µg); C, cell extract from *E. coli* MC1022 (not diluted). The band of PepA activity is arrowed.

shown). The extract from FI7894 was diluted 50-fold in 50 mM Tris/HCl, pH 7.5, prior to application onto non-denaturing gels and gave a similar level of activity to 10 µg purified PepA on the zymogram (stained by dianisidine oxidation). In both the protein-stained gel (Fig. 1a) and the zymogram (Fig. 1b) a band was clearly observed in FI7894 that corresponded to the purified PepA from *L. lactis* and this was not present in the MC1022 extracts. *E. coli* MC1022 had very little Glu-pNA-hydrolysing activity and the presence of the non-PepA oxidation bands observed in Fig. 1(b) for *E. coli* MC1022 extracts probably reflects the use of an undiluted sample. The strong expression of PepA in FI7894 is clearly shown in Fig. 1(a), where it is the major protein present in the 50-fold diluted crude extract.

Sequencing and analysis of the *EcoRI* fragment

Both strands of the 4.2 kb *EcoRI* DNA fragment were sequenced (only 2.1 kb shown here) using a 'walking' sequencing strategy in which synthetic primers were designed from the determined sequence. Analysis of the sequenced region revealed the presence of one complete and two incomplete ORFs (Fig. 2). ORF 1 has an ATG start codon at nucleotide 642 and a stop codon (TAA) at

1	ttcccataattccccactcgatagcaagatccatgtactcatcacggtctacttcaacaaatctaaattctggattctctgtttcaattt	90
	AAGGGTATTAAGGGGTGAGCTATCGTTCTAGGTACATGAGTAGTCCAGATGAAGTTGTTAGATTTAAGACCTAAGAGACAAAGTTAAA	
	G M I G W E I A L D M Y E D R D V E V F R F E P N E T E I	
	PvuII	
91	ctggcatttttaggtttgataaaattacaatctccacaccaaccagctgtgaaaaagaagactgtttttctgggctttgacaagctcag	180
	GACCGTAAAATCCAAACTATTTTAAATGTTAGAGGTGTGGTGGTGCACACTTTTCTTCTGACAAAAAGACCCGGAAACTGTTTCGAGTC	
	E P M K P K I F N C D G C W G A T F F F V T K E P G K V L E	
181	ctaaattttcaatattttctggaataatcattctgtatttcctttctgtatttttcaaatggattctaaccattttctaaagaagttcttc	270
	GATTTAAAGTTATAAAGACCTTATTAGTAagacataaaggaaagcataaaaagtttacctaagattggtaaagatttcttcaagaag	
	A L N E I N E P I I M	
	<--- orf 3	
271	ttttttttcaaaaacttctaagtttttggcattgatttcaaaagcaatagatttatcattactgacaattccccctcaaaaagggcacc	360
	aaaaaaaagtttttgaagatttcaaaaccgtaactaaagttttcgttatctaataagtaagactgttaagggggaagttttcccggtgg	
361	agaagtgtggtcatcaaaaatccaaaagcatcaattttttttagaanaaatgtgtcgactctatcagtaagctcatgaatcatttc	450
	tcttcaaccgagtagtttttaggtttttcgtagtttaaaaaaacatctttttacaacagcctgagatagtcatttcgagtacttagtaag	
451	ttcttccatgtttttataaccatgataagctgcgtgagcaacggctgctccgcgacaattcccaaaccaaacttttagtattttttcat	540
	aagaaggtacaaaaatattgttactattcgacgcactcggtgcgcagcaggcgctgttaagggtttggtttgaaatcataaaaaagta	
	16bp spacing	
541	atggaaattTTAACAattttcttgggtataatTATAGTgtaaatcatcaaaagaattactgacagatttgcagtaaaattttcagtatcc	630
	tacctttaaaattgtataaagaacatattaatatcacatttagtagttttcttaatgactgtctaacagtcatttataaaagtcagtagg	
631	cCGAGGagaaaaATGGAACCTATTTGACAAAGTAAAAGCACTGACAGAAATTCAGCCACTTCTGGTTTGAAGGTCCCGTTCGCGACTAT	720
	gcctcctctttttaccttgataaactgtttcattttcgtgactgtcttaagttcggtgaagacaaaaacttccagggcaagcgtgata	
	M E L F D K V K A L T E I O A T S G F E G P V R D Y	
	pepA --->	
721	TTAAAGCTCGTATGGTIGAGCTAGGCTATCAACCAGAATTGACGGACTTGGTGGTATTTTGTGACAAAAGCTTCAAAAGTCGAAAAT	810
	aattttcgagcataccaactcgatccgatagttgggtcttaaaactgcctgaaccaccataaaaacactgttttcgaagttttcagctttta	
	L K A R M V E L G Y Q P E F D G L G G I F V T K A S K V E N	
811	GCCCCACGAATCATGGTTCGGCTCACATGGATGAGGTCCGATTATGGTCAGCTCAATCAAAGCTGACGGAACATTCCGCGTTGTTCCT	900
	cggggtgcttagtaccacgacgagtgactactccagcctaaataaccagtcgagtttagtttcgactgccttgaaggcgcaacaagga	
	A P R I M V A A H M D E V G F M V S S I K A D G T F R V V P	
901	CTTGGTGGTTGGAACCCCTTAGTCGTTTCTGGTCAAGTMTTACCCTATTTACACGTACAGGTAAAAAGATTCCGGTTGTAACCTGGTGGT	990
	gaaccaccaaccttgggaatcagcaagaccagttgcataaaggatggaataatgtcatgtccatttttctaaggccaacattgaccacca	
	L G G W N P L V V S G Q R F T L F T R T G K K I P V V T G G	
991	CTTCCTCCACACCTTCTTCGCGGAACCTGGTGTAAACACCTCAAATCCCAGCAATCTCTGATATCATCTTTGATGGTGCATTGAAAATGCT	1080
	gaaggaggtgtggaagaagcgccttgaccacattgtggagtttagggctcgtagagactatagtagaaactaccacgtaaaccttttacga	
	L P P H L L R G T G V T P Q I P A I S D I I F D G A F E N A	
1081	GCCGAAGCTGCCGAATTTGGAATCGCCCAAGGTGATTTAATTATTCAGAAACAGAAACAATCTGTGACGAAATGGGAAAAATATCATT	1170
	cggcttcgacggttaaaccttagcgggttcactaaatttaataaggtctttgtctttgttaagacagtcgtttaccctttttatagtaa	
	A E A A E F G I A Q G D L I I P E T E T I L S A N G K N I I	
1171	TCFAAGCTTGGGATAATCGCTATGGTTCGTTAATGATTCTTGAACCTTCTTGAATTTTGTAGCTGACAAAGAATTACCTGTAACATTAATT	1260
	agattttgaacctatttagcgataccaacgaattactaagaacttgaagaacttaaaaatcgactgtttcttaatggacattgtaattaa	
	S K A W D N R Y G C L M I L E L L E F L A D K E L P V T L I	
1261	ATTGGAGCGAATGTTCAAGAGGAAGTCGGAATTCGTTGGTGCAAAAGTTCCACAACCAAAATTCATCTGATTTATCTTTGCGCTTGAC	1350
	taacctcgcttacaagtttctccttcagcctgaagcaccagcttttcaaggtgttggtttaaagtttaggactaaataagaacggcaactg	
	I G A N V Q E E V G L R G A K V S T T K F N P D L F F A V D	

Fig. 2. For legend see facing page.

1351	TGTTCAACAGCAAGCGATACCTTCGGTGACGATAATGGTCGCCTCGGAGAAGGAACAACGCTCCGTTTCTTCGACCCAGGTCATATCATG acaagtggctcggtcgctatggaagccactgctattaccagcggagcctcttccttgttgcgaggcaagaagctgggtccagtatagtac C S P A S D T F G D D N G R L G E G T T L R F F D P G H I M	1440
1441	CTTCCTGGAATGAAGAACTTCTTACTTGACACTGCAAAATCATGCCAAAGTTAAACACAAGTTTACATGGCCAAAGGGGGAACCGATGCA gaaggaccttacttcttgaagaatgaactgtgacgttttagtacggtttcaattttgtgttcaaatgtaccggtttcccccttggtacgt L P G M K N F L L D T A N H A K V K T Q V Y M A K G G T D A	1530
1531	GGTCCGCTCATTAGCAAAATGGAGGAGTTCCTTCAACAACAATCGGTGTGGTAGCCCGCTATATCCATAGCCATCAAACCTATTTTCAAC ccacggcgagtaaatcggttacctcctcaaggaagtgtgttagccacaccatcgggcgatataggtatcggtagtttgataaaagttg G A A H L A N G G V P S T T I G V V A R Y I H S H Q T I F N	1620
1621	ATTGATGATTTTCTTCAAGCTCAAACCTTCTTGAGAGCGATTATTACTTCACTTAATACTGAAAAAGTAGCAGAAATCAAAAACCTACTAA taactactaaaagaagttcgagtttgaaagaactctcgctaataatgaagtgaattatgactttttcatcgctcttagtttttgatgatt I D D F L Q A Q T F L R A I I T S L N T E K V A E I K N Y *	1710
1711	ttctaataaaaaaacactaaggaattatcctaagtggttttttagttactttttgatgaggaagctgactaaaattgaaagaaggtaaaa aagattatcttttttgatccttaaatagatcacaaaaaAATCAATGAAAACTACTCCTTCAGACTGATTTTAACTTTCTTCCATTTT * N S K S S S T Q S F N F S P L	1800
1801	ttttcttccaaccagcagctatatcactagctgacccacctatagagaatcataggtagtagtaggtgctcctgcttttagtagccaga AAAAGAAGGTTGGTCGTCGATATAGTATCGACTGGGTGGATATCTCTTAGTATCCATCATCACCAGGAGACGAAATCATCGGGTCT I K K W G A A I D S A S G G I S F D Y T T T P A G A K T A W	1890
1891	agctagtcggtgtttctcgcgccaaggttgacttcttgagcttaccatttgctagtttaactgtgactttccctggtttttctcctgtag TCGATCAGCAACAAGAGCGGTTCCAACCTGAAGAACTCAGAATGGTAAACGATCAAATTGACACTGAAAGGACCAAAAAGAGGACATC F S T T T E G G L N V E Q T K G N A L K V T V K G P K E G T	1980
1981	aaaccaatactttacttttagtaacatttgatcagttattggatcagtaaatttttgtccagggccaaaatggtttggatcagctgcat TTTGGTTATGAAATGAAATCATTTGTAACCTAGTCATAACCTAGTCATTTAAAAACAGGTCCCGGTTTTCACAACTAGTCGACGTA S V L V K S K T V N P D T N P D T F K Q G P G F I N P D A A	2070
2071	taatcgcatataaccaattgtgcaataataag ATTAGCGTAATTGGTTAACACGTTATATTC N I A N V L Q A I Y <--- orf 2	2100

Fig. 2. Nucleotide sequence of 2.1 kb of the lactococcal insert of pF1872. The upper strand contains the coding sequence of *pepA* (nt 643–1710) and the lower strand those of two incomplete ORFs. The deduced amino acid sequences are given below the nucleotide sequence. Six base pairs upstream of the putative *pepA* ATG start codon is a putative ribosome-binding site GGAGG (underlined); sequences that resemble promoter –10 and –35 elements (van de Guchte *et al.*, 1992) are underlined and in upper-case letters. An inverted repeat stretching from nt 1717 to nt 1753 with a ΔG value of $-21.4 \text{ kcal mol}^{-1}$ ($-89.5 \text{ kJ mol}^{-1}$) may act as a transcription terminator for both *pepA* and ORF2. A number of relevant restriction sites are indicated. The N-terminal 25 amino acids of PepA (whose sequence was also determined experimentally) are also underlined.

nt 1710, giving it a total length of 1065 bp. It has the potential to specify a protein of 38.1 kDa. The N-terminal 25 amino acids of the translated sequence of ORF 1 were identical to those determined experimentally from purified PepA (underlined in Fig. 2). There was a potential Shine–Dalgarno sequence (Shine & Dalgarno, 1974) at nt 632–636 and putative lactococcal promoter sequences (van de Guchte *et al.*, 1992) at nt 572–577 and 550–555. The incomplete ORF downstream of *pepA* (ORF 2) has a TAA stop codon at nt 1753. The region between these two ORFs from nucleotides 1717 to 1753 has the potential to form a stem-loop of 16 bp (containing four G–C pairs and no mismatches). It is possible that this could act as a

bidirectional transcription terminator for both ORFs. There was also a third, incomplete ORF (ORF 3) upstream of *pepA* with an ATG start codon at nt 211. Analysis of all three putative ORFs (using the program CODONPREFERENCE) with lactococcal codon usage tables (van de Guchte, 1991), predicted that these ORFs are likely to be efficiently expressed in *L. lactis*.

The purified PepA that provided the N-terminal sequence data presented in this paper was isolated from an intracellular extract (Niven, 1991). However, the initial characterization of the enzyme (Exterkate & de Veer, 1987) indicated that it was a membrane-bound enzyme.

Endo	M E E E D M L L	K K F G V G V	N E E I E A I	I E E I K C V D E	40
Ecu	. . . M N I E L L	Q Q C A S V	D E Q E F I	I N T L E P C V N E	36
Gap	. . . M E L K V	K A Q T	F F G P Y	K A R M V L G Y Q	37
Consensus	- - - - -	- - - - -	- - - - -	- - - - -	40
Endo	I K V T N L I	A V K G . . . G	K K . . .	I V T Y I D	77
Ecu	I T S F V	A R G N F . . . G	K V A V G	I V T H I D	73
Gap	P E G I F	V T K A S V E N A	R . . .	I V T S S I K	77
Consensus	- - - - -	- - - - -	- - - - -	- - - - -	80
Endo	D K F L F S A V	V S R Y D C I	V K F K N . . .	. . . G V V A V	110
Ecu	E S F L F F T T I	G G W N Q S M L N	H V I R H K	V G V I S V	113
Gap	A D T F V V P L	G G N P L V V S	F F L F R T	K V V T G L	117
Consensus	- - - - -	- - - - -	- - - - -	- - - - -	120
Endo	Y Y E E K L E D M K	N L Q L . S . . K M	Y I I G A R S R E	L K M . V N I	146
Ecu	A A T E K Q K	Q Q P L . S F D E M	F I I G A N S R E	E V E K R V E I	152
Gap	P L R G T G V	T P I P A I S D I	I F G A F E N A A	E A E F I A Q	157
Consensus	- - - - -	- - - - -	- - - - -	- - - - -	160
Endo	V A C F V G D . A	V L Q G D T . V	K A L D N F S G A	V V V K A I K E K	184
Ecu	N F S A N F A	C W G E D . V V G	K A L D N F I G A	M A Q T V N	191
Gap	L I T E T I	L S A N G N I	K A W D N F Y G L	I L E F A	197
Consensus	- - - - -	- - - - -	- - - - -	- - - - -	200
Endo	K T D . . N E I Y F	V F T E E V I	E G A T A A F S I	K I I A I I V	222
Ecu	N P . . I Y G	V G S E E E V I	E G A Q T A E H I	K P I V V I V L T	229
Gap	D K L P V I I	G A N E E E V I	E G A V T T K F	N P I L F F I C	237
Consensus	- - - - -	- - - - -	- - - - -	- - - - -	240
Endo	T M T G P E S H	P M E . . V K C G	P A I K V K R S	V C H E V R K L	260
Ecu	A V G I V P I	N I K Y P L K Q	G P G M L D K R	Y F P N Q K L V A A	269
Gap	S P S F D D	N . . . G R D E	T T R F D P G	H M L G M K N F	273
Consensus	- - - - -	- - - - -	- - - - -	- - - - -	280
Endo	E E S A K R N N I	P Y V L E I L E A	S P S I S T	A G G I F G A S	300
Ecu	L K S C A A N D L	P L Q F S T K T	A D G G R Y N V M	G G G R F V V A L C	309
Gap	L L D T A N A K V	K T Q V . Y A K	D A G A A A	N G G V F T T G	312
Consensus	- - - - -	- - - - -	- - - - -	- - - - -	320
Endo	I P V R V H P V	E T A S M S L I N N	A V K L	 V E A	329
Ecu	L P T R Y L H A N S	G M I S K A D Y E	L L L I G F L	T T A M N A F	349
Gap	V V A R Y I H H Q	T I F N I D D F L	Q . F A I I	S N T M A E	351
Consensus	- - - - -	- - - - -	- - - - -	- - - - -	360
Endo	C *				330
Ecu	S Q F R Q V D				356
Gap	K N Y				354
Consensus	- - - - -				367

Fig. 3. Comparison of the deduced PepA amino acid sequence (Gap [glutamyl aminopeptidase]) with that of an endoglucanase from *Clostridium thermocellum* (Endo) and the translation of an ORF from the *E. coli* chromosome from 87.2 to 89.2 min (Ecu). Identical amino acids in all three of the aligned sequences are boxed in black and those identical to PepA in either one or the other sequences in dark grey. Spaces indicated by dots were introduced in order to obtain maximum alignment.

More recent immunohistochemical localization studies (Baankreis, 1992) also suggested a peripheral/extracellular location for PepA, although cellular fractionation experiments in the same study showed that PepA activity was largely associated with the cytosolic

fraction, with some activity (higher than found with other intracellular peptidases) associated with the solubilized cell wall fraction. The *pepA* gene sequence has no recognized N-terminal signal sequences, and hydrophobicity plots (Kyte & Doolittle, 1982) of the PepA

Fig. 4. (a) Schematic representation of Campbell-like integration of pFI950 into the chromosomal *pepA* gene in *L. lactis* MG4695 and (b) Southern hybridization of chromosomal DNA from the parental strain MG4695 and the integrant(s) FI8362, FI8364 and FI8365 digested with *PvuII*. The truncated *pepA* from pFI950 was used as a probe. The sizes of the expected hybridizing restriction fragments are indicated in (a). Lanes: 1, 100 bp ladder; 2, FI8362 digested with *PvuII*; 3, FI8364 digested with *PvuII*; 4, FI8365 digested with *PvuII*; 5, MG1363 digested with *PvuII*; 6, MG4695 digested with *PvuII*; 7, phage lambda DNA digested with *HindIII*.

protein (not shown) indicated no potential hydrophobic transmembrane domains. These findings suggest that PepA is an intracellular peptidase. This conclusion is supported by a recent study which showed that the lactococcal extracellular cell wall proteinase can, from a growth medium where β -casein (supplemented with the leucine and histidine) is the only nitrogen source, provide sufficient transportable peptides to support maximal growth, no extracellular peptidases being required (Kunji *et al.*, 1995). Indeed all lactococcal peptidases that have been isolated thus far are intracellular, leading to the conclusion that *L. lactis* does not require any extracellular peptidases (Pritchard & Coolbear, 1993).

Homology comparisons

The predicted amino acid sequence from ORF 1 (*pepA*) was compared to the translated DNA sequences in the EMBL database using the program TFASTA (Pearson & Lipman, 1988). The analysis showed that the *pepA* gene product (PepA) has 30.3% identity, in a 294 amino acid overlap, with an endoglucanase from *Clostridium thermocellum* (Romaniec *et al.*, 1992). It also has 32.8% identity, in a 357 amino acid overlap, with the gene product from a region of the *E. coli* chromosome from 87.2 to 89.2 min which specifies an ORF of unknown function (Plunkett *et al.*, 1993) (Fig. 3).

Construction of PepA-deficient lactococcal strains

To investigate whether PepA is essential for the growth of *L. lactis* in milk, the *pepA* gene was inactivated. The suicide vector pFI950 is based on the plasmid pCRII (see Methods). It has an internal, truncated region of the *pepA*

gene (0.76 kb, generated by PCR) which lacks both the first 136 bases and the last 111 bases of *pepA* cloned into the *EcoRI* site of pCRII. In addition to this, an erythromycin resistance gene was cloned into the *BamHI* site in the multiple cloning site of pCRII. Insertional inactivation of *pepA* was achieved by transformation of *L. lactis* MG4695 (a derivative of MG1363 carrying the *Lac⁺ Prt⁺* plasmid pLP712: Gasson, 1983) with pFI950. This plasmid has no Gram-positive origin of replication and selection for erythromycin-resistant transformants facilitated integration of pFI950 into the *pepA* gene in the chromosome of MG4695 by Campbell-like single cross-over recombination. This resulted in the presence of two incomplete copies of the *pepA* gene, one lacking the first 136 bases and the other lacking the last 111 bases (Fig. 4a).

Erythromycin-resistant colonies were analysed by Southern hybridization for integration of pFI950 and interruption of the *pepA* gene. A *pepA* probe was hybridized with *PvuII*-digested chromosomal DNA from erythromycin-resistant colonies. Strains containing the integrated plasmid were predicted to contain two fragments (1.64 kb and 2.5 kb) which would hybridize with the probe; wild-type strains would have only one band (1.98 kb) capable of hybridization. Strains FI8362, FI8364 and FI8365 contained the two hybridizing fragments that were expected, indicating that integration of pFI950 into *pepA* had indeed occurred in these strains (Fig. 4b).

Cell extracts were prepared from the isolated clones and assayed for PepA activity. The three strains (FI8362, FI8364 and FI8365) were all deficient in PepA activity (data not shown). Their ability to grow in milk was examined. The PepA deficiency in all three mutants isolated resulted in a delay in the onset of the exponential

Fig. 5. Growth of the *pepA* mutant F18362 in milk, as shown by acidification rates (a) and by OD₆₀₀ measurements (b). ▲, Mutant F18362 (proteinase positive, PepA negative); ●, MG1363 (proteinase negative, PepA positive); ■, MG4695 (proteinase positive, PepA positive).

phase of growth (shown for F18362 in Fig. 5). Although the delay was only slight it was reproducible across three separate cultures; each time point was the mean of three readings from each culture. At 3.5 h there was no significant difference but between 4 and 9 h the difference was significant at the 99% confidence level. At 10.5 h this dropped to 95% confidence, and then the difference became insignificant. Thus the difference is highly significant throughout the exponential phase and probably arises from a longer lag phase in the mutant strains. Variation in the lag phase as a consequence of environmental history is an established concept in microbial growth modelling (Baranyi *et al.*, 1995) and it is conceivable that loss of a peptidase has a similar effect. As has been found with other peptidases reported thus far (Mierau *et al.*, 1993a), PepA was also not an essential peptidase for ultimate growth of *L. lactis* in milk. This is, perhaps, not unexpected as lactococci contain at least three other intracellular peptidases, namely a dipeptidase (Hwang *et al.*, 1981), a tripeptidase (Bosman *et al.*, 1990) and PepC (Neviani *et al.*, 1989), that are reported to hydrolyse glutamate-containing peptides or chromogenic substrates. Therefore, it may be anticipated that these enzymes would be able to substitute for PepA in the deficient strains, although the efficiency may be reduced, resulting in the delay in early growth. As the transport of free glutamate is pH dependent in lactococci, with increased translocation as acidification occurs (Poolman *et al.*, 1987), it is likely that greater emphasis may be placed on peptides, either from the non-protein nitrogen or as the result of proteolysis of milk proteins, supplying essential glutamate at the higher pH found in the early growth phase, for which the glutamyl-cleaving peptidases would then be required. It may, therefore, be anticipated that any effect of a *pepA* deletion supplying essential glutamate would be more prominent in the early phase of growth.

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