Lactobacilli carry cryptic genes encoding peptidase-related proteins: characterization of a prolidase gene (*pepQ*) and a related cryptic gene (*orfZ*) from *Lactobacillus delbrueckii* subsp. *bulgaricus*

Terhi Rantanen^{1,2} and Airi Palva¹

Author for correspondence: Airi Palva. Tel: +358 3 4188 277. Fax: +358 3 4188444.
e-mail: airi.palva@mtt.fi

¹ Agricultural Research
Centre of Finland, Food
Research Institute,
Jokioinen 31600, Finland

² University of Turku,
Department of
Biochemistry and Food
Chemistry, Turku 20014,
Finland

Two genes, *pepQ* and *orfZ*, encoding a prolidase and a prolidase-like protein, respectively, were cloned and characterized from *Lactobacillus delbrueckii* subsp. *bulgaricus*. The identity of the *pepQ* and *orfZ* genes with the *Lactobacillus delbrueckii* subsp. *lactis* prolidase gene (*pepQ*) was shown to be 98% and 60%, respectively. Both *pepQ* and *orfZ* were preceded by a putative promoter region. Northern analysis of *pepQ* mRNA revealed a 1.1 kb transcript indicating that *pepQ* forms a monocistronic transcriptional unit. Under the growth conditions used, no evidence was obtained that *orfZ* was expressed, either by mRNA size determination in Northern analysis or by primer extension analysis. With reverse transcription-PCR, however, the presence of monocistronic *orfZ* transcripts was established. The *orfZ* gene could also be overexpressed in *E. coli* using the vector pKK223-3. The size of the protein synthesized, 41 kDa, confirmed the molecular mass of OrfZ calculated according to DNA sequence analysis. In contrast to *PepQ*, which showed a substrate specificity characteristic of prolidase enzymes, no enzymic activity for the *orfZ*-encoded protein was found with the peptide substrates tested. These results indicate that *orfZ* is a cryptic gene, which is expressed at a very low level under the growth conditions used. It is noteworthy that homologues of the *Lb. delbrueckii* subsp. *bulgaricus* *orfZ* and *pepQ* genes appeared to be present in both *Lb. delbrueckii* subsp. *lactis* and *Lactobacillus helveticus*.

Keywords: *Lactobacillus delbrueckii* subsp. *bulgaricus*, prolidase, peptidase, *pepQ*

INTRODUCTION

Lactic acid bacteria (LAB) have a limited capacity to synthesize amino acids and are, therefore, dependent on exogenous nitrogen sources for optimal growth. When growing in milk, LAB utilize complex proteolytic systems for the degradation of milk proteins. The biochemical and genetic characterization of these systems from lactococci and lactobacilli have been

investigated in a number of laboratories (Kok, 1990; Pritchard & Coolbear, 1993; Tan *et al.*, 1993; Visser, 1993; Kok & De Vos, 1994), and shown to comprise a cell-envelope-associated proteinase, membrane-bound transport systems and several cytoplasmic peptidases. The manufacture of fermented food by LAB would benefit from a more detailed understanding of proteolysis. In cheese manufacturing, the proteolytic activity of LAB contributes to the ripening process and is known to be essential for the formation of flavour and texture. Peptidases are considered to be particularly important for flavour development. The milk proteins contain a high amount of proline residues which, in free form, give a sweet flavour to cheese (Biede & Hammond, 1979; Fox, 1989). Thus, proline-specific peptidases play

Abbreviation: RT, reverse transcription.

The EMBL accession numbers for the nucleotide sequences of the *Lactobacillus delbrueckii* subsp. *bulgaricus* *orfZ* and *pepQ* genes reported in this paper are Z93945 and Z93944, respectively.

a very important role in cheese ripening by degrading proline-containing peptides, which often are bitter, and by making peptides accessible to the action of other peptidases by removal of proline residues (Baankreis & Exterkate, 1991).

Several proline-specific peptidases with distinct substrate specificities have been found in LAB. To date prolinase, prolidase, proline iminopeptidase, aminopeptidase P and X-prolyl dipeptidyl aminopeptidase activities have been detected and genes responsible for these activities characterized (Fernández-Esplá *et al.*, 1997; Kunji *et al.*, 1996). In this work, we describe two new genes, *pepQ* and *orfZ*, from *Lactobacillus delbrueckii* subsp. *bulgaricus* B14. The *pepQ* gene encodes prolidase activity, whereas *orfZ*, even though showing high homology to *pepQ*-like genes, was found to have a cryptic function under the experimental conditions used.

METHODS

Bacterial strains, plasmids and culture conditions. *Lb. delbrueckii* subsp. *bulgaricus* B14 (Federal Dairy Research Centre, Kiel, Germany) was grown in MRS broth (Difco) at 42 °C. For pH-controlled cultivation, a Biostat B (B. Braun Biotech International) bioreactor was used. The pH was maintained at 6.0 by addition of 1 M aqueous ammonia solution with slow agitation. *Lactobacillus helveticus* 53/7 (Valio) was grown in milk or MRS at 42 °C. *Lactobacillus delbrueckii* subsp. *lactis* DSM 20072 (DSMZ) was grown in MRS at 37 °C. *Escherichia coli* strains DH5 α F' (Woodcock *et al.*, 1989), JM105 (Pharmacia) and NM514 (Amersham) were grown in Luria broth (L broth) and *E. coli* strain CM89 (Miller & Schwartz, 1978) in L broth supplemented with 0.3 mM thymine and 0.05 mM thiamin. Erythromycin (0.3 mg ml⁻¹) and ampicillin (0.05 mg ml⁻¹) were added when using the vectors pJDC9 (Chen & Morrison, 1987) and pKK223-3 (Pharmacia), respectively, in *E. coli*.

DNA isolation, synthesis and transformation methods. *Lb. delbrueckii* and *Lb. helveticus* chromosomal DNA was isolated as described by Vidgren *et al.* (1992) without a guanidine hydrochloride treatment. Phage DNA was isolated according to the instructions of the cDNA rapid cloning module (λ gt10; Amersham). Plasmid DNA was isolated from *E. coli* by alkaline lysis with the Wizard (Promega) and FlexiPrep (Pharmacia) DNA purification systems. Oligonucleotides were synthesized with an Applied Biosystems DNA/RNA synthesizer model 392 and purified by ethanol precipitation or with NAP-10 columns (Pharmacia Biotech). For DNA synthesis, performed by PCR, the Dynazyme DNA polymerase (Finnzymes) was used under the reaction conditions recommended by the manufacturer. Other DNA techniques were as described by Sambrook *et al.* (1989). *E. coli* strains were transformed by electroporation using a Bio-Rad Gene Pulser.

Cloning of the *Lb. delbrueckii* subsp. *bulgaricus* *orfZ* and *pepQ* genes and Southern analyses. An *Lb. delbrueckii* subsp. *bulgaricus* genomic library was constructed in λ gt10 using λ DNA *in vitro* packaging and cDNA rapid cloning modules (λ gt10; Amersham). Instead of the Amersham cDNA rapid ligation module, a RiboClone *Eco*RI Linker Ligation System (Promega) was employed. During a screening of the *Lb. delbrueckii* subsp. *bulgaricus* library for the operon of the

oligopeptide transport system, a hybridization-positive clone was found showing DNA homology to the *Lb. delbrueckii* subsp. *lactis* DSM 7290 prolidase gene (*pepQ*) (Stucky *et al.*, 1995). For further elucidation and cloning of the missing region of this *pepQ*-like gene, called here *orfZ*, a Southern blot analysis of the *Lb. delbrueckii* subsp. *bulgaricus* chromosomal DNA was performed using a *orfZ*-specific 0.5 kb fragment amplified by PCR as a hybridization probe. The primer pair used for PCR was 5' TTTGACTTGGGGATCATG 3' and 5' TTGGGTATGACCAAATAGC 3', and the λ clone, carrying part of the *orfZ* gene, served as the template. The hybridization-positive fragment pool was cloned in *E. coli* using pJDC9 followed by screening of the *orfZ*-specific transformants.

For cloning of *pepQ*, the upstream and coding regions of the *pepQ* gene were cloned in pJDC9 as a PCR product synthesized with the primer 5' TTGTGCAATCGCTTACAG 3' (p290), designed according to the upstream region of *Lb. delbrueckii* subsp. *lactis* *pepQ* (Stucky *et al.*, 1995) and with the *Lb. delbrueckii* subsp. *bulgaricus* *pepQ*-specific primer 5' TTTGGCTGCACCGTGTTTC 3' (p288, which was derived from a sequenced PCR fragment generated with *Lb. delbrueckii* subsp. *lactis*-specific primers from the chromosomal DNA of *Lb. delbrueckii* subsp. *bulgaricus*). The missing 3' end and the downstream region of *pepQ* was identified by Southern hybridization using the above *pepQ* PCR fragment as the hybridization probe. The resulting hybridization positive 1.3 kb *Hind*III–*Sal*I fragment pool was ligated with pJDC9 and the ligation mixture was directly used as the template in PCR with the *pepQ*-specific primer 5' ACACG-GTGCAGCCAAATG 3' (p287) and a biotinylated vector-specific primer. After PCR sequencing of the 3' end obtained, the entire *pepQ* was synthesized by PCR with the specific primers designed for the upstream and downstream regions of the gene (p290 and 5' ACGATTCAGCTAACGGCG 3', p318) and cloned in pJDC9.

The probes for Southern blots were labelled with digoxigenin-dUTP (DIG, Boehringer Mannheim). Hybridizations were performed under high stringency conditions and when necessary with different stringencies (68 °C, 60 °C and 50 °C overnight, followed by washes with 0.5 $\times$ SSC, 0.1% SDS at 68 °C, 55 °C and 45 °C). A DIG Luminescent Detection Kit (Boehringer Mannheim) was used for hybrid detection.

DNA sequencing and sequence analysis. Sequencing was performed on an A.L.F. DNA Sequencer (Pharmacia). The dideoxy sequencing reactions (Sanger *et al.*, 1977) were performed according to the AutoRead Sequencing Kit manual (Pharmacia). Both DNA strands were sequenced using pUC19-specific primers and sequence-specific oligonucleotides for primer walking. DNA sequences were assembled and analysed with the PC/GENE programs (release 6.85; IntelliGenetics). For homology searches, the databases of EMBL and SWISS-PROT were used both as a CD-ROM version (release 15.0, June 1995) and directly by e-mail with the EMBL BLITZ server.

RNA methods. Total RNA from different phases of growth was isolated from *Lb. delbrueckii* subsp. *bulgaricus* cells essentially as described by Palva *et al.* (1988) and Vesanto *et al.* (1994). RNA gel electrophoresis and Northern blots were performed as described previously (Hames & Higgins, 1985). Hybridization probes were labelled with [α -³²P]dCTP [>3000 Ci mmol⁻¹ (111 TBq mmol⁻¹); Amersham] with a random-primed DNA labelling kit (Amersham) or with nonradioactive DIG. The primer extension of *orfZ* was performed with total RNA using an A.L.F. DNA Sequencer essentially as described by Myöhänen & Wahlfors (1993) and

Vesanto *et al.* (1995) with 10 pmol of the oligonucleotide 5' TTCATTGATCGCCGGAGC 3' labelled with fluorescein.

Reverse transcription (RT)-PCR of *orfZ* mRNA was performed as follows. Two cDNA preparations were synthesized by RT (AMV reverse transcriptase, Promega) using 1.5 µg total *Lb. delbrueckii* subsp. *bulgaricus* RNA. The oligonucleotides used in the first-strand synthesis were 5' TTCCCGGCTCCAGATTTCAC 3' (p459) and 5' AGCAGCAAAGCCATCTTACTG 3' (p174), being complementary to the *orfZ*-specific mRNA derived from ORF2 and its downstream region (ORF3), respectively. These cDNA samples were phenol-extracted, precipitated and dissolved in 20 µl H₂O. Amplifications by PCR were performed with 3 µl cDNA samples. The oligonucleotides 5' ATGTGGCAGAGCCAGTGGAC 3' (p458) and 5' CAAGTGGGTGATTATCAGC 3' (p234), binding to *orfZ* (ORF2) and the upstream region of ORF2, respectively, were used with the *orfZ*-specific primer 459. A PCR reaction was also performed with the *orfZ* primer 458 and the downstream ORF3 primer 174. As a control for the absence of contaminating DNA, 5 µg total RNA was directly amplified by PCR using the primer pair 458/459.

Peptidase activity assays. Peptidase activity was determined in liquid cultures of *E. coli* by a coupled enzyme reaction containing peroxidase, L-amino-acid oxidase and o-dianisidine (Wohlrab & Bockelmann, 1992). Cells were disrupted with an Ultrasonic 2000 sonicator (Braun). The reaction volume was 1 ml and samples were incubated at 37 °C for 30–60 min. For characterization studies, enzyme activity was determined by two alternative methods: the iminopeptidase (proline-liberating) activity was determined using the modified method of Troll & Lindsley (1955) as described by Baankreis & Exterkate (1991). The hydrolysis of peptides not containing proline as the amino-terminal residue was assayed by measuring the release of α-amino acids by the modified cadmium-ninhydrin method described by Doi *et al.* (1981). All activity assays were performed with at least two parallel samples.

Construction of an overexpression system for the *Lb. delbrueckii* subsp. *bulgaricus* *orfZ*. For overexpression in *E. coli*, the *orfZ* gene was synthesized by PCR using primers 243 and 5' AACAAAGATGTCTCCAAGCCTG 3' (p179). The 1.7 kb PCR product was digested and ligated as a 1.5 kb *EcoRI*–*HindIII* fragment into the pKK223-3 vector and transferred into the *E. coli* JM105 host by electroporation. Expression of *orfZ* was characterized by growing the cells to a density of 50 Klett units (filter 66) followed by 1 mM IPTG induction and estimation of product accumulation over 5 h by SDS-PAGE.

RESULTS AND DISCUSSION

Cloning and sequencing of the *orfZ* region from *Lb. delbrueckii* subsp. *bulgaricus*

The 3' end of a gene, here referred to as *orfZ*, showing significant homology to the *Lb. delbrueckii* subsp. *lactis* prolidase gene (*pepQ*) (Stucky *et al.*, 1995) was found during cloning of genes of the oligopeptide transport system from an *Lb. delbrueckii* subsp. *bulgaricus* λ library. The sequenced part of *orfZ* encoded 138 amino acids and was found to show an identity of 65% at the amino acid level with the *Lb. delbrueckii* subsp. *lactis* *pepQ*. However, the expected identity of *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis*

prolidases should be higher considering the close relatedness of these two subspecies. This suggested that the *orfZ* gene product could be a novel prolidase. To localize and clone the entire *orfZ* gene, an *orfZ*-specific probe was synthesized by PCR for Southern analysis of the *Lb. delbrueckii* subsp. *bulgaricus* chromosomal DNA. A hybridization-positive 3.2 kb *Bam*HI–*Hind*III fragment was found, cloned into pJDC9 and designated pKTH2141, followed by DNA sequencing of the insert. The DNA sequence of the 3.2 kb insert revealed three open reading frames (ORF1, ORF2 and ORF3) of 735, 1119 and 885 bp, respectively. ORF2, hybridizing with the *orfZ* probe, had a capacity to encode a protein of 373 amino acids with a calculated molecular mass of 41.4 kDa. A putative promoter region (TTGGAT-16 nt-TATGAT) and a ribosome-binding site (gGGAGtg) were found 96 and 7 nucleotides upstream of the start codon (ATG) (Fig. 1a), respectively. An inverted repeat structure with a ΔG of –23.5 kJ mol^{–1} (Tinoco *et al.*, 1973) was detected 23 nucleotides downstream of the stop codon, representing a putative weak transcription terminator.

The predicted amino acid sequence of ORF2/*orfZ* showed 62% similarity with the *PepQ* of *Lb. delbrueckii* subsp. *lactis* DSM 7290 (Fig. 1b). At the nucleic acid level the corresponding identity was 60%. Since the identity of two proline iminopeptidases, characterized earlier from *Lb. delbrueckii* subsp. *bulgaricus* CNRZ 397 (Gilbert *et al.*, 1994) and *Lb. delbrueckii* subsp. *lactis* DSM 7290 (Klein *et al.*, 1994), is 98%, the result obtained here suggests that *orfZ* is not indeed the counterpart of the *Lb. delbrueckii* subsp. *lactis* *pepQ*. Further analysis of the putative *orfZ*-encoded protein with the PC/GENE set of programs revealed that the protein would not possess any transmembrane or membrane-associated helices or hydrophobic segments likely to be part of a signal peptide, thus suggesting that *OrfZ* would be a cytoplasmic protein.

Both ORF1 and ORF3 were found to be preceded by a putative promoter sequence upstream of the respective start codons. However, their deduced amino acid sequences did not show any significant similarity with other proteins found in the databases.

Sequencing of the *pepQ* gene from *Lb. delbrueckii* subsp. *bulgaricus*

For comparison of the *Lb. delbrueckii* subsp. *bulgaricus* prolidases, the expected counterpart of the *Lb. delbrueckii* subsp. *lactis* *pepQ* gene was cloned (see Methods) from *Lb. delbrueckii* subsp. *bulgaricus* by taking advantage of the published sequence of the *Lb. delbrueckii* subsp. *lactis* *pepQ* gene (Stucky *et al.*, 1995).

The plasmid clones obtained (pKTH2145, pKTH2146 and pKTH2147) were sequenced; *pepQ* was shown to be 1104 bp in length and to encode a protein of 368 amino acids with a calculated molecular mass of 41 kDa. A putative promoter had two identical –35 regions (TTTTCa) located 13 and 23 nucleotides upstream of

(a)

```

-35          -10
AGAATGATTTTTTGGATTGTGATATAAAATATATTATGATCTAGTGGCTTTGCCTAGCTAATTCATTAATAAA - 70

                                     RBS
AGCCTAGAAAAAGGAAGAAATATGAAAAATAGAGCAAGAACACTTTCTTGGGGAGTGAAAAAGCATGAAA - 140
                                     M K

-----
TTTTGGAGGAACAGTTAAAAAGTGAGCTATTGGTCTATACCAACCAACTTTTGTGTTTGAGCATGACCT - 1330
L          ----->          <-----

```

(b)

```

bulgpepz  MKEERKLTWEEHLAALTSTYLDEKGI DLAYVAEPVDIAIYLTGYSSWTEERVALLVSR TG - 59
bulgpepq  M-----NLDKLQNLQNGEMGVAVVSPTTINYFTGFTIDPEERIFKLF AFKDA - 49

* . . . * . . . * . . . * . . . * . . . * . . . * . . . * . . . * . . .

bulgpepz  QALLLAPAINEGEVKQTSWADRACFYQDGENPWEKAKKRMTAWPKGF--WALEARAVTL - 116
bulgpepq  EPFLFCFALNYEEAKASAWDGDVVGVLDSEDPWGKIAEEIHQSKDYQNNAWAVEKGLGTV - 108
          . . . * . . . * . . . * . . . * . . . * . . . * . . . * . . .

bulgpepz  SHYRAI KEVFPEANLASDISTWLARQRMIKSPAIEIKLQAAGRLAQDALDLAFALKKAG - 175
bulgpepq  AHYQALHAQFPDSDFSKDLSDFTIAHIRLFTKESELVKLRKAGEEADFQIGFEALR-- - 165
          . . . * . . . * . . . * . . . * . . . * . . . * . . . * . . .

bulgpepz  QGMSESELALELDYLK--KKGMGDLSPFLIVQAGESAASISGLPSQKGQVQAGDIVFDL - 233
bulgpepq  NGVTERAVVSQIEYQLKLQKQVMQTSFDTIVQAGKNAANPHQGQPSMNTVQPNELVFLFDL - 224
          . . . * . . . . . . . . . . . . . . . . . . . . . . . . . . .

bulgpepz  GIMKDGYSADVSRVTALVEI SPAKWEIYETVRLAQETAARAARPGMTAGELDQVARGI - 292
bulgpepq  GTMHGYASDSSRTVAYGEPTDKMRIEYVNRTAQQAADAAPGMTASELDGVARKII - 283
          . . . * . . . * . . . * . . . * . . . * . . . * . . . * . . .

bulgpepz  EEAGYGYQFYTHRLGHGIGMQVHEPVLNLEPGSKQKLEAGMCFSIIEPGIYLPVGGVVRIED - 351
bulgpepq  TDAGYGEYFIHRLGHGIGMEVHEPFSIANGDVVLEEGMCFSIIEPGIYIPGFAGVRIED - 342
          . . . * . . . * . . . * . . . * . . . * . . . * . . . * . . .

bulgpepz  CGWLGEDGFHPFTTKRKL D L L L L --- - 373
bulgpepq  CGVLTKGFKPPTHTSKELKVLVPKE - 368
          * . . . * . . . * . . . * . . .

```

Fig. 1. Partial nucleotide sequence of the *Lb. delbrueckii* subsp. *bulgaricus* *orfZ* gene and alignment of the deduced amino acid sequences of the *Lb. delbrueckii* subsp. *bulgaricus* OrfZ and PepQ. (a) The predicted -35 and -10 regions of the putative promoter and stop codon of the *orfZ* gene are shown in bold type. RBS indicates the putative ribosome-binding site. The deduced transcription terminator is marked with dashed arrows. (b) Identical and similar amino acids in the OrfZ (bulgpepz) and PepQ (bulgpepq) proteins are indicated by asterisks and dots, respectively. The four amino acids which differed between *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis* PepQ are shown in bold and boxed. The conserved amino acid residues of the putative catalytic site with the zinc-binding domains of metalloproteases are underlined (Jongeneel et al., 1989).

the putative -10 region (TATACT). The ribosome-binding site (AGGAGaA) was found seven nucleotides upstream of the start codon ATG. An inverted repeat structure with ΔG of $-71,1 \text{ kJ mol}^{-1}$ was detected 13 nucleotides downstream of the stop codon, representing the putative transcription terminator. At the nucleic acid level, the *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *lactis pepQ* genes showed an identity of 98% and only four amino acids differed in the predicted amino acid sequences of the two PepQ proteins (Fig. 1b). Two of the amino acid changes were between similar amino acids: Glu-34 and Ser-93 in the *Lb. delbrueckii* subsp. *bulgaricus* PepQ were replaced by Asp and Thr in the *Lb. delbrueckii* subsp. *lactis* PepQ, respectively. In addition, Gly-83 and Gln-91 in the *Lb. delbrueckii* subsp. *bulgaricus* PepQ were replaced by Ser and Lys, respectively, in the *Lb. delbrueckii* subsp. *lactis* PepQ. The upstream regions of these two *pepQ* genes were also very similar, containing only two different nucleotides over the region extending 91 bp upstream of ATG. In contrast, the downstream regions were found to be quite different, with the 93 bp region downstream of the stop codon showing only 42% identity.

mRNA analyses

Several attempts were made to determine the size of *Lb. delbrueckii* subsp. *bulgaricus* *orfZ* mRNA by Northern blot analysis of samples taken at different phases of growth. The *orfZ*-specific probe used was labelled either with digoxigenin or with the more sensitive radioactive [α - 32 P]dCTP. No *orfZ*-derived transcripts could be

detected, even though RNA samples of up to 100 µg were tested (data not shown). However, the presence of *orfZ*-specific mRNA could be identified by RT-PCR (data not shown). The only PCR product detected was with internal *orfZ*-specific primers (p458 and p459, see Methods). The primer pair used was *orfZ*-specific and did not give a PCR product with the isolated *pepQ* plasmid pKTH2147. No PCR products were amplified by RT-PCR using primers based on sequences located external to *orfZ* (data not shown). The absence of contaminating DNA in the RNA preparations was also confirmed. These results indicate that *orfZ* is transcribed as a monocistronic unit. However, the 5' end mapping by primer extension and a subsequent analysis by an A.L.F. DNA Sequencer failed to detect *orfZ* transcripts (data not shown), further confirming that the expression level of *orfZ* was extremely low under the growth conditions employed. Instead, the expression of *pepQ*, and the size of the *pepQ* mRNA was determined as a function of growth from *Lb. delbrueckii* subsp. *bulgaricus* cells by Northern blotting, which showed a 1.1 kb transcript and confirmed that the *pepQ* gene also forms a monocistronic transcriptional unit (Fig. 2). Furthermore, the highest level of *pepQ* transcripts per unit dry weight of cells was obtained at the mid-exponential phase followed by a slow decrease in the relative amount of *pepQ* mRNAs throughout the remainder of the growth phase (Fig. 2).

Characterization of the OrfZ and PepQ proteins

To study the enzymic activity of OrfZ, the *orfZ* gene in pJDC9 was transferred into the peptidase-negative *E. coli* strain CM89, resulting in plasmid pKTH2142.

Fig. 2. Expression of the *Lb. delbrueckii* subsp. *bulgaricus* B14 *pepQ*. (a) Cell density shown as a function of growth. (b) Northern blot analysis of *pepQ* mRNA from samples taken 4, 8, 12 and 16 h after inoculation. For quantification of *pepQ* transcripts, volumes of total RNA were adjusted to equalize the differences in cell dry weights, except for the RNA sample taken at 4 h where an eightfold lower amount of total RNA was available for analysis. The level of *pepQ* transcripts was determined by densitometric quantification. Numbers on the left hand side refer to RNA molecular mass markers (BRL).

However, no enzymic activity against the 19 substrates listed in Table 1 could be demonstrated. To test the functionality of OrfZ, the *orfZ* gene in pKK223-3 was overexpressed in *E. coli* JM105(pKTH2143) and the expected 41 kDa product was obtained (Fig. 3). The overproduced OrfZ in JM105 cell extracts did not, however, hydrolyse the peptide substrates tested, leaving the specificity of OrfZ unelucidated (Table 1). Thus, OrfZ could either require a very specific substrate or it could represent a degenerated form of a prolidase that has lost its original activity. The expression level of *orfZ* is likely to be very low since, in addition to the observed transcript levels, the codon usage of *orfZ*, unlike that of *pepQ*, has a bias typical of poorly expressed genes (Pouwels & Leunissen, 1994). It is also noteworthy that the amino acid sequence homology between OrfZ and

Fig. 3. SDS-PAGE analysis of the *orfZ* gene product in *E. coli*. Lane 1, molecular mass markers; lanes 2 and 3, crude extracts isolated 1 h after IPTG induction from *E. coli* JM105 with the pKK223-3 vector and from *E. coli* JM105 with pKK223-3 carrying the *orfZ* gene (pKTH2143), respectively.

PepQ is substantially lower in the amino-terminal half than that in the carboxy-terminal half. Interestingly, Klein *et al.* (1997) have also recently reported a gene (*orfW*) that is highly homologous to the aminopeptidase genes *pepC* and *pepG*, but does not encode a measurable function.

The enzymic activity of PepQ was studied after cloning of the *pepQ* gene in pJDC9 in *E. coli* CM89 to form pKTH2140. PepQ hydrolysed all dipeptides tested containing proline at the carboxy-terminal end, with the exception of Pro-Pro. The other peptides tested were not degraded (Table 1). This is in accordance with the classification of *pepQ* as an X-Pro dipeptidase gene. Degradation of peptide substrates by the *Lb. delbrueckii* subsp. *lactis* PepQ (Stucky *et al.*, 1995; indicated by an asterisk in Table 1) suggests that the substrate specificity of *Lb. delbrueckii* subsp. *bulgaricus* PepQ is much more stringent than that of *Lb. delbrueckii* subsp. *lactis*. However, this could be due to the differences in the assay systems used for determination of peptidase activities rather than true differences in the specificities of these two enzymes. As with other prolidases characterized, EDTA was strongly inhibitory to *Lb. delbrueckii* subsp. *bulgaricus* PepQ, whereas PMSF had no effect on PepQ activity. The effect of thiol inhibitors on PepQ enzymes has been shown to be in most cases moderate (Booth *et al.*, 1990; Fernández-Esplá *et al.*, 1997;

Table 1. Peptide substrate specificities of *Lb. delbrueckii* subsp. *bulgaricus* PepQ

+ and – indicate hydrolysis and lack of hydrolysis of the substrate tested, respectively.

Substrate	Hydrolysis	Substrate	Hydrolysis	Substrate	Hydrolysis	Substrate	Hydrolysis	Substrate	Hydrolysis
Ala-Pro	+	Leu-Leu	–	Ile-Val	–	Pro-Pro	–	Ile-Pro-Leu	–†
Leu-Pro	+	Met-Leu	–	Leu-Val	–	Pro-Ala	–	Val-Pro-Leu	–
Met-Pro	+	Phe-Leu	–	Phe-Val	–	Pro-Val	–†	Pro-Gly-Gly	–†
Val-Pro	+	Val-Leu	–	Ala-Met	–			Leu-Leu-Leu	–†

* Substrate hydrolysed by the *Lb. delbrueckii* subsp. *lactis* PepQ (all X-Pro substrates are hydrolysed by this enzyme) (Stucky *et al.*, 1995).

† Data for the *Lb. delbrueckii* subsp. *lactis* PepQ not available.

Kaminogawa *et al.*, 1984), whereas an almost complete inhibition of *Lb. delbrueckii* subsp. *bulgaricus* PepQ was obtained with 1 mM *p*-hydroxymercuribenzoate in this study.

Distribution of *orfZ* and *pepQ*

To test the distribution of *orfZ* and *pepQ* in *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. helveticus*, Southern blots with different hybridization stringencies were performed using DIG-labelled *orfZ* and *pepQ* probes. The Southern blot analyses showed that, in addition to *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. helveticus* also possesses both a *pepQ* gene and a *pepQ*-like *orfZ* gene (data not shown). The presence of *orfZ* was also identified in *Lb. delbrueckii* subsp. *lactis* DSM 20072 by PCR (data not shown). These results suggest that *orfZ* may be commonly found in the genus *Lactobacillus*. This might argue in favour of a specific function for OrfZ.

ACKNOWLEDGEMENTS

The authors are grateful to Mrs Anneli Virta for the running of the A.L.F. Sequencer and Ms Jaana Jalava for technical assistance.

REFERENCES

- Baankreis, R. & Exterkate, F. (1991). Characterization of a peptidase from *Lactococcus lactis* ssp. *cremoris* HP that hydrolyses di- and tripeptides containing proline or hydrophobic residues as the aminoterminal amino acid. *Syst Appl Microbiol* **14**, 317–323.
- Biede, S. L. & Hammond, E. G. (1979). Swiss cheese flavour. 1. Chemical analysis. *J Dairy Sci* **62**, 227–237.
- Booth, M., Jennings, V., Fhaolain, I. N. & O'Cuinn, G. (1990). Prolidase activity of *Lactococcus lactis* subsp. *cremoris* AM2: partial purification and characterization. *J Dairy Res* **57**, 245–254.
- Chen, J. & Morrison, D. (1987). Cloning of *Streptococcus pneumoniae* DNA fragments in *Escherichia coli* requires vectors protected by strong transcriptional terminators. *Gene* **55**, 179–187.
- Doi, E., Shibata, D. & Matoba, T. (1981). Modified colorimetric ninhydrin methods for peptidase assay. *Anal Biochem* **118**, 173–184.
- Fernández-Esplá, M. D., Martín-Hernández, M. C. & Fox, P. F. (1997). Purification and characterization of a prolidase from *Lactobacillus casei* subsp. *casei* IFPL 731. *Appl Environ Microbiol* **63**, 314–316.
- Fox, P. (1989). Proteolysis during cheese manufacture and ripening. *J Dairy Sci* **72**, 1379–1400.
- Gilbert, C., Atlan, D., Blanc, B. & Portalier, R. (1994). Proline iminopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397: purification and characterization. *Microbiology* **140**, 537–542.
- Hames, B. & Higgins, S. (1985). *Nucleic Acid Hybridisation: a Practical Approach*. Oxford, Washington, DC: IRL Press.
- Jongeneel, C. V., Bouvier, J. & Bairoch, A. (1989). A unique signature identifies a family of zinc-dependent metallopeptidases. *FEBS Lett* **242**, 211–214.
- Kaminogawa, S., Azuma, N., Hwang, I.-K., Suzuki, Y. & Yamauchi, K. (1984). Isolation and characterization of a prolidase from *Streptococcus cremoris* H61. *Agric Biol Chem* **48**, 3035–3040.
- Klein, J. R., Schmidt, U. & Plapp, R. (1994). Cloning, heterologous expression, and sequencing of a novel proline iminopeptidase gene, *pepI*, from *Lactobacillus delbrueckii* subsp. *lactis* DSM 7290. *Microbiology* **140**, 1133–1139.
- Klein, J. R., Schick, J., Henrich, B. & Plapp, R. (1997). *Lactobacillus delbrueckii* subsp. *lactis* DSM7290 *pepG* gene encodes a novel cysteine aminopeptidase. *Microbiology* **143**, 527–537.
- Kok, J. (1990). Genetics of the proteolytic system of lactic acid bacteria. *FEMS Microbiol Rev* **87**, 15–42.
- Kok, J. & De Vos, W. (1994). The proteolytic system of lactic acid bacteria. In *Genetics and Biotechnology of Lactic Acid Bacteria*, pp. 169–210. Edited by M. Gasson & W. De Vos. Glasgow: Blackie Academic & Professional.
- Kunji, E. R. S., Mierau, I., Hagting, A., Poolman, B. & Konings, W. N. (1996). The proteolytic systems of lactic acid bacteria. *Antonie Leeuwenhoek* **70**, 187–221.
- Miller, C. G. & Schwartz, G. (1978). Peptidase deficient mutants of *Escherichia coli*. *J Bacteriol* **135**, 603–611.
- Myöhänen, S. & Wahlfors, J. (1993). Automated fluorescent primer extension. *BioTechniques* **14**, 16–17.
- Palva, A., Nyberg, K. & Palva, I. (1988). Quantitation of α -amylase mRNA in *Bacillus subtilis* by nucleic acid sandwich hybridization. *DNA* **7**, 135–142.
- Pouwels, P. H. & Leunissen, J. A. M. (1994). Divergence in codon usage of *Lactobacillus* species. *Nucleic Acids Res* **22**, 929–936.
- Pritchard, G. & Coolbear, T. (1993). The physiology and biochemistry of the proteolytic system in lactic acid bacteria. *FEMS Microbiol Rev* **12**, 179–206.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Stucky, K., Klein, J., Schüller, A., Matern, H., Henrich, B. & Plapp, R. (1995). Cloning and DNA sequence analysis of *pepQ*, a prolidase gene from *Lactobacillus delbrueckii* subsp. *lactis* DSM7290 and partial characterization of its product. *Mol Gen Genet* **247**, 494–500.
- Tan, P., Poolman, B. & Konings, W. (1993). Proteolytic enzymes of *Lactococcus lactis*. *J Dairy Res* **60**, 269–286.
- Tinoco, I., Borer, P. N., Dengler, B., Levine, M. D., Uhlenbech, O. C., Crothers, D. M. & Gralla, J. (1973). Improved estimation of secondary structure in ribonucleic acids. *Nature New Biol* **246**, 40–41.
- Troll, W. & Lindsley, J. (1955). A photometric method for the determination of proline. *J Biol Chem* **215**, 655–661.
- Vesanto, E., Varmanen, P., Steele, J. L. & Palva, A. (1994). Characterization and expression of the *Lactobacillus helveticus* *pepC* gene encoding a general aminopeptidase. *Eur J Biochem* **224**, 991–997.
- Vesanto, E., Savijoki, K., Rantanen, T., Steele, J. L. & Palva, A. (1995). An X-prolyl dipeptidyl aminopeptidase (*pepX*) gene from *Lactobacillus helveticus*. *Microbiology* **141**, 3067–3075.

Vidgren, G., Palva, I., Pakkanen, R., Lounatmaa, K. & Palva, A. (1992). S-layer protein gene of *Lactobacillus brevis*: cloning by polymerase chain reaction and determination of the nucleotide sequence. *J Bacteriol* **174**, 7419–7427.

Visser, S. (1993). Proteolytic enzymes and their relation to cheese ripening and flavor: an overview. *J Dairy Sci* **76**, 329–350.

Wohlrab, Y. & Bockelmann, W. (1992). Purification and characterization of a dipeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Int Dairy J* **2**, 345–361.

Woodcock, D., Crowther, P., Doherty, J., Jefferson, S., De Cruz, E., Noyer-Weidner, M., Smith, S., Michael, M. & Graham, M. (1989). Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic Acids Res* **17**, 3469–3478.

Received 23 April 1997; revised 21 August 1997; accepted 3 September 1997.