Characterization of a prolidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397 with an unusual regulation of biosynthesis

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***Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397 (*Lb. bulgaricus*) is characterized by a high level of peptidase activities specific to proline-containing peptides. A prolidase (PepQ, EC 3.4.13.9) was purified to homogeneity and characterized as a strict dipeptidase active on X-Pro dipeptides, except Gly-Pro and Pro-Pro. The values for K_m and V_{max} were, respectively, 2.2 mM and 0.33 mmol min⁻¹ mg⁻¹, with Leu-Pro as the substrate. The enzyme exhibited optimal activity at 50 °C and pH 6.0, and required the presence of Zn²⁺. Size exclusion chromatographies and SDS-PAGE analysis led to the conclusion that this prolidase was a homodimer. Antibodies raised against the purified protein allowed the detection of PepQ among several *Lactobacillus* species but not lactococci. The *pepQ* gene and the upstream region were isolated and sequenced. The deduced peptide sequence showed that PepQ belongs to the M24 family of metallopeptidases. The *pepR1* gene is located immediately upstream of *pepQ* and its product is homologous to the transcription factor CcpA, which is involved in catabolite repression of catabolic operons from Gram-positive bacteria. The *pepR1-pepQ* intergenic region contains a consensus catabolite-responsive element (CRE) which could be a target for PepR1 protein. Moreover, in contrast to other proline-specific enzymes from *Lb. bulgaricus*, PepQ biosynthesis was shown to be dependent on the composition of the culture medium, but not on the peptide concentration. A possible regulation mechanism is discussed.**

Keywords: prolidase, *pepQ* gene, *Lactobacillus delbrueckii* subsp. *bulgaricus*, CcpA

INTRODUCTION

Due to the unique structure of proline, the presence of this imino acid in a polypeptide chain restricts its susceptibility to proteolysis, so many proteases are unable to cleave a peptide bond involving proline. Caseins are proline-rich proteins which are hydrolysed during milk fermentation and cheesemaking conducted with lactic acid bacteria.

We have identified and characterized several proteolytic enzymes in *Lactobacillus delbrueckii* subsp. *bulgaricus*

CNRZ 397 (*Lb. bulgaricus*), a thermophilic lactic acid bacterium. The proline-rich oligopeptides resulting from casein hydrolysis by PrtB, a cell-surface endoprotease (Gilbert *et al.*, 1996), which are often bitter, are degraded by a set of proline-specific peptidases. The caseinolytic pathway leads to the liberation of free proline, which is essential to *Lb. bulgaricus* (Atlan *et al.*, 1989), characterized by a high level of several proline-specific activities. Prolyl aminopeptidase (EC 3.4.11.5, PepIP) is highly specific for di- or tripeptides with proline at the N-terminal position (Gilbert *et al.*, 1994). The *pepIP* gene encoding this serine peptidase has been expressed at a very high level in *Escherichia coli* (Atlan *et al.*, 1994). Another pathway of degradation of proline-containing peptides in *Lb. bulgaricus* involves an X-prolyl dipeptidyl aminopeptidase (EC 3.4.14.5, PepX)

Abbreviation: CRE, catabolite-responsive element.

The EMBL accession number for the sequence reported in this paper is Y13385.

which is able to catalyse the removal of X-Pro dipeptides from oligopeptides containing a proline residue at the penultimate position. This serine peptidase is a homodimer of 150 kDa (Atlan *et al.*, 1990). X-Pro dipeptides can then be hydrolysed by a proline dipeptidase (EC 3.4.13.9, prolidase, PepQ) that has already been purified from *Lactobacillus casei* subsp. *casei* and *Lactococcus lactis* subsp. *cremoris* (Fernandez-Espla *et al.*, 1997; Kaminogawa *et al.*, 1984; Booth *et al.*, 1990). The *pepQ* gene has recently been isolated from *Lactobacillus delbrueckii* subsp. *lactis* (*Lb. lactis*) and *Lb. bulgaricus* B14 (Stucky *et al.*, 1995; Rantanen & Palva, 1997). Little is known about the effectors controlling the biosynthesis of proline-specific enzymes (Meyer *et al.*, 1996).

In this paper, a broad investigation of PepQ from *Lb. bulgaricus* CNRZ 397 was undertaken. PepQ activity was determined after growth of *Lb. bulgaricus* cells in different media and the enzyme was purified and characterized. The *pepQ* gene was isolated and expressed in a PepQ-deficient strain of *E. coli*. In addition a survey of the occurrence of PepQ protein among lactic acid bacteria was performed.

METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Table 1. *E. coli* FM9001 is a RecA⁻ derivative from CM91 (Miller & Schwartz, 1978) and was obtained by recombination with strain MCL31 (Lorence & Rupert, 1983). Spontaneous mutants of FM9001 were selected on synthetic medium M63 (Miller, 1972) supplemented with glucose (2 g l⁻¹), amino acids (methionine, leucine and proline; 0.1 g l⁻¹) and 2 mM Val-Pro-Leu. Valine is toxic for *E. coli*, and only cells unable to hydrolyse the tripeptide were able to grow on this selective medium. The resulting mutant strain FM9003 selected for further study is deficient for prolidase (PepQ) and aminopeptidase P (PepP) activities. *E. coli* was grown aerobically at 37 °C in LB medium (Miller, 1972). When required, LB medium was supplemented with 100 µg ampicillin ml⁻¹, 100 µg erythromycin ml⁻¹ or 50 µg kanamycin ml⁻¹. *Lactobacilli* were routinely grown anaerobically in MRS broth (Difco) (de Man *et al.*, 1960) at 40 °C for thermophilic strains (*Lb. bulgaricus*, *Lb. lactis* and *Lactobacillus helveticus*) and 30 °C for *Lb. casei*. *Lc. lactis* was grown at 30 °C in M17 broth supplemented with 5 g glucose l⁻¹ (Terzaghi & Sandine, 1975). Reconstituted Gamma milk (10%) was made by adding sterile water to skim milk powder sterilized by γ radiations (Prolait, Niort, France). Growth was estimated by measuring OD₆₀₀. One OD₆₀₀ unit corresponds to 0.5 mg and 0.25 mg bacterial dry weight ml⁻¹ for *E. coli* and *Lb. bulgaricus*, respectively.

Preparation of cell extracts. For enzyme purification experiments, *Lb. bulgaricus* cells were collected at the end of the exponential growth phase, washed in 50 mM sodium phosphate buffer pH 7.2 and resuspended in distilled water (4 × 10⁹ cells ml⁻¹). For other total cell extracts, lactic acid bacteria were grown in 50 ml medium to exponential phase, washed and resuspended in 5 ml 0.2 M Tris buffer pH 7.0. *E. coli* cells were grown in 50 ml LB medium supplemented with kanamycin, washed and resuspended in 5 ml 0.1 M sodium citrate buffer pH 6.0. Cells were disrupted in a French pressure cell (138 MPa). The homogenate was centrifuged at 15000 g for

45 min or at 25000 g for 20 min. The resulting supernatant (total cell extract) was used for further enzyme studies.

Protein and enzyme assays. Proteins were estimated by the method of Bradford (1976) with crystalline bovine serum albumin (Sigma) as a standard. PepQ activity was routinely assayed by the method of Yaron & Mlynar (1968). Unless otherwise stated, the enzyme reaction was performed at 40 °C in 0.25 ml 0.1 M sodium citrate buffer pH 6.0, containing 2.5 mM ZnSO₄ and 6.4 mM Leu-Pro. The reaction was stopped by the addition of 0.62 ml ninhydrin reagent (6 g ninhydrin dissolved in 200 ml of a 6 M phosphoric acid/glacial acetic acid mixture, 80:120, v/v) and 0.62 ml glacial acetic acid. The solution was heated at 100 °C for 30 min and the A₄₈₀ measured.

PepP activity was estimated by the assay of tyrosine released from human casomorphin. The reaction was carried out at 40 °C in 0.1 M sodium citrate buffer pH 6.0 containing 5 mM ZnSO₄ and 0.5 mM human casomorphin, and was stopped by rapid cooling. Products were analysed at room temperature by reverse-phase chromatography on a Pep RPC column (Pharmacia) equilibrated in 0.1% trifluoroacetic acid (TFA) in water (mobile phase A). The samples to be analysed were made up to 0.2 ml with A and injected onto the column, which was eluted as follows: from 0 to 3 min, an isocratic elution with A, and from 3 to 20 min, a linear gradient of 100% A to 100% B (mobile phase B: 0.1% TFA in acetonitrile). The flow rate was 0.7 ml min⁻¹ and the UV detector was adjusted at 278 and 215 nm. Under these conditions, tyrosine is eluted at 7.4 min and tyrosine-depleted human casomorphin and casomorphin at 14 and 15.25 min, respectively. Amino acids were separated by thin-layer chromatography on silica gel 60F254 thin-layer plates (Merck). Acetic acid/n-butanol/water (1/4/1, by vol.) was used as the developing solvent, and 0.25% (w/v) ninhydrin in acetone as the locating agent. The spots were developed by heating at 100 °C for 5 min.

PepX, PepIP and PepN activities were assayed as described previously with respectively Ala-Pro-, Pro- and Lys-p-nitroanilide as the substrate (Gilbert *et al.*, 1997). One unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol product (proline, tyrosine or p-nitroanilide) min⁻¹.

PepQ enzyme purification. A total extract (197 ml) from *Lb. bulgaricus* was treated with (NH₄)₂SO₄ at 60% saturation. The 60% supernatant was brought to 100% saturation. Fractions obtained at 60% and 100% (NH₄)₂SO₄ were pooled and applied to a column (12 × 2.6 cm) of DEAE-Trisacryl equilibrated in 20 mM Tris/HCl pH 7.4, 1 mM ZnSO₄. The column was then washed with 50 ml of the equilibration buffer. Elution was performed using a linear gradient from 0 to 500 mM NaCl (total volume 300 ml) at a flow rate of 1.5 ml min⁻¹. Fractions of 6 ml were collected and two peaks of UV-absorbing material, named A and B, were eluted at 250 mM and 400 mM NaCl concentrations, respectively. Peak A contained the enzyme activity; it was then chromatographed on a Source 15Q column (7.5 × 0.75 cm) equilibrated in 20 mM sodium acetate pH 5.5, 1 mM ZnSO₄. Chromatography was developed at a flow rate of 0.75 ml min⁻¹ as follows: from 0 to 5 min, the equilibration buffer, then for 60 min, a linear gradient from 0 to 300 mM NaCl; 1.5 ml fractions were collected and the active fraction was loaded onto a Mono Q HR 5/5 column (Pharmacia) equilibrated in 20 mM sodium acetate pH 4.8, 1 mM ZnSO₄. Chromatography was developed at room temperature as follows: from 0 to 3 min, the equilibration buffer, then for 30 min, a linear gradient from 0 to 300 mM NaCl; the flow rate was set at 1 ml min⁻¹ and 1 ml

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristics	Source or reference
Strains		
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> CNRZ 397		INRA, Jouy en Josas, France
<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i> CNRZ 250		INRA, Jouy en Josas, France
<i>Lactobacillus helveticus</i> NCDO 766		Institute of Food Research, Reading, UK
<i>Lactobacillus casei</i> ATCC 4646		TNO Nutrition & Food Institute, Rijswijk, The Netherlands
<i>Lactococcus lactis</i> subsp. <i>lactis</i> NCDO 763		Yakult Central Institute for Microbial Research Kunitachi-shi, Tokyo, Japan
<i>Escherichia coli</i> CM91	leu-9 Δ (<i>pro-lac</i>) <i>met thyA pepQ</i> 12	Miller & Schwartz (1978)
<i>Escherichia coli</i> MCL31	Hfr PO201, Δ (<i>srl-recA</i>)306::Tn10	Lorence & Rupert (1983)
<i>Escherichia coli</i> FM9001	RecA ⁻ derivative from CM91	This work
<i>Escherichia coli</i> FM9003	Val-Pro-Leu-resistant (PepP ⁻) derivative of FM9001	This work
Plasmids		
pUC18	2.7 kb, Amp ^r , <i>lacZ</i> α	Yanisch-Perron <i>et al.</i> (1985)
pJDC9	7.0 kb, Ery ^r , <i>lacZ</i> α	Chen & Morrison (1987)
pLG339	6.2 kb, Tet ^r Kan ^r	Stoker <i>et al.</i> (1982)
pQF6	pUC18 derivative containing the PCR fragment (1.2 kb) corresponding to <i>pepQ</i> of <i>Lb. bulgaricus</i>	This work
pQA3	pUC18 derivative containing the inverse PCR fragment (1.5 kb) corresponding to <i>pepQ</i> promoter region of <i>Lb. bulgaricus</i>	This work
pRF1	pJDC9 derivative containing the inverse PCR fragment (2.2 kb) corresponding to <i>pepR1</i> of <i>Lb. bulgaricus</i>	This work
pMS2	pLG339 derivative containing <i>pepQ</i> of <i>Lb. lactis</i>	Stucky <i>et al.</i> (1995)
pQLB18	pMS2 derivative containing <i>pepQ</i> of <i>Lb. bulgaricus</i> with promoter of <i>Lb. lactis</i>	This work
pQBB12	pQLB18 derivative containing <i>pepQ</i> of <i>Lb. bulgaricus</i> with its own promoter	This work

fractions were collected. The active fraction was treated by hydrophobic interaction chromatography at 20 °C, using a 1 ml phenyl Sepharose HP column (Pharmacia). The column was equilibrated in 20 mM sodium acetate pH 5.5, 1 mM ZnSO₄, 1 M (NH₄)₂SO₄, and eluted as follows: from 0 to 4 min, the equilibration buffer, and from 4 to 30 min, a descending linear gradient from 1 M to 0 M (NH₄)₂SO₄. Fractions of 0.8 ml were collected with a flow rate of 0.4 ml min⁻¹. Finally, the active fraction was treated by size-exclusion chromatography at room temperature on a Superose 12 HR column (Pharmacia) equilibrated in 20 mM sodium acetate pH 5.5, 1 mM ZnSO₄, 100 mM NaCl. The flow rate was 0.4 ml min⁻¹ and 0.8 ml fractions were collected. Cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (46 kDa), bovine serum albumin (69 kDa) and α -amylase (200 kDa) were the molecular mass standards.

Electrophoretic techniques, Western blotting and immunoblotting. SDS-PAGE was carried out by the method of Laemmli (1970). Proteins were visualized using the Quick Silver detection kit (Amersham) or by Coomassie blue.

Thin-layer gel electrofocusing was performed with the LKB2117-Multiphor II (LKB Produkter) in the pH 3.5–10

range; the gel was 0.5 mm thick. The detection procedure was the same as for SDS-PAGE.

Western blotting was performed by electrotransfer with the LKB2117-Multiphor II onto nitrocellulose membrane (Schleicher & Schuell). Immunoblots were carried out by the method of Harlow & Lane (1988) using antibodies raised against the purified *Lb. bulgaricus* PepQ protein and anti-rabbit IgG peroxidase conjugate (Sigma). Anti-PepQ polyclonal antibodies were provided by the Centre de Bioexpérimentation Valbex (IUT A, Université Lyon I).

N-terminal sequence determination. The N-terminal sequence of PepQ was determined by the method described by Edman & Begg (1967) at the Service Central d'Analyse CNRS (Vernaison, France).

Recombinant DNA procedures. Restriction endonucleases, Klenow DNA polymerase, T4 DNA ligase and T4 polynucleotide kinase were obtained from New England Biolabs and Life Technologies-Gibco-BRL, and used according to the manufacturers' instructions. Plasmid extraction and *E. coli* transformation were carried out as described by Sambrook *et al.* (1989). DNA fragments were purified with the UltraClean

Gen-apex DNA purification kit (Prolabo). DNA sequencing was performed using the T7-sequencing kit (Pharmacia).

PCR and inverse PCR amplifications, and plasmid construction. Plasmids used in this study are listed in Table 1. PCR was performed using the Advantage™ Genomic PCR kit (Clontech) and primers were provided by Life Technologies-Gibco-BRL. The kit contains a polymerase providing 3'–5' proof-reading activity, which ensures high fidelity. PCR amplification of the *pepQ* gene was carried out with chromosomal DNA from *Lb. bulgaricus* CNRZ 397 as a matrix and primers QStart (5' CCCATCGATTAGACAAATTACAAAC 3') and QStop (5' GCGCAAGGCATAATTGTATACGA 3') (see Fig. 2a). These primers were chosen according to the sequence of *pepQ* from *Lb. lactis* DSM 7290 (Stucky *et al.*, 1995). Primer QStart contains the ATG start-codon and two base changes allowed the insertion of a *Clal* restriction site. Primer QStop is located 76 nt downstream the stop-codon of the *pepQ* gene and contains a *Bst1107I* restriction site. PCR product was digested with *Clal* and *Bst1107I* restriction enzymes and inserted into the pUC18 vector (pQF6; see Table 1 and Fig. 2a). Inverse PCR, performed by the method of Ochman *et al.* (1990), was used to amplify the chromosomal region upstream of *pepQ*. For amplification of the promoter region, chromosomal DNA from *Lb. bulgaricus* was digested with *EcoRI*. Resulting fragments were circularized with T4 DNA ligase, and used for PCR amplification with primers InvQ1 (5' GTCCATCCCGTTTCCTGC 3') and InvQ2 (5' GAAGAAGCCAAGGCATCAGC 3') (see Fig. 2a). These primers have divergent directions and are located in the 5' part of the *pepQ* gene. They were chosen according to the sequence of *pepQ* from *Lb. bulgaricus* CNRZ 397. The PCR product was cloned into the pUC18 vector after Klenow and kinase treatments (pQA3, Table 1). For the amplification of *pepR1*, chromosomal DNA from *Lb. bulgaricus* was digested with *PstI*. Resulting fragments were circularized and used for PCR amplification with primers InvR1 (5' CCGTTTGATGAAGGAAGACGCCG 3') and InvR2 (5' ACATTGTTGTTGCCGTTGACTACCCG 3') (see Fig. 2a). These primers have divergent directions and are located in the 5' part of the *pepR1* gene. They were chosen according to the sequence of *pepR1* from *Lb. bulgaricus* CNRZ 397. The PCR product was cloned into the pJDC9 vector after Klenow and kinase treatments (pRF1, Table 1). Plasmid pQLB18 was constructed by replacing the 1.1 kb *PstI*–*XbaI* fragment of pMS2 with the 1.1 kb *PstI*–*XbaI* fragment of pQF6, corresponding to the *pepQ*-encoding region of *Lb. bulgaricus* (Table 1 and Fig. 2a). Plasmid pQBB12 was derived from pQLB18, in which the 0.6 kb *EcoRI*–*PstI* fragment was replaced by the 0.6 kb *EcoRI*–*PstI* fragment of pQA3, corresponding to the promoter region of *Lb. bulgaricus* (Table 1 and Fig. 2a).

RESULTS

Peptidase activities of *Lb. bulgaricus*

Activities of three proline-specific peptidases (PepQ, PepX, PepIP) and a general aminopeptidase (PepN) were assayed in total cell extracts from *Lb. bulgaricus*. Bacterial cells were grown in MRS, a peptide-rich medium, or in milk, which contains a very low amount of peptides and free amino acids (Table 2). PepX, PepIP and PepN activities did not significantly differ after bacterial growth in MRS or milk. By contrast, PepQ activity assayed from MRS cultures was twofold higher than that from milk cultures (2.6 and 1.3 U mg⁻¹, respectively). The addition of peptides (tryptone or Leu-

Table 2. Peptidase activities of *Lb. bulgaricus* CNRZ 397 after growth in different media

Activities are in units (mg bacterial dry weight)⁻¹ and are the mean values from at least three independent assays.

Medium	PepQ	PepX	PepIP	PepN
MRS	2.6	177	16.8	26.0
Milk	1.3	140	17.6	26.8
Milk + tryptone 1 %	1.1	137	21.4	24.7
Milk + Leu-Pro 5 mM	1.0	120	17.3	21.6

Pro) to milk cultures did not significantly modify PepQ activity.

PepQ purification and properties

PepQ purification was achieved by a six-step procedure described in Methods; quantitative results are summarized in Table 3. The purified protein was electrophoretically pure (Fig. 1) and was used for all the enzyme characterizations. In order to keep the prolidase activity at a reasonable level, it was necessary to carry out all the purification steps in the presence of 1 mM ZnSO₄.

Under our assay conditions, PepQ activity was optimal in the range 40–50 °C and fell abruptly beyond 50 °C and 55 °C. The optimal pH was 6.0. The isoelectric point of PepQ was pH 4.5. At 40 °C, the substrate Leu-Pro was hydrolysed with a *K_m* of 2.2 mM and a *V_{max}* of 0.33 mmol min⁻¹ (mg protein)⁻¹.

PepQ is not a serine enzyme since it was inhibited neither by 1 mM *p*-aminophenylmethylsulfonyl-fluoride (APMSF) nor by 1 mM 3,4-dichloroisocoumarin. Complete inhibition occurred in the presence of 1 mM bestatin or 1 mM DTT. PepQ activity was inhibited by 1 mM EDTA, and in order to reverse this inhibition or to activate the prolidase, Zn²⁺ was found to be the best of the divalent cations tested (Zn²⁺, Ca²⁺, Co²⁺, Cu²⁺, Mg²⁺, Mn²⁺, Ni²⁺).

Studies of substrate specificity showed that the purified enzyme is highly specific for dipeptides of the X-Pro type, except for Pro-Pro and Gly-Pro, which underwent very low hydrolysis (if any) as compared with the dipeptides made of Arg, Leu, Phe, Val as N-terminal amino acid (Table 4). Therefore, the purified enzyme is strictly a proline dipeptidase with no detectable PepP activity.

On SDS-PAGE, the enzyme migrated like a molecule of about 50 kDa (Fig. 1). On size-exclusion chromatography and under non-denaturing conditions [20 mM sodium acetate, 1 mM ZnSO₄, 100 mM (or 300 mM) NaCl pH 5.5 buffer], the apparent molecular mass ranged from 68 to 70 kDa; under denaturing conditions (5 M urea), the apparent molecular mass dropped to approximately 45 kDa, a value very close to that determined from the deduced amino acid sequence

Table 3. Distribution of protein and prolidase activity upon purification of the total extract

Purification step	Protein (mg)	Enzyme activity ($\mu\text{mol min}^{-1}$)	Specific activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	Recovery*	Purification factor
Total extract	354	2730	7.7	100	1
$(\text{NH}_4)_2\text{SO}_4$, 60% sat.	99	284	2.86	10	0.37
$(\text{NH}_4)_2\text{SO}_4$, 100% sat.	202	2280	11.2	83	1.45
DEAE-Trisacryl	71	1900	26	69	3.37
Source 15Q	13	1340	103	49	13.3
Mono Q	2.8	710	253	26	33
Hydrophobic interaction chromatography	1.3	412	316	15	41
Size-exclusion chromatography	0.38	170	446	6.2	58

* Recovery is the enzyme activity in the fraction expressed as a percentage of that in the total extract.

Fig. 1. SDS-PAGE analysis of the fractions obtained in the course of the PepQ purification. Lane A, total extract; lane B, $(\text{NH}_4)_2\text{SO}_4$ precipitate; lane C, DEAE-Trisacryl chromatography; lane D, chromatography on Source 15Q; lane E, chromatography on Mono Q; lane F, hydrophobic interaction chromatography; lane G, size-exclusion chromatography. Myosin (200 kDa), phosphorylase b (92 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa) and carbonic anhydrase (29 kDa) were the molecular mass standards.

encoded by *pepQ* (41 kDa). Taken together, the results strongly suggest that PepQ is a homodimer.

Cloning and analysis of the *pepQ* gene region

QStart and QStop primers were used for the PCR amplification of *pepQ* from the *Lb. bulgaricus* chromosome. Considering the high DNA sequence identity between *Lb. delbrueckii* subspecies, these primers were chosen according to the sequence of the *pepQ* gene from *Lb. lactis* DSM 7290 (Stucky *et al.*, 1995). A single 1.2 kb PCR product was obtained and cloned into the pUC18 vector (pQF6, Table 1). Inverse PCR was used to amplify the chromosomal region upstream of the *pepQ* gene, with primers chosen according to the *Lb. bulgaricus* CNRZ 397 sequence. A single inverse-PCR product of 1.5 kb was obtained with InvQ1 and InvQ2 primers, and cloned into the pUC18 vector (pQA3, Table 1). A second 2.2 kb inverse PCR product was obtained with InvR1

Table 4. Substrate specificity of purified PepQ

Substrate	Activity
Leu-Pro*	100
Arg-Pro*	130
Pro-Pro*	0
Val-Pro*	82
Ala-Pro*	15
Gly-Pro*	0
Phe-Pro*	75
Leu-Gly†	—
Leu-Ala†	—
Leu-Ala-Pro*	0
Gly-Pro-Ala†	—
Ala-Pro-Gly†	—
Val-Pro-Leu†	—
Human casomorphin‡	0

* Prolidase activity was measured by assaying the release of proline (Yaron & Mlynar, 1968); relative activity is a percentage of the activity obtained with Leu-Pro (446 U mg^{-1}).

† Thin-layer chromatography on silica gel 60F254 was used to look for a release of the N-terminal amino acid. —, No release of amino acid observed.

‡ The susceptibility of human casomorphin to the prolidase action was studied by measuring the appearance of tyrosine by reverse phase chromatography.

and InvR2 primers, and cloned into the pJDC9 vector (pRF1, Table 1).

The sequence of the overall region (3553 nt) was determined from DNA inserts of pQF6, pQA3 and pRF1. Nucleotide sequence comparison with the corresponding region from *Lb. lactis* DSM 7290 showed 98% identity (Stucky *et al.*, 1995, 1996). DNA sequence analysis revealed two divergent open reading frames (ORFs) (Fig. 2a). One ORF encodes 368 amino acid residues and corresponds to *pepQ*. Immediately upstream, a second ORF (333 codons) was named *pepR1*

Fig. 2. Genetic map of the chromosomal region of *pepQ* and comparison of putative RBS sequences of *pepR1*. (a) Genetic map. Restriction sites: E, *EcoRI*; H, *HincII*; S, *SmaI*; P, *PstI*; Hd, *HindIII*; X, *XbaI*; B, *Bst1107I*. The *pepQ* and *pepR1* genes are indicated by striped zones and arrows. Details of intergenic region: putative -10 and -35 sequences of both genes are boxed, and the CRE sequence is presented with the consensus CRE sequence above. Position and direction of primers used for direct and inverse PCR are indicated by small arrows: 1, InvR1; 2, InvR2; 3, QStart; 4, InvQ1; 5, InvQ2; 6, QStop. The 1.1 kb *PstI*-*XbaI* fragment of pQF6 and the 0.6 kb *EcoRI*-*PstI* fragment of pQA3 are represented by white and black bars, respectively. (b) Comparison of putative RBS sequences of *pepR1*: nucleotides according to the consensus RBS sequence are in bold letters (Matern *et al.*, 1994); nucleotides of the 16S rRNA from *E. coli* that could hybridize to the RBS sequence of *pepR1* from *Lb. delbrueckii* are underlined (Shine & Dalgarno, 1974).

in reference to *Lb. lactis* DSM 7290. The *pepQ-pepR1* intergenic region contains a putative -35 box, -10 box and RBS promoter sequences of both genes (Fig. 2a). The two -35 sequences are separated by 25 bp. The *Lb. bulgaricus* RBS sequence of *pepR1* differs in one base from that of *Lb. lactis* DSM 7290 (Fig. 2b). A well-conserved consensus catabolite-responsive element (CRE) overlaps the -35 box of *pepR1*.

Amino acid sequence analysis of PepQ protein

The deduced amino acid sequence of PepQ is in agreement with the first 20 amino acids of the N-terminal sequence of the purified enzyme (data not shown). Comparison of PepQ sequences from *Lb. bulgaricus* CNRZ 397 and B14 revealed only one different residue at position 93 (Thr instead of Ser) (Rantanen & Palva, 1997); thus, the *Lb. bulgaricus* CNRZ 397 and *Lb. lactis* DSM 7290 PepQ sequences only differ in three residues (Stucky *et al.*, 1995).

A computer search in protein databases revealed significant similarity between the amino acid sequence of *Lb. bulgaricus* PepQ and aminopeptidases P (PepPs), methionyl aminopeptidases (PepMs) and creatinases, particularly in the C-terminal two-thirds of the sequences. According to Rawlings & Barrett (1995), prolidases, PepPs and PepMs constitute the M24 family of metallopeptidases. Previous reports concerning PepQ

from *Lb. lactis* DSM 7290 and *Lb. bulgaricus* B14 claimed that these enzymes contain a Zn-binding site (HEXXH motif) characterizing Zn-metalloproteinases (Stucky *et al.*, 1995; Rantanen & Palva, 1997). In contrast to this statement, proteins of M24 family are characterized by the absence of a consensus Zn-binding motif and contain metal atoms that are not necessarily zinc. The three-dimensional structure of PepM from *E. coli* shows that the active site consists of five residues binding two cobalt atoms (Roderick & Matthews, 1993). These five metal ligands (Asp-223, Asp-234, His-298, Glu-327 and Glu-341 in the PepQ sequence of *Lb. bulgaricus*) are well conserved as shown in Fig. 3.

The deduced amino acid sequence of PepR1 exhibits 50% identity with CcpA of *Lb. casei* ATCC 393 (Monedero *et al.*, 1997). PepR1 was found to be related to the LacI-GalR repressor family and showed a helix-turn-helix motif characteristic of DNA-binding proteins. Restricted to this domain, sequence identity between PepR1 and *Lb. casei* ATCC 393 CcpA was 95%.

Expression of *pepQ* in *E. coli* FM9003

Plasmids pMS2, pQLB18 and pQBB12 are pLG339 derivatives (Table 1). pMS2 and pQBB12 contain, respectively, *pepQ* genes from *Lb. lactis* DSM 7290 and *Lb. bulgaricus* CNRZ 397 with their own promoters. In

	223	234	298	327	341
1	ELVLFDLGTMEHGYASDSSRTV		GHGIGMEVHEFPSI	VLEEGMCFSEPGIY	FAGVRIEDCGVLTK
2	RSFLLDAGAEYNGYAADLTRTW		GHPLGLQVHDVAGF	ILQPGMVLTIPEGIY	FGGRIEDNVVIHE
3	DMCLFDMGGEYYSVASDITCSF		GHFLGIDVHDVGGY	HLQPGMVLTVPGIY	FGGVRIEDDVVID
4	DLVLI DAGCEYKGYAGDITRTF		SHWLGLDVHDVGVI	ILEPGMVLTVPEGLY	GIGIRIEDDIVITE
5	DPVTIDFGCYEYHYASDMTRTI		GHGLGLDVHEIPYF	QLRSGMVITDEPGIY	FGGVRIEDDLLVTE
6	DIVNIDVTVIKDFHGDTSKMF		GHGIGRGFHEEPQV	VLKPGMTFTIPEPMVN	SLSAQYEHITIVVTD
7	DIISIDIGAKLNGYHGDSAWTY		GHGVGQDLHEDPQI	RLKPGMVLAIPEPMVN	KKCAHFETHTIAITE
	*	*	** *	* ** *	*

Fig. 3. Multiple sequence alignment of M24 family peptidases: lines 1 and 2, PepQs from *Lb. bulgaricus* (Y13385) and *E. coli* (P21165) (Nakahigashi & Inokuchi, 1990), respectively; line 3, human prolidase (PepD) (P12955) (Tanoue et al., 1990); lines 4 and 5, PepPs from *E. coli* (P15034) (Yoshimoto et al., 1989) and *Lc. lactis* (Y08842) (Matos et al., 1998), respectively; lines 6 and 7, PepMs from *E. coli* (P07906) (Fujita et al., 1994) and *Bacillus subtilis* (P19994) (Nakamura et al., 1990), respectively. Database accession numbers are listed above in parentheses after each species. The five conserved metal ligands are shown in bold letters; numbering corresponds to the sequence of *Lb. bulgaricus* PepQ; conserved amino acids are indicated by an asterisk.

Table 5. Expression of *pepQ* from *Lb. bulgaricus* in *E. coli* FM9003

Activities are in units (mg bacterial dry weight)⁻¹ and are the mean values from at least three independent assays.

Plasmid	10 ³ × PepQ activity	PepN activity
pLG339	< 0.1	2.1
pMS2	20.8	2.2
pQLB18	23.4	1.7
pQBB12	49.9	2.1

pQLB18, the *Lb. bulgaricus* gene was cloned downstream of the *pepQ* promoter from *Lb. lactis* DSM 7290.

E. coli FM9003, which is unable to hydrolyse the Leu-Pro dipeptide, was transformed with pLG339, pMS2, pQLB18 and pQBB12. PepQ and PepN activities were assayed in total cell extracts obtained from transformants. PepN was used as an internal standard of *E. coli*. No PepQ activity was present in the extract from the strain harbouring pLG339 (Table 5). PepQ activities encoded by pMS2 and pQLB18 were not significantly different (20.8×10^{-3} and 23.4×10^{-3} U mg⁻¹, respectively), indicating that the PepQ proteins from both *Lb. delbrueckii* subspecies have similar enzyme properties. By contrast, the presence of pQBB12 was associated with a 2.5-fold higher activity (49.9×10^{-3} U mg⁻¹). Taken together, these results suggested that the promoter region from *Lb. bulgaricus* led to a better transcription level of *pepQ* than that from *Lb. lactis* DSM 7290.

Immunodetection of PepQ in lactic acid bacteria

Western blot analysis of total cell extracts from *E. coli* transformants harbouring pLG339, pMS2, pQLB18 or pQBB12, and from *Lb. bulgaricus* CNRZ 397, *Lb. lactis* CNRZ 250, *Lb. helveticus* NCDO 766, *Lb. casei* ATCC 4646 and *Lc. lactis* NCDO 763 was performed after SDS-PAGE, with antibodies raised against the purified

Fig. 4. PepQ immunodetection in total extracts from transformants of *E. coli* FM9003 and from lactic acid bacteria: proteins from total extracts were separated by SDS-PAGE. Lanes A–C, *E. coli* FM9003 harbouring pLG339, pMS2 or pQBB12, respectively (amounts of total extracts used correspond approximately to 2×10^8 cells); lanes D and E, *Lb. bulgaricus* CNRZ 397 after growth in MRS or milk, respectively; lanes F–H, *Lb. lactis* CNRZ 250, *Lb. helveticus* NCDO 766, *Lb. casei* ATCC 4646, respectively after growth in MRS; lane I, *Lc. lactis* NCDO 763 after growth in M17-glucose. To obtain readable blots, amounts of total extracts correspond to about 10 µg proteins for lanes D and E, 20 µg for lanes F and G, and 80 µg for lanes H and I; SeeBlue prestained standard solution (Novex) was used for molecular mass markers.

PepQ from *Lb. bulgaricus* (Fig. 4). A single 48 kDa band was observed in extracts from *E. coli* strains carrying pMS2, pQLB18 or pQBB12, whereas no signal was observed with strains harbouring pLG339. This apparent size was in accordance with the SDS-PAGE migration of the purified protein (Fig. 1).

A major protein (about 48 kDa) corresponding to the PepQ protein was detected in total extracts from *Lb. bulgaricus* and *Lb. lactis* CNRZ 250. A single protein was also detected in *Lb. helveticus* NCDO 766 and *Lb. casei* ATCC 4646 extracts, but with a different molecular mass (45 and 42 kDa, respectively). No protein signal was detected in the *Lc. lactis* NCDO 763 extract. Thus, PepQ protein appeared well conserved within lactobacilli.

A minor band (about 40 kDa) was revealed in *Lb. bulgaricus* CNRZ 397 and *Lb. lactis* CNRZ 250 total extracts and a second major band (about 50 kDa) was

found in the *Lb. lactis* extract. These data suggested the presence of two other proteins with a structural domain closely related to a PepQ region. It is noteworthy that, recently, a second *pepQ*-like gene (*orfZ*) was detected in *Lb. delbrueckii* subspecies (Rantanen & Palva, 1997). Under pH-controlled conditions, *orfZ* was shown to be expressed at a very low level and to encode a 41 kDa protein without any enzyme activity. It cannot be excluded that in *Lb. bulgaricus* CNRZ 397, a minor band with a similar molecular mass corresponds to an *orfZ* gene product.

DISCUSSION

The PepQ enzyme we purified from *Lb. bulgaricus* CNRZ 397 exhibits a narrow specificity towards X-Pro dipeptides, as expected for a prolidase. Nevertheless, analysis of PepQ from *Lb. bulgaricus* revealed some significant differences as compared to prolidases already described in other micro-organisms. (i) It is the first time that a prokaryotic PepQ is described as a homodimer. (ii) In contrast to properties of prolidases from *Lc. lactis* subsp. *cremoris* AM2 and *Lb. casei* IFPL 731, we did not find any substrate inhibition (Booth *et al.*, 1990; Fernandez-Espla *et al.*, 1997). (iii) Zn^{2+} appeared to be essential to *Lb. bulgaricus* PepQ activity, whereas an inhibition of enzyme activity by this divalent cation was described for PepQ from *Lb. casei* IFLP 731 and, to a lesser extent, for PepQ from *Lc. lactis* subsp. *cremoris* H61 (Fernandez-Espla *et al.*, 1997; Kaminogawa *et al.*, 1984). Both enzymes were activated by other divalent cations such as Mn^{2+} or Co^{2+} . (iv) PepQ proteins from lactobacilli differ from the lactococcal ones, since the latter are not recognized by antibodies raised against the *Lb. bulgaricus* enzyme.

General features shared by *Lb. bulgaricus* PepQ and other described prolidases are the inhibition by chelating and reducing agents, and the requirement of a divalent cation for the enzyme activity. The active site of human prolidase PepD, which requires Mn^{2+} as cofactor, has been extensively studied (King *et al.*, 1989). In the proposed model, Mn^{2+} participates in the substrate positioning, and, in theory, this role could be effected by another divalent cation such as Zn^{2+} . The specificity of prolidases towards different cations remains to be elucidated.

Peptide sequence analysis showed that PepQ does not belong to the large family of Zn-metallopeptidases, but rather ranks among the M24 family of metallo-peptidases, which also comprises PepMs and PepPs (Rawlings & Barrett, 1995). Bazan *et al.* (1994) proposed that these enzymes, and also creatinases, share a common structure, in which five conserved residues bind two divalent cations. The metal ligands are located in the C-terminal part of the protein, and it was not surprising to find the highest sequence identity between *Lb. bulgaricus* and other M24-family-related proteins in this region.

Lb. bulgaricus and *Lb. lactis* DSM 7290 *pepQ* genes have been expressed in *E. coli* (PepQ⁻ PepP⁻ strain), and the transcription level appeared significantly higher with the *Lb. bulgaricus* promoter region. Some differences in the nucleotide sequences were observed between the intergenic regions of *Lb. lactis* DSM 7290 and *Lb. bulgaricus*, particularly in the RBS sequence of *pepR1*. The *Lb. bulgaricus* sequence could lead to a better binding of the 16S rRNA from *E. coli*, but the effect on the transcription of *pepQ* remains unclear (Shine & Dalgarno, 1974).

Peptidase regulation mechanisms have received little attention (reviewed by Gonzales & Robert-Baudouy, 1996; Meijer *et al.*, 1996). With regard to the proteolytic system of *Lb. bulgaricus* CNRZ 397, we have previously shown that biosynthesis of two aminopeptidases (API and APIII) was subject to a regulatory mechanism related to the composition of culture medium (Atlan *et al.*, 1989). Biosynthesis of the cell-surface proteinase (PrtB) was also dependent on the peptide concentration of the culture medium (Gilbert *et al.*, 1997). In the present paper, a regulation mechanism for PepQ biosynthesis has been demonstrated for the first time. The mechanism involved is probably different from that controlling biosynthesis of PrtB, API and APIII, as well as lactococcal PepN and PepX, because PepQ biosynthesis is not dependent on extracellular peptide concentration (its substrate Leu-Pro included). A possible control could be ascribed to the presence of the close location of the promoter region of *pepR1*, including a well-conserved CRE sequence. The *pepR1*-deduced sequence is significantly similar to CcpA protein, which is involved in catabolite repression in Gram-positive bacteria. CcpA is known to bind specifically to CRE sequences via its helix-turn-helix domain, and consequently to repress the expression of catabolic operons displaying CRE sequences (reviewed by Saier *et al.*, 1996). Such a CRE sequence was found in the *pepR1-pepQ* intergenic region and might be the target of a transcription factor such as *pepR1* itself.

Experiments related to the potential role of *pepR1* in catabolite repression and/or *pepQ* transcription are now in progress. The biosynthetic regulation of two peptidases by components implicated in catabolite repression has already been described in *Salmonella typhimurium* (Conlin *et al.*, 1994; Lombardo *et al.*, 1997). The physiological significance of such a mechanism is not known. The identification of mechanisms involved in the biosynthetic regulation of peptidases will provide a better understanding of the role of these enzymes in the caseinolytic pathway of lactobacilli.

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