PepR1, a CcpA-like transcription regulator of *Lactobacillus delbrueckii* subsp. *lactis*

Joachim Schick, Beate Weber, Jürgen R. Klein and Bernhard Henrich

Author for correspondence: Bernhard Henrich. Tel: +49 631 2052347. Fax: +49 631 2053799.
e-mail: henrich@rhrk.uni-kl.de

Universität Kaiserslautern,
Fachbereich Biologie,
Abteilung Mikrobiologie, PO
Box 3049, D-67653
Kaiserslautern, Germany

The PepR1 protein from *Lactobacillus delbrueckii* subsp. *lactis* DSM 7290 shares extensive homology with catabolite-control proteins from various Gram-positive bacteria. Expression of the subcloned *pepR1* gene allowed for partial complementation of a *ccpA* defect in *Staphylococcus xylosus*. The influence of PepR1 on transcription of the prolidase gene *pepQ*, which is located adjacent to *pepR1*, was examined by use of *lacZ* reporter gene fusions in *Escherichia coli*. PepR1 stimulated transcription initiation at the *pepQ* promoter about twofold, and this effect required the integrity of a 14 bp palindromic *cre*-like sequence located 74 nt upstream of *pepQ*. In gel-mobility-shift assays, PepR1 specifically interacted with the *pepQ* promoter region and also with DNA fragments covering the promoters of the *pepX*, *pepI* and *brnQ* genes of *Lb. delbrueckii* subsp. *lactis*, which encode two additional peptidases and a branched-chain amino acid transporter, respectively. *cre*-like elements were identified in each of these DNA fragments. Catabolite control of *PepQ* was demonstrated in *Lb. delbrueckii* subsp. *lactis*. During growth with lactose the enzyme activity was twofold higher than in the presence of glucose, and corresponding differences were also detected in the level of *pepQ* transcription.

Keywords: *Lactobacillus delbrueckii*, PepR1 transcriptional regulator, peptidase Q, *cre* sequence, catabolite control

INTRODUCTION

Thermophilic lactic acid bacteria of the species *Lactobacillus delbrueckii* are involved in the fermentation of various dairy products, especially in the production of Italian-type hard cheeses (Olson, 1990). Due to numerous amino acid auxotrophies, growth of these bacteria in milk depends on the effective utilization of milk proteins (Juillard *et al.*, 1995). This is achieved by the coordinate action of a cell-envelope-associated proteinase, various peptidases, and some peptide and amino acid transport systems (Mierau *et al.*, 1997). We have previously isolated a series of 10 genes of the proteolytic system of *Lb. delbrueckii* subsp. *lactis* DSM 7290, a strain originally isolated from Emmental cheese. These genes, which code for peptidases with different specificities and an amino acid transporter, have been cloned and sequenced, and biochemical properties of most of the respective proteins have been established (Klein *et al.*, 1995, 1997). From an economic point of view, it seems reasonable that the expression or activities of the various components of the proteolytic system are controlled in response to specific external or internal

stimuli. Little is known, however, about the underlying mechanisms and the genetic functions mediating them.

In the vicinity of *pepQ* (Stucky *et al.*, 1995a), one of the cloned peptidase genes of *Lb. delbrueckii* subsp. *lactis*, we recently detected an open reading frame (*pepR1*) which was suspected to encode a catabolite-responsive regulatory protein (Stucky *et al.*, 1996). The *pepR1* gene and its flanking regions were sequenced, and transcriptional start points were determined. The 152 bp intergenic region between the divergently running *pepQ* and *pepR1* genes contains a 14 bp palindromic sequence which partly overlaps the -35 region of the *pepR1* promoter. This palindrome is a highly conserved copy of the catabolite-responsive element *cre*, which is suspected to be involved in catabolite control of a number of genes in Gram-positive bacteria. The 37 kDa PepR1 protein has a typical helix–turn–helix DNA-binding motif and shares homology with a catabolite-control protein (CcpA), which is involved in the control of carbohydrate metabolism in Gram-positive bacteria. *cre* elements can be identified in the promoters of many CcpA-regulated genes, suggesting direct interactions of CcpA with these sequences (Hueck *et al.*, 1994). Recently, in *Strepto-*

coccus mutans (Simpson & Russell, 1998), *Lactococcus lactis* (Luesink *et al.*, 1998) and *Lb. delbrueckii* subsp. *bulgaricus* (Morel *et al.*, 1999), two genes encoding CcpA and PepQ homologues were found to be arranged similarly to *pepR1* and *pepQ* of *Lb. delbrueckii* subsp. *lactis*.

In the present communication we show that *PepR1* fulfils a CcpA-like physiological function, that it modulates transcription from the *pepQ* promoter, and that it specifically binds to DNA sequences derived from several genes of the proteolytic system.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Media used were LB medium (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl) for *Escherichia coli*, B medium (1 % peptone, 0.5 % yeast extract, 0.5 % NaCl, 0.1 % K_2HPO_4) for *Staphylococcus xylosus* and CDM (chemically defined medium; Ledesma *et al.*, 1977) or MRS medium (De Man *et al.*, 1960) for *Lb. delbrueckii*. Antibiotics and supplements were added to the media as required: kanamycin at 40 $\mu\text{g ml}^{-1}$,

ampicillin at 200 $\mu\text{g ml}^{-1}$, tetracycline at 30 $\mu\text{g ml}^{-1}$, chloramphenicol at 20 $\mu\text{g ml}^{-1}$, erythromycin at 2.5 $\mu\text{g ml}^{-1}$ and thymidine at 100 $\mu\text{g ml}^{-1}$.

Recombinant DNA techniques and DNA sequencing. Standard techniques were applied for the cloning, PCR amplification and Southern blotting of DNA, and for purification of DNA fragments from gels (Sambrook *et al.*, 1989). Plasmid DNA was prepared by the method of Birnboim & Doly (1979) except that, in the case of *Sta. xylosus*, lysostaphin (0.1 mg ml^{-1} final concentration) was added to the lysis mixture. Restriction endonucleases, nucleic acid modifying enzymes (Boehringer Mannheim and New England Biolabs) and ULTma DNA polymerase (Perkin Elmer) were used as recommended by the manufacturers. *Escherichia coli* and *Sta. xylosus* were transformed by electroporation (Dower *et al.*, 1988; Brückner, 1997) using a Gene Pulser (Bio-Rad). Primers for PCR and nucleotide sequencing were purchased from MWG-Biotech. Automated sequencing with an Applied Biosystems model 373A sequencer was performed on both strands of the respective DNA segment. Synthetic primers were designed from the known sequences of the vector and the DNA insert. Nucleotide and amino acid sequences were analysed by the Microgenie (Beckman), Husar (Geniusnet) and PC/Gene (IntelliGenetics) programs.

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant features	Reference/source
Strains		
<i>Escherichia coli</i>		
DH5 α	<i>endA recA hsdR gyrA</i>	Hanahan (1983)
CM89	$\Delta(\text{pepD-phoA})$ <i>pepA pepB pepN pepQ</i>	Miller & Schwartz (1978)
TB1	$\Delta(\text{lac-proAB})$ <i>rpsL</i> ($\Phi 80$ <i>lacZ</i> Δ M15) <i>hsdR</i>	Johnston <i>et al.</i> (1986)
<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i>		
DSM 7290	Isolated from Emmentaler cheese	Klein <i>et al.</i> (1995)
<i>Staphylococcus xylosus</i>		
C2a	Wild-type	Götz <i>et al.</i> (1983)
TX154	C2a <i>ccpA::ermB</i>	Egeter & Brückner (1996)
Plasmids		
pRB473	Ap^r Cm^r Km^r Bm^r , ColE1 origin, pUB110 origin	Egeter & Brückner (1996)
pR1473	pRB473 fused to 1330 bp fragment containing <i>pepR1</i> from pUR1	This study
pFN476	Ap^r , pSC101 origin	Sankar <i>et al.</i> (1993)
pFNP13	pFN476 fused to 1325 bp PCR product containing <i>pepR1</i>	Stucky <i>et al.</i> (1996)
pMS1	Km^r , <i>pepQ</i> ⁺ <i>pepR1</i> ⁺ , pSC105 origin	Stucky <i>et al.</i> (1995a)
pMC1871	Tc^r , 'lacZ', ColE1 origin	Shapira <i>et al.</i> (1983)
pMCPQ2	pMC1871 fused to 123 bp PCR product containing <i>pepQ</i> upstream region	This study
pMCPQ3	pMC1871 fused to 123 bp PCR product containing mutated <i>pepQ</i> upstream region	This study
pUR1	Ap^r , <i>pepR1</i> ⁺ , ColE1 origin	Stucky <i>et al.</i> (1996)
pMal-c2	Ap^r , $P_{\text{tac}}::\text{malE-lacZ}'$, ColE1 origin	di Guan <i>et al.</i> (1988)
pR1Mal	pMal-c2 fused to 1012 bp PCR product containing <i>pepR1</i>	This study
pBR322	Ap^r Tc^r , ColE1 origin	Sutcliffe (1979)
pJK433	Km^r , <i>pepX</i> ⁺ , pSC105 origin	Meyer-Barton <i>et al.</i> (1993)
pJK502	Km^r , <i>pepI</i> ⁺ , pSC105 origin	Klein <i>et al.</i> (1994)
pKS1	Km^r , <i>brnQ</i> ⁺ , pSC105 origin	Stucky <i>et al.</i> (1995b)

Plasmid construction. To subclone *pepR1*, the gene, together with 102 nt upstream and 229 nt downstream sequence, was excised from pUR1 as a 1330 bp *KpnI*–*Bam*HI fragment and inserted between the *Bam*HI and *KpnI* sites of the shuttle vector pRB473. The resulting plasmid, pR1473, initially identified in transformants of *E. coli* DH5 α , was introduced into *Sta. xylosus* C2a and TX154.

Transcriptional fusions of the *pepQ* promoter region (P_{pepQ}) with a promoterless *lacZ* reporter gene were constructed by cloning a 123 bp fragment, covering the first 18 nt of the *pepQ* coding sequence and 105 upstream nt (see Fig. 3b) and a variant of this fragment (P_{pepQ^*}) with a CG $\rightarrow$ AT exchange at positions 81/82 upstream of the *pepQ* start codon, into the *Sma*I site of the pMC1871 vector, leading to the plasmids pMCPQ2 and pMCPQ3. The 123 bp fragments were amplified by PCR from plasmid pMS1, using the primers 5'-TAATTTGTCTAAATTCATGGCAAATTC-3' and 5'-CATGAGAACATTTTGTGCAATCG($\rightarrow$ TA)CTTA-CAG-3' (underlined nucleotides were exchanged in the primer used for construction of pMCPQ3).

To construct a *pepR1::malE* gene fusion (di Guan *et al.*, 1988), a 1019 bp DNA fragment including the *pepR1* coding sequence together with 8 upstream and 12 downstream nt was amplified by PCR from plasmid pUR1 by using the primers 5'-cgggatccATGAATAAGCAAGATGTAACCATTTACG-3' and 5'-aaactgcagTTACTTGTTCAGGTACTTTGCTT-CTTG-3' (nucleotides in lower case were added to introduce the underlined *Bam*HI and *Pst*I sites) and, after *Bam*HI/*Pst*I digestion, was ligated to *Bam*HI/*Pst*I-cut plasmid pMAL-c2 (New England Biolabs). The integrity of the fusion gene in the resulting construct, pR1Mal, was examined by sequencing a segment of the resulting plasmid spanning the entire *pepR1* coding region and its upstream and downstream junctions with the vector. pR1Mal allows for transcriptional control of the *pepR1::malE* gene fusion by the *tac* promoter and for cleavage of its hybrid protein product by the factor Xa protease (Nagai & Thøgersen, 1987).

Enzyme assays. To determine the activity of α -glucosidase in strains of *Sta. xylosus*, bacteria were grown in B medium supplemented with appropriate sugars, and cell-free extracts were prepared and assayed as previously described by Egeter & Brückner (1996). The reactions contained 15–600 μ g total soluble protein, as determined by the method of Spector (1978). Specific enzyme activities were calculated as nmol nitrophenol released from the respective chromogenic substrates (mg protein) $^{-1}$ min $^{-1}$.

To assay PepQ, cell-free extracts were prepared from *Lb. delbrueckii* subsp. *lactis* after centrifugation, washing the cells with 50 mM Tris/HCl, pH 7.5, and resuspending them in two cell-wet-weight equivalents of the same buffer. After mixing with glass beads (5 cell-wet-weight equivalents, 0.17 mm diameter), cells were disrupted by agitation in a Vibrogen cell mill (Sauer) for 15 min at 4 °C and maximum speed. The extracts, obtained after removal of debris by centrifugation (15 min, 4 °C, 15000 g), contained about 10 mg total soluble protein ml $^{-1}$, as determined by the method of Spector (1978). PepQ activities in the extracts were measured with Leu-Pro as a substrate and ninhydrin reagent, according to Morel *et al.* (1999). The units (U) of specific PepQ activity were defined as nmol proline liberated (mg total protein) $^{-1}$ min $^{-1}$. Transformants of *E. coli* were assayed for β -galactosidase (Miller, 1972) after washing the cells once with 0.9% NaCl.

Purification of the PepR1 protein. Expression of the *pepR1::malE* fusion was induced in a growing culture of *E.*

coli TB1(pR1Mal) by the addition of IPTG (0.3 mM final concentration) at OD₆₀₀ 0.5. Two hours after induction, bacteria were harvested by centrifugation (20 min, 4 °C, 3000 g), washed in buffer A (20 mM Tris/HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA), resuspended in 10 ml of the same buffer per gram of cells (wet weight), and disrupted by ultrasonication. Debris was removed by centrifugation for 30 min at 4 °C and 10000 g, and the supernatant, after adjusting its protein content to 2.5 mg ml $^{-1}$ (Spector, 1978), was subjected to affinity chromatography through an amylose resin (New England Biolabs). The column (2.5 $\times$ 10 cm, 15 ml bed volume, equilibrated with buffer A) was loaded with the extract and washed with 8 vols buffer A. The fusion protein was eluted with 10 mM maltose in the same buffer at a flow rate of 1 ml min $^{-1}$ and, of 30 2 ml fractions collected, those containing the protein peak were pooled. The resulting solution, containing 15 mg protein at a concentration of 1.1 mg ml $^{-1}$, was treated with 0.5 mg factor Xa for 14 h at 22 °C to cleave the PepR1–MalE fusion. The sample was dialysed against 20 mM Tris/HCl, pH 8.0, 25 mM NaCl, and subjected to ion-exchange chromatography on a Q-Sepharose Fast Flow (Pharmacia Biotech) column (1 $\times$ 10 cm, 5 ml bed volume), equilibrated with buffer B (10 mM Tris/HCl, pH 8.0, 25 mM NaCl). After application of the protein solution, the column was washed with 5 vols buffer B and the proteins were eluted with a gradient of 25–500 mM NaCl (25 ml each) in 20 mM Tris/HCl, pH 8.0. Fractions (1 ml) containing the separated PepR1 protein were identified by analysing aliquots (5 μ l) on SDS-polyacrylamide gels.

Preparation of promoter-containing DNA fragments. DNA fragments covering the promoter regions of the *pepQ* gene (positions –153 to +17 from the first nt of the start codon), the *pepX* gene (positions –288 to –5 from the first nt of the start codon), the *pepI* gene (positions –127 to +59 from the first nt of the start codon), and the *brnQ* gene (positions –171 to +12 from first nt of the start codon) were amplified by PCR from the plasmids pMS1 (Stucky *et al.*, 1995a), pJK433 (Meyer-Barton *et al.*, 1993), pJK502 (Klein *et al.*, 1994) and pKS1 (Stucky *et al.*, 1995b) by using the primer pairs 5'-AATAACAGCCCTACCTATCTAAATAC-3'/5'-TAAT-TTGTCTAAATTCATGGCAAATTC-3', 5'-GTGTT-CAATTTATTTCTTGCAA-3'/5'-TACCTCATCTTTAT-ATCTTTACATCTATGG-3', 5'-ACCAGTTAAAGTCT-GTTTCA-3'/5'-CAGTAGGTTTGCCAATTTCC-3', and 5'-GCCTGGTACTTTTCAAAGGGG-3'/5'-GCTTTTC-TTTCATGTTGATCC-3', respectively. As a control, an internal fragment of the *bla* gene (positions +586 to +732 from the first nt of the start codon) was amplified from pBR322 by using the primers 5'-CTACTTACTCTAGCT-TCCCGGC-3' and 5'-CAATGATACCGGAGACCCACG-3'. PCR products were non-radioactively labelled as follows. To generate 3'-recessed ends, the DNA samples (1 μ g) were combined with 1 U T4 DNA polymerase in 20 μ l reaction volumes, containing 0.1 mM digoxigenin-11-dUTP (Boehringer Mannheim), 1 mM Tris/HCl, pH 7.5, 1 mM MgCl₂, 5 mM NaCl and 0.1 mM DTT, and incubated for 10 min at 37 °C. After addition of 1 μ l 1 mM (each) dATP, dCTP and dGTP, DNA ends were filled in during a further incubation at 37 °C for 10 min. Reactions were stopped by heating at 75 °C for 10 min, and the DNA was precipitated with ethanol and redissolved in 20 μ l water.

Gel-retardation assays. Of the individual promoter-containing DNA fragments, 2 ng were incubated with 1.6 μ g purified PepR1 protein for 20 min at room temperature in 20 μ l reaction volumes containing 20 mM Tris/HCl, pH 8.0, 20% (v/v) glycerol, 1 mM EDTA, 200 mM KCl, 1 mM DTT, 2 μ g

BSA and 1 ng sonicated herring sperm DNA. The samples were loaded at 100 V on 6% polyacrylamide gels in 40 mM Tris/HCl, pH 8.0, 20 mM acetic acid, 2 mM EDTA, 3% (v/v) glycerol and run at 150 V for 2.5 h. After blotting the DNA to positively charged nylon membranes, bands were visualized by chemiluminescence using a commercially available digoxigenin detection kit (Boehringer Mannheim).

Northern analysis. Total RNA (10 µg), prepared from culture aliquots of *Lb. delbrueckii* subsp. *lactis* according to Chomczynski (1994), was separated on 1.2% agarose, 1.1% formaldehyde gels and blotted to positively charged nylon membranes (Boehringer Mannheim) (Sambrook *et al.*, 1989). A *pepQ*-specific probe of 1107 bp was amplified by PCR from plasmid pMS1 by using the primers 5'-ATGAATTTAGAC-AAATTACAAAAGCTGGCTGCAGGAAAACGG-3' and 5'-TTATTCCTTAAGTGGCAGAACCTTCAATTCCTTGCTA-3'. After random-primed labelling with [α^{32} P]dCTP (High Prime kit; Boehringer Mannheim), the probe was used in Northern hybridizations as described by Church & Gilbert (1984).

RESULTS AND DISCUSSION

PepR1 complements a *ccpA* mutation

On the basis of sequence comparisons, PepR1 of *Lb. delbrueckii* subsp. *lactis* DSM 7290 falls into the LacI/GalR family of transcription regulators (Weickert & Adhya, 1992). PepR1 shares significant sequence identity with CcpA proteins from *Lactobacillus casei* ATCC 393 (50.5%) (Monedero *et al.*, 1997), *Bacillus megaterium* (44.8%) (Hueck *et al.*, 1995), *Sta. xylosus* (44.7%) (Egeter & Brückner, 1996), *Bacillus subtilis* (43.9%) (Henkin *et al.*, 1991), and with the CcpA homologue RegM from *Str. mutans* (49.6%) (Simpson & Russell, 1998). To check the functional significance of these similarities, we subcloned the *pepR1* gene into the *E. coli*/*Bacillus* shuttle vector pRB473. The resulting plasmid, pR1473, was used to perform complementation studies in a *ccpA* knock-out mutant of *Sta. xylosus*

TX154, and expression of the *malRA* operon, known to be subject to CcpA-dependent catabolite repression in this organism (Egeter & Brückner, 1996), was examined by assaying the α -glucosidase activity of MalA. A wild-type strain, *Sta. xylosus* C2a, showed about fivefold repression of α -glucosidase in the presence of 25 mM glucose; this repression was completely abolished in the *ccpA* mutant (Table 2). Expression of the cloned *pepR1* gene from plasmid pR1473 in the *ccpA* mutant was sufficient to relieve the deregulation of α -glucosidase to an extent that allowed for almost threefold repression of α -glucosidase by glucose. This clearly indicated that PepR1 of *Lb. delbrueckii* subsp. *lactis* DSM 7290 can interact in a CcpA-like manner with elements of the signal-transduction chain, which mediate catabolite control in Gram-positive bacteria (Hueck & Hillen, 1995). It is therefore possible that PepR1 is not only operative in *Sta. xylosus* but that it might be the functional equivalent of CcpA in *Lb. delbrueckii*. The fact that PepR1 achieved only partial complementation of the CcpA defect in *Sta. xylosus*, is most probably related to structural differences between the two proteins. These differences may account for less specific interactions of PepR1 with the HPr component of the phosphotransferase system (Deutscher *et al.*, 1995) and with DNA target sites (*cre* sequences) (Fujita *et al.*, 1995) in the heterologous *Sta. xylosus* host. In Fig. 3b, the upstream sequence of *Sta. xylosus malRA*, including the putative CcpA interaction site *cre* (Egeter & Brückner, 1996), is compared with upstream regions of *Lb. delbrueckii* genes that were found to specifically bind PepR1 (see below).

PepR1 regulates *pepQ* expression

From the fact that the *pepR1* and *pepQ* genes are closely linked, we previously suspected that PepR1 might exert a regulatory effect on the expression of *pepQ* (Stucky *et al.*, 1996). This was supported by the observation that in initial attempts to identify *pepQ* in a plasmid library of the DSM 7290 genome, only plasmids which, in addition to *pepQ*, contained at least the 5' part of *pepR1* (nt 1–470) were able to complement a prolidase mutation in *E. coli* (Stucky *et al.*, 1995a). This part covers the region (nt 19–84) that encodes the predicted DNA-binding motif of PepR1. To investigate a possible influence of PepR1 on *pepQ* transcription, we fused a DNA fragment (position –77 to +45 from the transcription-start site; see Stucky *et al.*, 1995a), carrying the assigned *pepQ* promoter (P_{pepQ}), with the promoterless *lacZ* gene of the vector pMC1871. Transcription of the $P_{\text{pepQ}}::\text{lacZ}$ fusion from the resulting construct pMCPQ2 was monitored by measuring β -galactosidase activity in transformants of *E. coli* expressing *pepR1* from its own promoter in the compatible plasmid pFNP13 (Stucky *et al.*, 1996). Fig. 1 shows that PepR1 activated the expression of the $P_{\text{pepQ}}::\text{lacZ}$ fusion about twofold in this system, as compared with a control culture containing the unmodified vector pFN476 instead of pFNP13. This effect remained nearly constant throughout the growth of the bacteria and therefore

Table 2. Complementation of a *ccpA* defect in *Sta. xylosus* by *pepR1* of *Lb. delbrueckii* subsp. *lactis*

Cells were grown at 37 °C in B medium or in B medium containing 25 mM glucose. At an OD₅₇₈ 2.5, cell-free extracts were prepared from 40 ml culture aliquots and used to determine α -glucosidase activities with *p*-nitrophenyl α -D-glucopyranoside (10 mM) as a substrate. Specific activities [nmol nitrophenol released min⁻¹ (mg protein)⁻¹] and standard deviations represent the results of at least three independent experiments. Repression factors (RF) were calculated as ratios of specific α -glucosidase activities measured in the absence and in the presence of glucose, respectively.

<i>S. xylosus</i> strain (plasmid)	α -Glucosidase specific activity		RF
	– Glucose	+ Glucose	
C2a(pRB473)	12.7 ± 1.6	2.40 ± 0.3	5.3
TX154(pRB473)	11.36 ± 0.5	11.16 ± 0.5	1.02
TX154(pR1473)	11.98 ± 0.6	4.30 ± 0.1	2.8

Fig. 1. Influence of PepR1 on transcription from variants of the *pepQ* promoter. Transformants of *E. coli* CM89 (Δlac), carrying pMCPQ2 ($P_{pepQ}::lacZ$) and pFN476 (■), pMCPQ2 ($P_{pepQ}::lacZ$) and pFN476 (●), pMCPQ3 ($P_{pepQ}::lacZ$) and pFN476 (○), or pMCPQ3 ($P_{pepQ}::lacZ$) and pFN476 (□), were grown at 37 °C in LB broth supplemented with ampicillin and tetracycline. Transcription from P_{pepQ} and P_{pepQ}^* was monitored by assaying β -galactosidase in aliquots of the cultures.

appeared to be independent of the growth phase. To check the significance of the *cre*-like palindrome in the *pepQ* promoter as a site of potential PepR1–DNA interaction, we constructed a pMCPQ2 analogue, pMCPQ3, in which the central dinucleotide 5′-CG-3′ of the original *cre*-like sequence was changed to 5′-AT-3′. Due to this exchange, the modified sequence differed in 3 of 14 nt from the *cre* consensus, 5′-TGWNANCG-NTNCA-3′, established by Weickert & Chambliss (1990). In the *lacZ* reporter-gene assay, this exchange almost completely abolished transcription activation from the modified promoter region P_{pepQ}^* by PepR1. From these results, we concluded that PepR1 modulates the initiation of transcription at the *pepQ* promoter and that the *cre*-like element plays an essential role in this interaction. In addition, *cre* may also be involved in some kind of autoregulation of PepR1, since the element was found to overlap the assigned –35 region of the *pepR1* promoter (Stucky *et al.*, 1996).

PepR1 interacts with promoter regions

To investigate whether PepR1 can directly interact with susceptible promoters or genes, we purified the protein by constructing an N-terminal fusion with MalE (di Guan *et al.*, 1988) (Fig. 2). Verification of the fusion by sequencing the respective region of the hybrid plasmid pR1Mal revealed a T at position 212 of the *pepR1* coding sequence instead of the previously reported C (Stucky *et al.*, 1996). After cleavage of the fusion protein and removal of the MalE moiety (Fig. 2), purified PepR1 was used to perform gel-mobility-shift assays with a 172 bp DNA fragment containing the *pepQ* promoter region. This DNA–protein complex was almost completely retarded in the gel, whereas an arbitrary control DNA fragment was not shifted (Fig. 3a).

Fig. 2. Purification of the PepR1 protein. Purification steps were (lane 1) preparation of crude extract of *E. coli* TB1, carrying the *pepR1::malE* fusion plasmid pR1Mal, (lane 2) affinity chromatography on amylose resin, (lane 3) cleavage with factor Xa, and (lanes 4 and 5) ion exchange chromatography on Q-Sepharose (lane 4, purified PepR1; lane 5, purified MalE). After each individual step, samples were subjected to SDS 12% polyacrylamide gel electrophoresis. Sizes of protein standards (lane M) are given in kDa.

Since CcpA proteins of other Gram-positive bacteria are global regulators, modulating the expression of numerous genes or operons (Hueck *et al.*, 1995), we also checked the affinity of PepR1 for the upstream and 5′-terminal regions of other genes of the proteolytic system of *Lb. delbrueckii* subsp. *lactis* DSM 7290. In gel-retardation assays (Fig. 3a), PepR1 was found to specifically interact with promoter-containing fragments of two additional peptidase genes, *pepX* and *pepI* (Meyer-Barton *et al.*, 1993; Klein *et al.*, 1994), and of the *brnQ* gene, which encodes a branched-chain amino acid transporter (Stucky *et al.*, 1995b). Under the conditions used, PepR1 showed distinct affinity for the *pepI* and *brnQ* fragments whereas the *pepX* fragment was only partially shifted.

Interaction of PepR1 with multiple promoter regions from the chromosome of *Lb. delbrueckii* strongly suggested that the protein is involved in the coordinate transcriptional control of a set of susceptible genes. This set seems to include various functions related to the production and uptake of protein cleavage products. Searching for a physical basis for specific PepR1–DNA recognition, we found *cre*-like sequences in all of the PepR1-binding DNA fragments (Fig. 3b). They match the *cre* consensus for *B. subtilis* (Weickert & Chambliss, 1990) in at least 10 out of 14 positions and are located at distances of 16, 39 and 22 nt upstream of the assigned –35 regions of the *pepQ*, *pepI* and *brnQ* promoters, or between the –35 and –10 regions of the *pepX* promoter. An additional *cre*-like element overlaps the translation-start codon of *pepI*. Together with the clear effect of altering the *cre*-like sequence upstream of *pepQ* on PepR1-dependent control of the *pepQ* promoter (see Fig. 1), the occurrence of potential *cre* sites in additional PepR1-binding DNA sequences indicates that *cre* is directly involved in PepR1–DNA interactions. Differences between the primary sequences of individual *cre* elements and between their locations relative to the transcription-start sites of the respective genes may contribute to determining the efficiency of PepR1 binding and action. In general, *cre* sites of genes activated by CcpA proteins are located upstream of the

Fig. 3. (a) Gel retardation of promoter-containing DNA fragments by PepR1. Digoxigenin-labelled DNA fragments (2 ng), covering the assigned promoters of *pepQ* (lanes 3, 4), *pepX* (lanes 5, 6) and *brnQ* (lanes 9, 10) were mixed with 2 ng of a labelled negative-control fragment ('bla') and incubated at room temperature for 20 min in the absence (lanes 3, 5, 7, 9) or in the presence (lanes 4, 6, 8, 10) of purified PepR1 protein (1.6 µg). As a control, the 'bla' fragment alone (2 ng) was incubated without (lane 1) or with (lane 2) PepR1 (1.6 µg). After 6% polyacrylamide gel electrophoresis, the bands were blotted to nylon membranes and visualized by chemiluminescence. (b) *cre*-like sequences in promoter-containing DNA fragments. The nt sequences of sections of the DNA fragments shown in (a) were aligned by the previously determined transcription-start sites (indicated by the angled arrow) of the *pepQ*, *pepX*, *pepI* and *brnQ* genes (Matern *et al.*, 1994). Numbers of the first and last residues of the entire fragments used in gel retardation assays (a) are given. Numbering refers to the respective GenBank entries Z54205 (*pepQ*), Z14230 (*pepX*), Z26948 (*pepI*) and ZW48676 (*brnQ*). For comparison, the corresponding upstream section of the *malRA* genes of *Sta. xylosus* (X78853) (Egter & Brückner, 1996) is also shown. Potential *cre* sequences are displayed in black boxes; -10 and -35 regions are indicated with open boxes; translation-initiation codons and putative ribosome-binding sites are underlined.

promoters, whereas for genes repressed by CcpA they are found within the promoters or at the 5' end of the respective genes or operons (Henkin, 1996). The location of a *cre*-like sequence upstream of the *pepQ* promoter (Fig. 3a) and the stimulating effect of PepR1 on *pepQ* transcription (Fig. 1) are in agreement with this general principle. From this, one might expect that *pepX* (where *cre* is within the promoter) would be repressed whereas *brnQ* (where *cre* is upstream of the promoter) would be activated by PepR1.

PepQ is subject to catabolite control

To examine whether the expression or the activity of *pepQ* or its product are effectively modulated by the nature of the carbon source, we cultivated *Lb. del-*

brueckii subsp. *lactis* DSM 7290 in different media in the presence of either glucose or lactose (Fig. 4a). Fig. 4b shows the specific PepQ activities measured in cell-free extracts prepared at different times during growth in chemically defined medium supplemented with 2% Casamino acids (Difco). During the exponential, late-exponential and stationary phases, the specific PepQ activities determined in the presence of glucose were 1.8-, 2- and 1.7-fold higher than those measured with lactose. This effect was reproducible in a number of experiments, and it was similar when other culture media (chemically defined medium without Casamino acids or MRS medium) were used. As estimated from Northern blots (Fig. 4c), the amount of *pepQ* transcripts correspondingly varied in response to the carbon source used. This indicated that the observed differences in

Fig. 4. Catabolite effects on PepQ activity and expression. (a) *Lb. delbrueckii* subsp. *lactis* DSM 7290 was grown at 42 °C in chemically defined medium supplemented with 2% Casamino acids and 2% glucose (solid line) or lactose (broken line), respectively. (b) At the times indicated by arrows, specific PepQ activities were determined in cells harvested from the glucose-grown (dotted bars) and the lactose-grown (hatched bars) cultures. (c) RNA prepared from the respective culture aliquots was used in Northern hybridizations with a *pepQ* probe.

PepQ activity were at least partly due to a modulation of *pepQ* transcription. In the exponential and stationary phases, the catabolite effects on *pepQ* transcription and PepQ activity, however, were not of the same magnitude, suggesting that post-transcriptional steps might be involved in regulation of PepQ expression.

It is therefore tempting to speculate that catabolite control of PepQ activity in *Lb. delbrueckii* is linked to the observed interaction of PepR1 with the *pepQ* promoter and to the glucose repression that PepR1 mediated in *Sta. xylosus*. Definitive proof that PepR1 is directly involved in catabolite effects in *Lb. delbrueckii*, however, might have to await the construction of appropriate *pepR1* mutants.

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