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In vitro DNA binding of purified CcpA protein from *Lactococcus lactis* IL1403★

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During this study His-tagged CcpA protein purified under native conditions to obtain a biologically active protein was used for molecular analysis of CcpA-dependent regulation. Using electrophoretic mobility shift assays it was demonstrated that CcpA of *L. lactis* can bind DNA in the absence of the HPr-Ser-P corepressor and exhibits DNA-binding affinity for nucleotide sequences lacking *cre* sites. However, purified HPr-Ser-P protein from *Bacillus subtilis* was shown to slightly increase the DNA-binding capacity of the CcpA protein. It was also observed that CcpA bound to the *cre* box forms an apparently more stable complex than that resulting from unspecific binding. Competition gel retardation assay performed on DNA sequences from two PEP:PTS regions demonstrated that the *ybhE*, *bglS*, *rheB*, *yebE*, *ptcB* and *yecA* genes situated in these regions are most probably directly regulated by CcpA.

Keywords: CcpA, DNA binding, EMSA, competition EMSA, carbon catabolite repression

INTRODUCTION

In Gram-positive bacteria carbon catabolite repression is exerted through binding of the CcpA protein to *cis*-acting catabolite responsive element (*cre*). The *cre* site was first identified in *Bacillus subtilis* (Nicholson *et al.*, 1987; Miwa & Fujita, 1990). The 14-nucleotide consensus sequence TGWNANCGNTNWCA (W, adenine or thymine) was proposed based on site-directed mutagenesis studies (Weickert & Chambliss, 1990). CcpA interacts with the HPr-Ser-P protein, which distinguishes this regulator from other regulators of the LacI/GalR family that interact with low molecular mass compounds (Schumacher *et al.*, 2004). In *B. subtilis,* the complex of CcpA and seryl-phosphorylated HPr protein can bind to *cre* elements located in the vicinity of regulated genes. The physiological effect depends on the position of the *cre* site in respect to the regulated gene (Titgemeyer & Hillen, 2002). CcpA acts as an activator when the *cre* sequence is located upstream of the promoter sequence of the regulated gene. On the other hand, binding of this regulator to *cre* located either in the promoter region of catabolic genes or situated within the coding sequence prevents, respectively, the initiation of transcription or transcript elongation.

Crystal structures for the *B. megaterium* apoCcpA protein and the CcpA-(HPr-Ser-P)–DNA complex were reported recently (Schumacher *et al.*, 2004). These results showed that CcpA has a three-dimensional structure similar to the regulators of the LacI/GalR family and confirmed the presence of a helix-turn-helix (HTH) DNA-binding N-terminal domain,

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Abbreviations: BSA, bovine serum albumin; CcpA-His-tagged, CcpA protein with 6 His residues at its N-terminus; *cre*, catabolite responsive element; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assays; FBP, D-fructose-1,6-bisphosphate; HPr-Ser-P, serine-46-phosphorylated HPr protein; HTH, helix-turn-helix; PEP:PTS, phosphoenolpyruvate-dependent phosphotransferase system.

an effector binding site and a C-terminal dimerisation domain (Henkin *et al.*, 1991; Weickert & Adhya, 1992; Schumacher *et al.*, 2004).

The *Lactococcus lactis* subsp. *lactis ccpA* gene (Gene Bank accession No. AF106673) was identified and sequenced in our group (Aleksandrzak *et al.*, 2000). The 37 kDa gene product shares 50% identity with CcpA from *B. subtilis* (Henkin *et al.*, 1991; UniProtKB/Swiss-Prot P25144), while the identity to CcpA from *L. lactis* ssp. *cremoris* (Luesink *et al.*, 1998; UniProtKB/TrEMBL Q9ZAE0) reaches 96%. Most data on the CcpA proteins and their interactions come from studies of CcpA in the *Bacillus* species. To our knowledge, there are no data describing interaction of *L. lactis* CcpA with DNA.

In this study, we used His-tagged CcpA protein from *L. lactis* subsp. *lactis* IL1403, which was overproduced in *Escherichia coli*, purified under native conditions (Kowalczyk & Bardowski, 2003), and subsequently used for molecular analysis of its interactions with DNA.

To analyse the DNA-binding ability of the CcpA protein from *L. lactis* we used electrophoretic mobility shift assays (EMSA). Our results showed that CcpA of *L. lactis* can bind DNA in the absence of the HPr-Ser-P corepressor and is able to bind DNA lacking *cre* sites. However, it seems that CcpA has a higher affinity for *cre*-containing fragments and that HPr-Ser-P slightly increases the DNA-binding ability of the CcpA protein.

In the *L. lactis* IL1403 genome several thousand *cre*-like sequences can be detected, yet most of them have no biological significance because of their unsuitable location (Guédon *et al.*, 2002). We found that CcpA is involved in the regulation of several genes coding for proteins engaged in cellobiose and lactose transport or catabolism in *L. lactis* (Kowalczyk *et al.*, unpublished). These genes are localised in two different regions within the *L. lactis* genome, which for the purpose of this publication were termed "PEP:PTS regions". A search for the *L. lactis cre* TGNNANCGNTNNCA motif proposed by E. Guédon and coworkers allowing one or two mismatches except for the central CG base pairs (Guédon *et al.*, 2002), showed several *cre*-like sites in the PEP:PTS regions. To elucidate whether the CcpA effect is direct or indirect, binding of CcpA to *cre*-containing DNA fragments located within two PEP:PTS regions was studied (Fig. 1). The obtained results demonstrated that some of the analysed genes might be directly regulated by the CcpA protein.

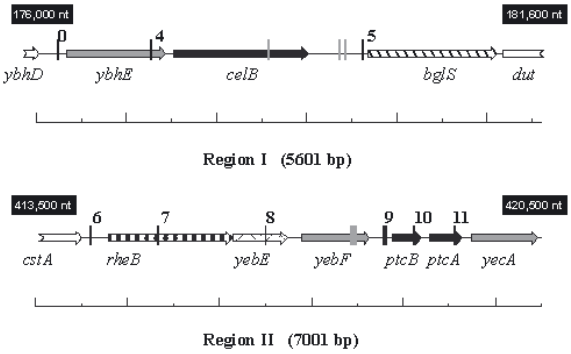

Figure 1. The genetic organisation of two PEP:PTS regions in the *L. lactis* IL1403 genome.
Sequences are derived from the *L. lactis* IL1403 genome (AE005176). The potential *cre* elements deviating from the *cre* consensus TGNNANCGNTNNCA by a maximum of two mismatches, but not in the central CG base pairs, were identified by sequence analysis. The *cre* elements that were further analysed in this study are marked as vertical black lines whereas other *cre* sites are marked as vertical grey lines. Genes encoding proteins homologous to the A, B and C components of the PEP:PTS system are denoted in black and those homologous to hypothetical regulators in grey. Numbering of *cre* sites given here refers directly to the numbering of lanes in Fig. 7. Point marked as "0" represents *ybhE cre*.

MATERIALS AND METHODS

Recombinant DNA and PCR methods. General recombinant DNA methods were as described previously (Sambrook *et al.*, 1989). DNA fragments were amplified by PCR with 1 U of *Taq* polymerase (Polgen) using appropriate primers presented in Table 1.

The PCR reaction was performed for 30 cycles at 95°C for 1 min, 55°C for 30 s and 72°C for 30 s. PCR products were purified with Montage PCR Centrifugal Filter Devices (Millipore). DNA was quantified by gel electrophoresis with Smart Ladder (Eurogentec) and also spectrophotometrically at 260 nm (1 A_{260}= 50 μg/ml).

The oligonucleotides for electrophoretic mobility shift assay were obtained from the DNA Sequencing and Oligonucleotide Synthesis Lab of our Institute. To produce double-stranded DNA fragments, complementary oligonucleotides at 100 ng each were mixed and incubated at 95°C for 3 min in a water bath and then cooled down slowly to the room temperature.

DNA-binding assays. Various amounts of purified His-tagged CcpA protein (Kowalczyk & Bardowski, 2003) were incubated with DNA in buffer A (12 mM Hepes, 4 mM Tris/HCl, 12 mM KCl, 1 mM EDTA, 1 mM DTT) with 25 mM D-fructose-1,6-bisphosphate and 10% glycerol (pH 7.9) or buffer B (100 mM Tris/HCl, 1 mM EDTA) with 25 mM D-fructose-1,6-bisphosphate (FBP) and 10% glycerol (pH 7.5), for 20 min at 30°C in a final volume of 20 μl and the probes were immediately loaded onto the gel.

For the DNA binding in the presence of HPr-Ser-P and competition experiments, DNA was 5′-

Table 1. Oligonucleotide primers used for Electrophoretic Mobility Shift Assay

Name	Oligonucleotide*
ybhEcref	5′-ATCAGGCGATTCTTCGTGAATA-3′
ybhEcrer	5′-TGAGCGTTCTGGATAGATAGAT-3′
nocref2	5′-ACCTCCAATTGCTCCAGAAA-3′
nocrer2	5′-GTCAAGGCCTTAAACTC-3′
ocreybhf	5′-TTTTAAGATGATATCGCTTGCAAAAATAAA-3′
ocreybhr	5′-TTTATTTTTGCAAGCGATATCATCTTAAAA-3′
okcref	5′-CTAATAAAATTAATCATTTTGAAAGCGCTA-ACAAAGTTTTATACGAAG-3′
okcrer	5′-CTTCGTATAAAACTTTGTTAGCGCTTTCA-AAATGATTAATTTTATTAG-3′
onocref	5′-GAGGTCATTAGCAAGATTGAAGACTCCACC-3′
onocrer	5′-GGTGGAGTCTTCAATCTTGCTAATGACCTC-3′
celBcref	5′-GCTTATGCGAGCGAAGAAGAAC-3′
celBcrer	5′-TCATCTGCGATACGTCCAACGA-3′
bglScr1f	5′-CTTATTGCACGCAGCCAA-3′
bglScr1r	5′-TGCCTGGAATACGGACAA-3′
rheBcref	5′-CTGGCCTAGTAAGCTTGA-3′
rheBcrer	5′-GCCTGAGAGAACAATTGG -3′
yebEcref	5′-ACCAGGTCGAATCCATGACT-3′
yebEcrer	5′-GGCGGTACATCACCATGAAT-3′
yebFcref	5′-TACTCAAGAGCGGCTAGA-3′
yebFcrer	5′-CAAGGCTCGGTAAAGATG-3′
ptcBcref	5′-TGACCACGCAACTCCCAGTA-3′
ptcBcrer	5′-CCCGTTATCTGCTGCTGCTT-3′
ptcAcref	5′-TGTGCAGCCGGTATGTCAAC-3′
ptcAcrer	5′-CTCCGCGCATCATTCCATAA-3′
yecAcref	5′-CCTTCCAAGCCATTCAAC-3′
yecAcrer	5′-ACGGACTCGCATATAAGG-3′

*Bolotin *et al.*, 2001.

labelled using T_4 polynucleotide kinase and [γ-^{32}P] ATP (Fermentas), and purified with the QIAquick PCR purification kit (Qiagen). Binding was carried out in 20 µl of buffer A with 25 mM D-fructose-1,6-bisphosphate and 2 µg/ml of sonicated calf thymus DNA to reduce non-specific binding. In competition experiments, after 10 min of incubation at 30°C unlabelled competing DNA was added and incubated for another 10 min. Samples were mixed with 2 µl of 50% glycerol containing 0.1% bromophenol blue and loaded onto a 5% polyacrylamide gel.

Lyophilised HPr-Ser-P of *B. subtilis* was kindly provided by S. Poncet (Laboratoire de Microbiologie et Genetique Moleculaire, INRA-INAPG-CNRS, Thiverval-Grignon, France) and was resuspended in 50 mM Tris/HCl, 300 mM NaCl, pH 8.0 buffer.

DNA electrophoresis. For general purposes, DNA electrophoresis was performed in TAE buffer on agarose gels supplemented with ethidium bromide. The gel concentration and agarose type varied according to the sizes of separated DNA fragments (Sambrook *et al.*, 1989).

For EMSA at 4°C, 1.5% MetaPhor agarose supplemented with ethidium bromide in TBE buffer was used. In the case of EMSA with oligonucleotides the concentration of MetaPhor agarose was increased to 3%. After electrophoresis, gels were photographed under UV light for documentation.

For DNA binding in the presence of HPr-Ser-P and competition experiments the EMSA in 5% polyacrylamide (acrylamide/bis-acrylamide, 82:1) gel was performed. Horizontal gels were pre-run in 0.5×TBE buffer for 30 min at 3 V/cm. After electrophoresis in the same buffer for 60 to 90 min at 10 V/cm, the gel was dried onto filter paper, and radiolabelled bands were visualised by autoradiography.

RESULTS

Model structure of CcpA in complex with both DNA and HPr

As the identity of *B. megaterium* CcpA and *L. lactis* CcpA sequences is lower than 60%, the already determined three-dimensional structures of *B. megaterium* CcpA (apo-form and in complex with HPr and DNA) (Schumacher *et al.*, 2004) cannot be directly extrapolated onto the lactococcal CcpA spatial structure. Yet, the crystallographic data obtained for *B. megaterium* CcpA were used as a basis to create a hypothetical model of the three-dimensional structure of the CcpA protein of *L. lactis*.

Atomic coordinates of the *L. lactis* CcpA 3D model were obtained using the SWISS-MODEL server (Peitsch, 1995; Guex & Peitsch, 1997; Schwede *et al.*, 2003). A model structure of the *L. lactis* CcpA dimer interacting with DNA and HPr was created by superposition of two modelled lactococcal CcpA monomers on the *B. megaterium* CcpA dimer (Fig. 2).

According to the model, the N-terminal domains of the CcpA dimer interact with DNA. Fragments I8–N27 (HTH motif) and A53–S60 (hinge helix) are proposed to directly contact DNA. As the first six N-terminal residues are located outside the protein–DNA interface, the His-tag at the N-terminus should not disturb the CcpA–DNA interactions (Fig. 2B).

These suppositions were recently confirmed by a collaborative study between our group and the group of W. Sanger through analysis of the *L. lactis* CcpA crystal structure (Loll *et al.*, in press). This analysis showed that CcpA residues which are involved in the interaction with DNA or with the HPr protein as well as the overall structure of the protein are conserved between bacilli and lactococci.

Binding of lactococcal CcpA to DNA

It has previously been shown that catabolite repression depends on the CcpA protein and a *cis*-

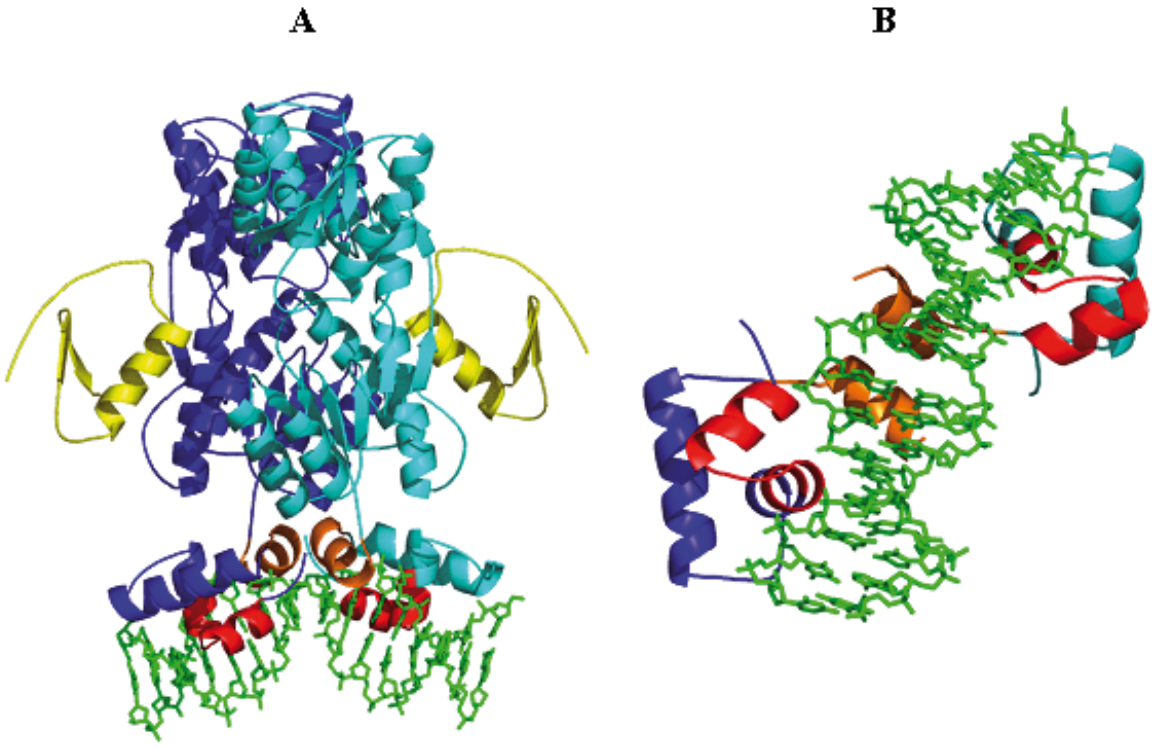

Figure 2. Ribbon representation of the *L. lactis* CcpA model structure in complex with DNA and HPr (A). *L. lactis* CcpA DNA-binding domain (B).
The N-terminus of this domain does not interact with DNA. CcpA monomers are shown in dark and light blue, DNA, green; HPr, yellow. Fragments of CcpA interacting with DNA are coloured: red, HTH motifs; orange, hinge helices.

acting catabolite responsive element (*cre*) located near the promoter region of the regulated gene (Nicholson *et al.*, 1987; Weickert & Chambliss, 1990; Henkin *et al.*, 1991). CcpA has a helix-turn-helix motif responsible for DNA binding and its binding to DNA in *B. subtilis*, *B. megaterium* and lactobacilli was demonstrated by several authors (Miwa *et al.*, 1994; Kim *et al.*, 1995; Jones *et al.*, 1997; Schick *et al.*, 1999; Aung-Hilbrich *et al.*, 2002; Mahr *et al.*, 2002). In this work, the ability of the *L. lactis* CcpA protein to bind DNA was analysed using EMSA (Fig. 3). For this analysis two PCR-generated DNA fragments were synthesised — one of 400 bp (AE005176, 175977..176376 bp) containing a *cre* site consistent with the *cre* consensus (*ybhE cre*) and the second one of 316 bp (AE005176, 2107756..2108071 bp) without *cre* (no-*cre*). Both DNA fragments were separately incubated in buffer A with 2 μg of CcpA or 2 μg of BSA (bovine serum albumin) as a control protein. After electrophoresis of the reaction mixtures both fragments — without *cre* (Fig. 3; lanes 4, 5) and with the *ybhE cre* (Fig. 3; lanes 8, 9) were shifted by the CcpA protein. However, much lower amounts of DNA were retarded in the case of the no-*cre* DNA fragment, for which a smear was visible below the DNA/CcpA complex. The use of BSA in place of CcpA did not lead to formation of any detectable complex (Fig. 3; lanes 3, 7) nor did it significantly change the formation of the DNA/CcpA complex when added at the same time as CcpA (Fig. 3; lanes 5, 9).

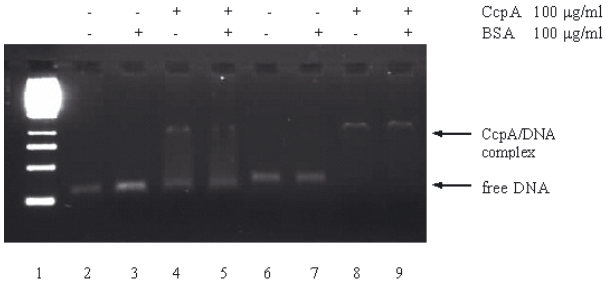

Figure 3. Electrophoretic mobility shift assay using DNA fragments and purified CcpA-His-tagged protein from *L. lactis*.
DNA fragments (25 ng) containing no-*cre* (lanes 2–5) and *ybhE cre* (lanes 6–9), were incubated in buffer A. Lane 1, molecular mass standard 1 kb (Fermentas).

Since retardation of DNA fragments was observed for both no-*cre* and *ybhE cre*, a similar experiment was performed in buffer B used by Aung-Hilbrich *et al.* (2002). In this buffer, discrimination between specific and unspecific binding was expected to be observed. Both DNA fragments, no-*cre* and the *ybhE cre* sequence, were retarded in the presence of CcpA (not shown). However, the amount of DNA shifted was lower than that detected in buffer A.

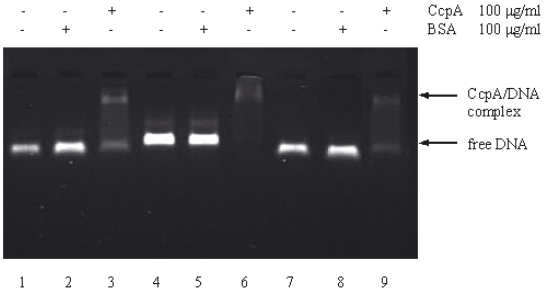

Figure 4. Electrophoretic mobility shift assay using DNA oligonucleotide fragments and purified CcpA-His-tagged protein from *L. lactis*.
Oligonucleotide fragments (200 ng) ono*cre*, not containing *cre* (lanes 1–3), ok*cre*, sequence used by Aung-Hilbrich and coworkers (lanes 4–6) and o*creybhE* containing *cre* sequence from the promoter region of *ybhE* gene (lanes 7–9), were incubated in buffer A.

Hence, to minimise the unspecific binding of CcpA to DNA, short oligonucleotides were used (Fig. 4). Double-stranded oligonucleotides: ono*cre*, containing no *cre* (Fig. 4, lanes 1–3), ok*cre*, the sequence used by Aung-Hilbrich and coworkers (Fig. 4, lanes 4–6), and o*creybhE* containing the *cre* sequence from the promoter region of the *ybhE* gene (Fig. 4, lanes 7–9) were used. All the oligonucleotides tested were shifted (Fig. 4, lanes 3, 6, 9), suggesting that the unspecific binding of CcpA to DNA does not depend on the length of DNA.

Effect of HPr-Ser-P on CcpA binding to DNA

The phosphorylated HPr corepressor protein was shown to be necessary for catabolite repression and formation of a specific ternary complex with *cre*-containing DNA in *B. megaterium* (Jones *et al.*, 1997; Schumacher *et al.*, 2004). Therefore, in order to increase the specific binding of lactococcal CcpA to DNA containing *cre* sites the HPr-Ser-P protein of *B. subtilis* was used. For binding, the concentrations of the CcpA protein that enabled observing partial

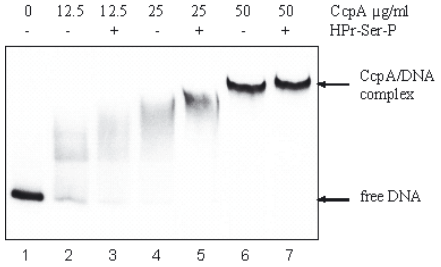

Figure 5. Electrophoretic mobility shift assay using *L. lactis* DNA fragment containing *ybhE cre* with purified *L. lactis* CcpA-His-tagged protein and HPr-Ser-P of *B. subtilis*.
DNA fragment (10 ng) was incubated in buffer A supplemented with 25 mM D-fructose-1,6–bisphosphate, 2 µg/ml sonicated calf thymus DNA and with purified *L. lactis* CcpA protein and HPr-Ser-P of *B. subtilis* (lanes 3, 5, 7).

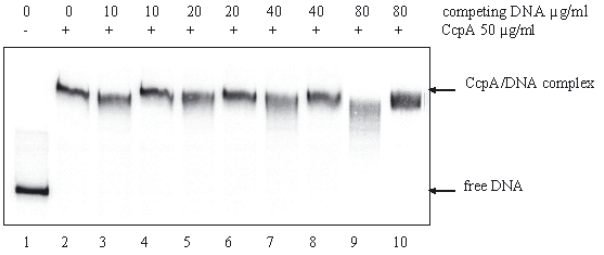

Figure 6. Competition gel retardation assay (competition EMSA).
Unlabelled *ybhE cre* site (lanes 3, 5, 7, 9) and no-*cre* fragments (lanes 4, 6, 8, 10) were tested for their ability to sequester CcpA from labelled *ybhE cre* probe (10 ng) in the presence of 2 µg/ml of sonicated calf thymus DNA.

shifting of DNA and an excess of HPr-Ser-P which binds to the surface of CcpA at the stoichiometry of 2 molecules per 1 CcpA dimer were used (Fig. 5).

In a control experiment it was observed that the addition of HPr-Ser-P to the reaction mixture containing CcpA and the DNA fragment without *cre* sequence did not change the efficiency of CcpA binding to DNA (not shown). In contrast, addition of HPr-Ser-P from *B. subtilis* to reaction mixtures containing CcpA and the DNA fragment with *cre* sequence slightly increased the CcpA binding (Fig. 5).

Competition EMSA with *cre*-containing DNA fragments located within two PEP:PTS regions

As the specific and unspecific DNA binding of the CcpA protein was difficult to discriminate, the affinity of CcpA for various *cre* sites and to no-*cre* fragments was examined by competition EMSA. First, unlabelled *ybhE cre* site (Fig. 6, lanes 3, 5, 7, 9) and a no-*cre* fragment (Fig. 6, lanes 4, 6, 8, 10) were tested for the ability to sequester CcpA from the labelled *ybhE cre* probe. The CcpA/*ybhE cre* complexes (Fig. 6, lanes 4, 6, 8, 10) were observed to migrate very similarly in all samples irrespective of the presence of the non-labelled no-*cre* fragment. In contrast, the labelled *ybhE cre* fragments (Fig. 6, lanes 3, 5, 7, 9) migrated faster, as more dispersed bands than the control (Fig. 6, lane 2) when challenged with the non-labelled *ybhE cre* competitor. Altogether, these results demonstrated that the unlabelled *ybhE cre* fragment, unlike no-*cre*, was able to replace labelled *ybhE cre* from the CcpA/*cre* complex.

Competition EMSA was also performed with several other unlabelled *cre*-containing fragments located within two PEP:PTS regions (Figs. 1 and 7). The fragments for further analysis were chosen based on the position of *cre* in the genome. These DNA fragments were used to sequester the CcpA protein from the CcpA/DNA complex containing a labelled no-*cre* fragment (Fig. 7). The CcpA/ no-*cre* complexes migrated very similarly when unla-

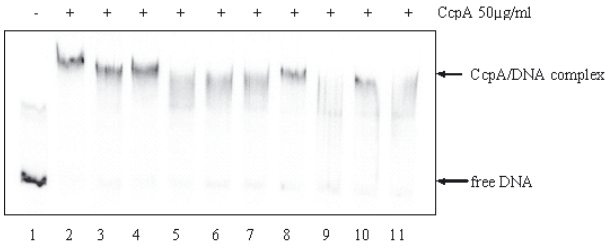

Figure 7. Competition EMSA.
Chosen unlabelled *cre* fragments from two PEP:PTS regions and a no-*cre* fragment were tested for their ability to sequester CcpA from labelled no-*cre* probe (10 ng) in the presence of 2 µg/ml sonicated calf thymus DNA. Lane 2, no competing DNA. The unlabelled competitor DNA was: no-*cre* sequence (lane 3), *celB cre* (lane 4), *bglS cre* (lane 5), *rheB cre* (lane 6), *yebE cre* (lane 7); *yebF cre* (lane 8); *ptcB cre*s (lane 9); *ptcA cre* (lane 10) and *yecA cre* (lane 11).

belled no-*cre* (Fig. 7, lane 3), *celB cre* (Fig. 7, lane 4), *yebF cre* (Fig. 7, lane 8) and *ptcA cre* (Fig. 7, lane 10) fragments were added to the binding mix. In contrast, the complexes migrated faster and as more dispersed bands when unlabelled fragments: *bglS cre*, *rheB cre*, *yebE cre*, *ptcB cre* and *yecA cre* (Fig. 7, lanes 5, 6, 7, 9, 11) were used for CcpA sequestration. Experiments showed that the *cre*-containing DNA fragments located in the vicinity of promoters of the *bglS*, *rheB*, *yebE*, *ptcB* or *yecA* genes were able to sequester CcpA from the CcpA/no-*cre* complex more efficiently than the unlabelled no-*cre* fragment or three other *cre*-containing fragments (*celB cre*, *yebF cre*, *ptcA cre*).

DISCUSSION

The CcpA protein plays an important role in the control of gene expression in low-GC Gram-positive bacteria (Henkin *et al.*, 1991). CcpA is postulated to exert its regulatory functions through direct interactions with target DNA sequences, called *cre* sites. The majority of these data come from studies on the CcpA protein from *Bacillus* species, while there is no such data from lactic acid bacteria, a group of biotechnologically important bacteria.

In this work, interactions of *L. lactis* CcpA regulatory protein with *cre* sites were tested. To determine the role of CcpA in the genetic regulation in *L. lactis* the DNA-binding ability of this regulator to particular *cre* sites was studied. For the initial analysis the *ybhE cre* sequence, which is consistent with the *cre* consensus and located near the promoter region of the *ybhE* gene reported to be CcpA-regulated (Kowalczyk *et al.*, unpublished) was chosen. This analysis was performed by EMSA with a non-*cre* containing region as a negative control. For this purpose the CcpA-His-tagged protein was overproduced and purified under native conditions to obtain a biologically active protein (Kowalczyk & Bardowski, 2003). The three-dimensional structural model demonstrates that the His-tag placed at the N-terminus of the CcpA protein is located outside the protein–DNA interface, thus most probably it does not interfere with the protein/DNA interactions (Fig. 2). Additionally, CcpA-His-tagged proteins were previously used for gel retardation analysis in *B. megaterium* (Deutscher *et al.*, 1995; Ramseier *et al.*, 1995).

Binding was done in a buffer containing FBP (fructose-bisphosphate) that was previously reported to enhance specific binding of CcpA to the *xyn cre* sequence in *B. subtilis* (Galinier *et al.*, 1999) and to be required for protection of *pta cre* sites of *B. subtilis* against DNase I digestion (Presecan-Siedel *et al.*, 1999). The analysis of the DNA-binding ability of the CcpA itself, in the absence of the HPr-Ser-P corepressor, showed that CcpA of *L. lactis* was able to bind DNA in these conditions, including DNA lacking *cre* sites (no-*cre* DNA) (Fig. 3). The unspecific binding of CcpA from *L. lactis* was not decreased in buffer B used by Hilbrich neither was it eliminated when short oligonucleotide DNA targets were used (Fig. 4). In contrast, the CcpA protein from *B. megaterium* did not show unspecific binding to DNA (Aung-Hilbrich *et al.*, 2002; Mahr *et al.*, 2002). However, it was previously reported that the CcpA protein without its corepressor could bind to *cre* that flanks the transcription start site of the α-amylase structural gene, *amyE* in *B. subtilis* (Kim *et al.*, 1995), and an unspecific interaction of CcpA with no-*cre* DNA was also reported in this bacterium (Miwa *et al.*, 1994).

HPr-Ser-P was found to interact with the His-tagged CcpA from *B. megaterium* immobilized on a Ni-NTA column (Deutscher *et al.*, 1995) and to form a specific ternary complex with CcpA and *cre* (Jones *et al.*, 1997; Schumacher *et al.*, 2004). Circular dichroism studies showed that the DNA duplex and the CcpA dimer presented a moderate change in the DNA spectrum compared with free DNA (unspecific interaction), however, the DNA spectrum in the presence of CcpA and HPr-Ser-P exhibited a significant change consistent with a structural change in the DNA (Jones *et al.*, 1997). Despite the strong evidence for the CcpA:HPr-Ser-P interaction, different hypotheses were proposed for the role of the CcpA corepressor in DNA binding and catabolite repression. Some studies suggest that HPr-Ser-P is necessary for specific binding of CcpA to *cre* (Fujita *et al.*, 1995; Kraus *et al.* 1998; Aung-Hilbrich *et al.*, 2002). However, specific binding was also observed in the absence of the corepressor in the case of PepR1, a *Lactobacillus delbrueckii* homologue of CcpA that specifically interacted in the absence of HPr-Ser-P with

pepQ, *pepX*, *pepI* and *brnQ* promoter regions (Schick *et al.*, 1999).

The presence of HPr-Ser-P of *B. subtilis* was found in this work to slightly enhance the CcpA binding to the *ybhE cre* fragment (Fig. 5). This weak effect of the protein/protein interaction could be due to the failure of cross communication between these two components of carbon catabolite repression. A similar effect was reported for the interaction of *L. casei* CcpA with *B. megaterium* HPr-Ser-P, which was 5-fold weaker than with its own HPr-Ser-P (Mahr *et al.*, 2002). However, successful *in vivo* heterologous complementations were also previously shown (Davison *et al.*, 1995; Egeter & Brückner, 1996; Monedero *et al.*, 1997). Another possible explanation could be that HPr-Ser-P together with FBP is not sufficient to cause an increase of CcpA binding to *cre* sites. This could suggest that HPr-Ser-P barely increases the DNA-binding ability of the CcpA protein in *L. lactis*. The role of HPr-Ser-P in modulating the contact between CcpA and the *cre* sequence could be explained by the fact that HPr-Ser-P could stimulate the activity of the CcpA/HPr-Ser-P/cre instead of participating in the formation of the *cre* DNA/protein complex.

We also analysed binding of CcpA to various *cre*-containing DNA fragments located within the PEP:PTS regions by competition EMSA (Figs. 6 and 7). These results suggest that the *ybhE*, *bglS*, *rheB*, *yebE*, *ptcB* and *yecA* genes could be directly regulated by CcpA, which needs to be confirmed by further analysis of gene expression.

CcpA was shown not to bind specifically to three *cre*-containing fragments (*celB cre*, *yebF cre*, *ptcA cre*). The obtained results suggest that these genes are not regulated by CcpA. In the case of the *ptcA* gene there is no promoter upstream of *ptcA* and the chosen *cre* sequence is in fact placed in the vicinity of the gene but not of its promoter. The lack of CcpA-binding can also be explained by the fact that the *ptcA* gene probably forms an operon with *ptcB* and could be regulated by binding of CcpA to *cre* within the promoter region of *ptcB*. The lack of specific CcpA-binding to the *celB cre* fragment could alternatively be explained by an indirect regulation by the CcpA protein. However, as the *celB* gene is directly preceded by the *ybhE* gene and *celB* can be transcribed together with *ybhE* (T. Aleksandrzak-Piekarczyk *et al.*, unpublished), a direct regulation by binding of CcpA to *cre* within the promoter region of the *ybhE* gene can also be suggested.

The *yebE cre* fragment was also found to bind CcpA with a higher affinity then the no-*cre* fragment. However, as the putative promoter of the *yebE* gene is difficult to identify, it is not possible to definitely confirm the role of this *cre* site in the regulation of the *yebE* gene. It is also likely that the *yebE* gene is not regulated by CcpA or can be transcribed with *rheB* as one operon.

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