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Characterization of the Catabolite Control Protein (CcpA) Gene from *Leuconostoc mesenteroides* SY1

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Abstract The *ccpA* gene encoding catabolite control protein A (CcpA) of *Leuconostoc mesenteroides* SY1, a strain isolated from kimchi, was cloned, sequenced, analyzed for transcript, and overexpressed in *Escherichia coli*. The *ccpA* ORF (open reading frame) is 1,011 bp in size, which can encode a protein of 336 amino acid residues with a molecular mass of 36,739 Da. The transcription start site was mapped at a position 49 nucleotides upstream of the start codon, and promoter sequences were also identified. The putative *cre* site overlapped with the −35 promoter sequence. The deduced amino acid sequence of the CcpA contained the helix-turn-helix motif found in many DNA-binding regulatory proteins. CcpA from *L. mesenteroides* SY1 had 54.6% identity with CcpA from *Lactobacillus casei*. The Northern blot experiment showed that *ccpA* was transcribed as a single 1.1 kb transcript, and transcription was repressed when grown on media containing glucose. CcpA was overproduced in *E. coli* BL21(DE3) cells using the pET expression vector, and purified to an apparent homogeneity. Gel Mobility Shift Assay with purified CcpA and a DNA fragment containing the *cre* sequence of the α-galactosidase gene (*aga*) from *L. mesenteroides* SY1 revealed that CcpA bound specifically to the *cre* site of *aga*.

Key words: *Leuconostoc mesenteroides* SY1, catabolite repression, CcpA, *cre*

The catabolite control protein, CcpA, is a global regulator controlling carbon catabolite repression (CCR), glycolysis, fermentative metabolism, and fixation of ammonium in low-G+C-content Gram-positive bacteria [31]. Bacteria carrying a defect in *ccpA* exhibit deregulated CCR and reduced growth rates [18]. The molecular mechanism of CcpA function in bacilli is well understood (for a review, see Ref. 14). When *Bacillus subtilis* is grown on a preferred carbon source, such as glucose or fructose, the metabolite-activated HPr kinase/phosphatase (PtsK) phosphorylates the proteins HPr and Crh at a seryl residue via ATP [6, 17, 27]. Both seryl-phosphorylated proteins activate CcpA by forming a complex with catabolite responsive element (*cre*) found within promoter or coding regions of catabolite-controlled genes [2]. This results in gene repression at the level of mRNA synthesis, which has been demonstrated for the *gnt* operon of *B. subtilis* and the *xyl* operon of *Bacillus megaterium* [5, 8]. Aside from its repressor function, CcpA operates as a pleiotropic activator, as has been reported for the *B. subilis* genes encoding acetate kinase, α-acetolactate synthase, phosphotransacetylase, and glutamate synthase, and for the *Lactococcus lactis las* operon encoding phosphofructokinase, pyruvate kinase, and L-lactate dehydrogenase [4, 9, 20, 28, 30].

Kimchi is the most famous Korean fermented vegetable food. During kimchi fermentation, diverse LAB (lactic acid bacteria) appear at different stages of fermentation and contribute to the development of the unique kimchi taste by producing various metabolites. Organisms belonging to the genus *Leuconostoc*, including *Leuconostoc mesenteroides*, are regarded as the most dominant and important organisms during the early and middle stages of fermentaion [11]. During these stages, *L. mesenteroides* cells produce various metabolites such as lactic acid, acetic acid, alcohol, CO_2, mannitol, and dextran, which are directly responsible for the taste of kimchi, and the cell number reaches the highest level during the optimum ripening period [1, 11, 29]. It is important and necessary to understand CCR and the functions of genes involved, if methods to produce high-quality kimchi are to be developed by controlling sugar

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utilization precisely in *L. mesenteroides*. In this paper we report the characterization of the *ccpA* gene from *L. mesenteroides* SY1, an isolate from kimchi, and show its regulatory role by confirming the binding of purified CcpA to a *cre* sequence of the α-galactosidase gene, which is under CCR. As far as we are aware, this is the first report on the *ccpA* gene of *L. mesenteroides*.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Leuconostoc mesenteroides SY1 was isolated from regular cabbage kimchi purchased from a local supermarket. Biochemical properties and 16S rDNA sequencing data were used to identify SY1 (results not shown). *Leuconostoc mesenteroides* SY1 was grown in MRS broth containing different carbon sources (1%) or on MRS plates (1.5% agar) at 30°C. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth at 37°C with vigorous agitation. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was supplemented at a concentration of 40 μg/ml and IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the final concentration of 0.5 mM when necessary. Antibiotics were used at the following concentrations: ampicillin (Ap), 100 μg/ml; kanamycin (Km), 30 μg/ml for the *E. coli*.

DNA Amplification, Cloning, and Sequencing

Chromosomal DNA was isolated from *L. mesenteroides* SY1 as described previously [25]. The PCR was performed with *L. mesenteroides* SY1 genomic DNA as a template using a GeneAmp PCR system 2400 (PE Applied Biosystems, Foster City, U.S.A.). A primer pair of [Leu]ccpAll-F (5'-CGGAGGATCCGTTGTCATAGTAGACTGACTAATCG-ACA-3') and [Leu]ccpAll-R (5'-CTTTGGATCCTAATAT-TCTTGTTGAGTGACCAACAAAACT-3') were synthesized based on the sequence of the *L. mesenteroides* ATCC 8293 *ccpA* gene in the database (GenBank accession number: NZ_AABH02000017). The *Bam*HI site introduced is underlined. Ex-*Taq* polymerase (TaKaRa Shuzo Co., Otsu, Shiga, Japan) was used, and the denaturation, annealing, and extension conditions were 94°C for 45 s, 63°C for 45 s, and 72°C for 4 min, respectively. A total of 30 cycles followed by a final 7 min extension at 72°C was employed. The amplified fragment was digested with *Bam*HI and ligated with pBluescript KS(+) (Stratagene, CA, U.S.A.). DNA sequences were determined by the dideoxy-chain terminaton method using an ABI-PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, U.S.A.) [19]. The primers for the sequencing were synthesized at Bionics (Seoul, Korea). The homology of the deduced amino acid sequence was analyzed using the Blast program at NCBI [32]. The sequence alignment was performed with the ClustalW program [13] using a PAM250 matrix. An amino acid sequence identity matrix was drawn using the BioEdit program version 4.7.8. [10]. A primary structure analysis was performed using the ProtParam program [7].

Northern Hybridization

L. mesenteroides SY1 cells were grown until the mid-exponetial growth phase (A_{600} of 0.7) in MRS medium containing different carbon sources (1%). Total RNA was extracted from a 15-ml culture using FastRNA® Pro Blue Kit (Q-biogene, Montreal, Canada), according to the protocol provided by the manufacturer. Cells were disrupted using the Mini-Beadbeater™-8 Cell Disrupter (BioSpec, Bartlesville, U.S.A.). RNA (20 μg) was separated on 1.2% agarose-formaldehyde gel, transferred to Hybond™-XL nylon membrane (Amersham Bioscience, Uppsala, Sweden) [26], and hybridized at 65°C with a ^{32}P-labeled 645-bp *ccpA* probe. The 645-bp probe was PCR amplified using a primer set of ccpa-probeF (5'-GATTGATGTTAGCGCAGGAC-3') and ccpa-probeR (5'-TACTATTCAGAGCTGGCACG).

Primer Extension

The oligonucleotide primer, CCPA-Extension1 (5'-CACT-CGCGATACTGTTGCTAGTGATACACC-3';), was 5' end labeled with [γ-^{32}P]dATP using T4 DNA polynucleotide kinase (Promega, Madison, U.S.A.) [24]. Then, the oligonucleotide was hybridized with 50 μg of total RNA extracted from *L. mesenteroides* SY1 cells grown on 1% raffinose, and the annealed primer was extended with an AMV reverse transcriptase (Promega, Madison, U.S.A.) according to the method provided by the manufacturer. The extended product was analyzed on a sequencing gel (6% acrylamide gel) adjacent to a DNA sequencing ladder generated from the CCPA-Extension1 primer with pLCCP53 as a template.

Production and Purification of CcpA in *E. coli*

For overexpression of *ccpA* in *E. coli*, the *ccpA* gene was amplified by PCR and subcloned into a pET26b(+) (Novagen, Madison, U.S.A.). Two oligonucleotide primers containing a unique *Nde*I site (ccpA-expF3: 5'-GGAATTCCATATGC-AAAAAAAGCAAACGGCTA-3') and *Xho*I site (ccpA-expR1: 5'-CCCCTCGAGATTAATTGTTGTTCCTCGTT-TTTC-3') were used for the amplification. The amplified fragment was digested with *Nde*I and *Xho*I, and ligated with pET26b(+). The resulting recombinant plasmid, pLCCPE1, was introduced into *E. coli* BL21(DE3) (Novagen, Madison, U.S.A.). BL21(DE3) cells harboring pLCCPE1 were grown overnight at 37°C and inoculated into fresh medium (1%) and cultured at 37°C. When A_{600} of the culture reached about 0.8, IPTG was added to the final concentration of 1 mM, and the culture was further incubated for 3 h at 30°C [16]. Cells were recovered by centrifugation at 5,000 ×*g* for 20 min, washed twice with 20 mM phosphate buffer (pH 7.4), and resuspended in 2 ml of the same buffer. Cells were disrupted by sonication for 1 min

followed by cooling on ice for 1 min, and this cycle of sonication-cooling was repeated 4 times. Disrupted cells were centrifuged and the supernatant was obtained as protein extract. His-tagged CcpA was purified by affinity column chromatography with a HiTrap Chelating HP column (Amersham Biosciences, Uppsala, Sweden), according to the protocol provided by the manufacturer.

Gel Mobility Shift Assay

A 388-bp DNA fragment containing the *cre* site of the α-galactosidase gene (*aga*) from *L. mesenteroides* SY1 (GenBank accession number: AY753204) was amplified by PCR using ^{32}P-labeled aga-cre-F (5'-GCACTAACGA-CGGTAGATCT-3') and aga-cre-R (5'-CCGCCCTCTTC-AATTTGTATC-3') as primers. The labeled probe DNA was incubated with 1 μg of purified CcpA in binding buffer [20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.05% Igepal CA-630 (Sigma, St. Louis, U.S.A.), 5% glycerol, 1 mM DTT, 25 μg/ml salmon sperm DNA] for 30 min at 30°C. After incubation, the sample was loaded directly onto a prerun 6% polyacrylamide gel prepared in TGOE buffer (25 mM Tris, 190 mM glycine-2.5% glycerol-0.5 mM DTT). The gel was run at 200 V for 1 h in a SE400 Sturdier Vertical Slab gel electrophoresis unit (Amersham Bioscience, Uppsala, Sweden), dried on Model 583 Gel Dryer (BioRad, Hercules, U.S.A.), and exposed to Hyperfilm™ MP (Amersham Biosciences, Uppsala, Sweden) for autoradiography.

Nucleotide Sequence Accession Number

The DNA sequence reported here was deposited in the GenBank database under accession number AY615196.

RESULTS AND DISCUSSION

Cloning and Sequence Analysis of *ccpA*

When PCR was performed with *L. mesenteroides* SY1 chromosomal DNA as a template and the primer set of [Leu]ccpAll-F and [Leu]ccpAll-R, as described in the Materials and Methods, a 5,342-bp fragment was amplified. The amplified fragment was cloned into pBluescript II KS(+), resulting in a recombinant plasmid, pLCCP53 (8.3 kb). DNA sequencing of the 5.3-kb fragment showed that the sequence was very similar to that of the *ccpA* gene locus of *L. mesenteroides* ATCC 8293 (98%, nucleotide sequence level); it also showed the presence of four ORFs (open reading frame, see Fig. 1). ORF1 was 585 bp in size and capable of encoding a putative metal-dependent membrane protease of 194 amino acids with a calculated molecular mass of 22,043 Da. The pI value for the putative metal-dependent membrane protease was calculated to be 9.34. ORF2 was 1,101 bp in size and capable of encoding a dipeptidase of 366 amino acids with a calculated molecular mass of 39,844 Da. Dipeptidase specifically cleaves X-prolyl peptide bonds, and the gene was accordingly designated *pepQ* [21]. The pI value for the dipeptidase was calculated to be 9.34. ORF3 was 1,011 bp in size and capable of encoding CcpA of 336 amino acids with a calculated molecular mass of 36,739 Da. The gene was designated *ccpA*. The pI value for the CcpA was calculated to be 4.74. ORF4 was 1,866 bp in size and capable of encoding acyltransferase of 621 amino acids with a calculated molecular mass of 71,042 Da. The pI value for the acyltransferase was calculated to be 10.28.

ccpA started with an ATG codon [2,187 nc (nucleotide)] and a potential RBS (AGAGGAA) located 7 bp upstream

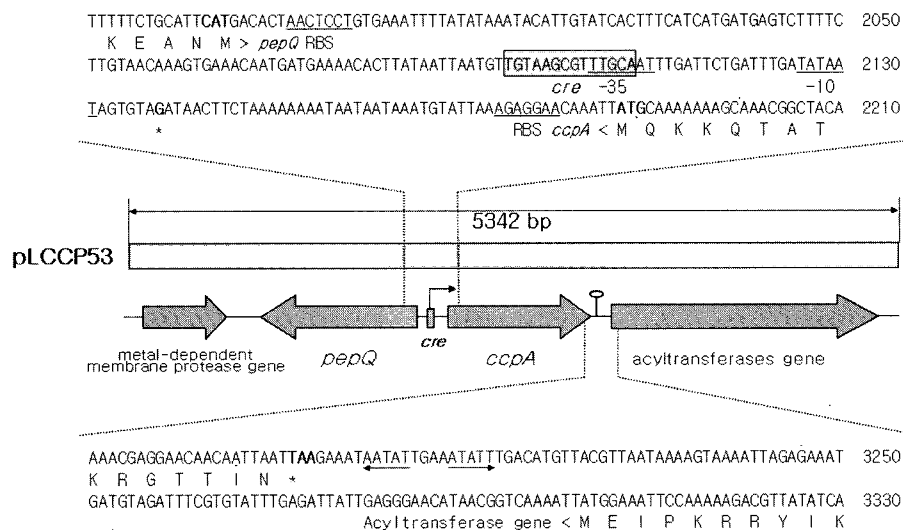

Fig. 1. Genetic organization of the *ccpA* and surrounding genes of *L. mesenteroides* SY1.
The size and orientation of ORFs were deduced from the nucleotide sequence. The nucleotide sequence of the upstream regulatory region of *ccpA* is shown. The transcriptional start site is in bold face and is marked by an asterisk. RBS and putative RNA polymerase binding sites (−10 and −35 region) in the sequence are underlined. The *cre* motif is shown as a gray-colored box. The N-terminal amino acid sequences of PepQ and CcpA are shown.

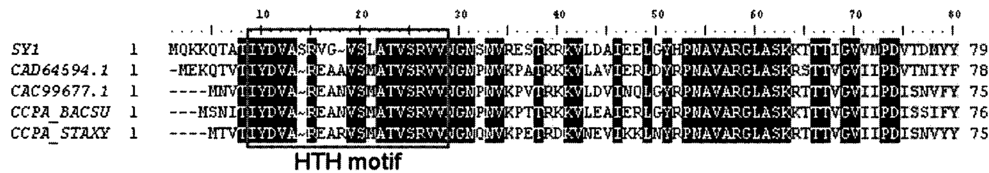

Fig. 2. Multiple sequence alignment of the N-terminus of CcpA from various Gram+ bacteria.
Helix-turn-helix motifs are indicated as a box. The sequence of *L mesenteroides* SY1 CcpA (SY1) was compared with those of *L. plantarum* WCFS (CAD64594.1), *L. monocytogenes* (CAC99677.1), *B. subtilis* (CCPA_BACSU), and *S. xylosus* (CCPA_STAXY).

of the start codon (Fig. 1). A putative promoter region with characteristic features, such as AT-rich regions and potential −10 (TATAAT, 2,126 nc) and −35 (TTGCAA, 2104 nc) regions, were also found (Fig. 1). This region coincided with the transcriptional start site determined by primer extension (see below). A *cre* site (TGTAAGCGTTTGCA) was found near the −35 region. A putative rho-independent transcription terminator (AATATTGAAATATT) was found 5 nc downstream from the stop codon of *ccpA* with ΔG of −0.6 kcal/mol. The *ccpA* sequence was compared with those of other *ccpA* genes in the DNA database. *ccpA* from *L. mesenteroides* SY1 was similar to those of other known *ccpA* on the amino acid sequence level. The identity scores of the amino acid sequence were 54.6%, 50.7%, and 46.8% for *Lactobacillus casei* [22], *L. plantarum* [23], and *Bacillus subtilis* [12], respectively. The helix-turn-helix (HTH) motif was found in the N-terminal region of CcpA from *L. mesenteroides* SY1. All published CcpA have the HTH motif (Fig. 2). The presence of the HTH motif indicates that CcpA is a transcriptional regulator with DNA-binding activity. *ccpA* and upstream *pepQ* are transcribed divergently from each other (Fig. 1), and this genetic organization is common among all published *ccpA* from LAB [21].

Transcriptional Analysis of *ccpA*

The size of the *ccpA* transcript was analyzed by Northern blot with a radiolabeled 645-bp *ccpA* probe. The autoradiogram (Fig. 3) showed that a single 1.1 kb transcript hybridized with the probe, which was in good agreement with the size of the *ccpA* transcript predicted from the DNA sequence

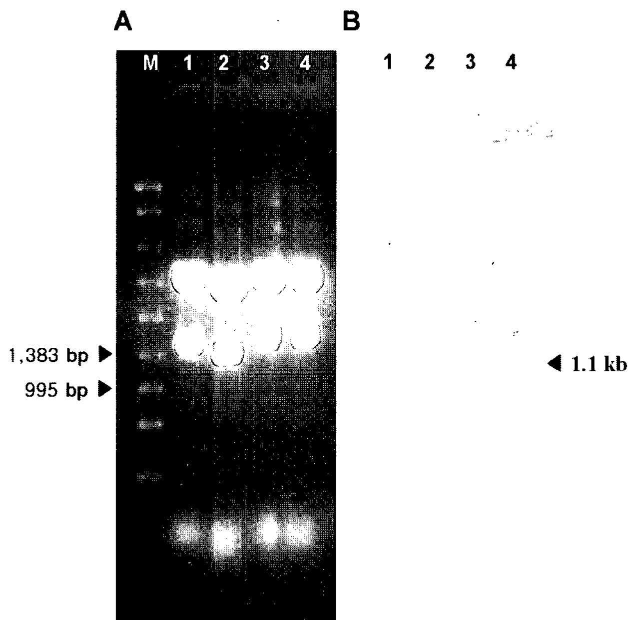

Fig. 3. Northern blot of *L. mesenteroides* total RNA (15 μg) separated on a 1.2% agarose-formaldehyde gel and hybridized with a ^{32}P-labeled 645-bp *ccpA* probe.
Total RNA from cells grown on MRS medium with 1% glucose (lane 1), fructose (lane 2), sucrose (lane 3), and raffinose (lane 4). A, agarose-formaldehyde gel; B, autoradiogram.

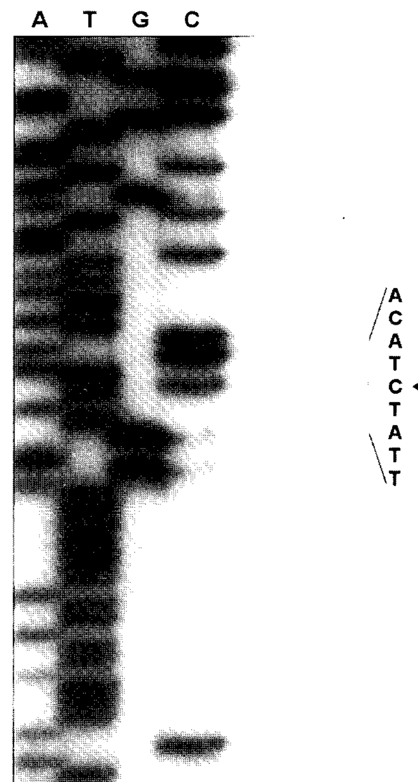

Fig. 4. Primer extension of the *ccpA* gene with avian myeloblastosis virus (AMV) reverse transcriptase and ^{32}P-labeled oligonucleotide complementary to the *ccpA* transcript.
Fifty μg of total RNA from cells grown on MRS medium with 1% raffinose was used for the hybridization.

data. The result confirmed that *ccpA* was a monocistronic gene, as expected from its sequence data, and the transcription terminator downstream of the stop codon really functions *in vivo*. The amount of *ccpA* transcripts was seemingly constant on different carbon sources (1% each of fructose, sucrose, and raffinose), although it was obviously less in cells grown on glucose (1%). Similar results were reported for *L. casei* [22] and *L. plantarum* [23].

The transcriptional start site of the *ccpA* gene, which was determined by primer extension experiments, was located 49 nucleotides upstream of the start codon (Fig. 4). This result agreed well with the location of the putative promoter sequences mentioned above (Fig. 1). *cre* overlapped partially with the −35 promoter region. In *S. xylosus*, *ccpA* is transcribed from two promoters; one is weak and constitutive, and the other possesses a *cre* sequence and is repressed by CcpA itself [3]. *L. mesenteroides* SY1 has only one promoter, which overlaps with a *cre* site. Therefore, it can be inferred that *ccpA* transcription in *L. mesenteroides* SY1 is likely to be repressed on glucose, leading to a reduction in CcpA level in cells grown on glucose. This could possibly be an adaptation, to regulate the level of available CcpA depending on the carbon sources [22].

Purification and *cre* Binding Activity of CcpA

ccpA was overexpressed in *E. coli* BL21(DE3) by the pET vector (Fig. 5, lane 1). C-terminal his-tagged CcpA was purified as described in Materials and Methods. The sample-loaded column was washed with 10 mM imidazole-containing phosphate buffer, pH 7.4 (Fig. 5, lane 2), and then his-tagged CcpA was eluted with 300 mM imidazole-containing phosphate buffer, pH 7.4 (Fig. 5, lane 3). As shown in Fig. 5, a total 14.74 mg of highly purified CcpA was obtained from the 250-ml culture of *E. coli* BL21(DE3) harboring pLCCPE1. The size of the purified protein, 37 kDa as judged by SDS-PAGE, was in good agreement with that (37,777 kDa) of His-tagged CcpA calculated from the amino acid sequence.

To confirm the *cre* binding activity of purified CcpA, we performed a gel mobility shift assay as described in Materials and Methods. The α-galactosidase gene (*aga*) expression in *L. mesenteroides* SY1 is under carbon catabolite repression, and thus the α-galactosidase activity is much lower in cells grown on glucose, when compared with cells grown on melibiose or raffinose. A putative *cre* sequence (5'-AGTAACCGTTTACA-3') is located upstream of the *aga* ORF. Mutagenesis studies with the *cre* sequences from several *B. subtilis* genes and operons revealed a palindromic sequence with the consensus 5'-TGWAARCGYTWNCW-3' (W=A or T, R=A or G, Y=C or T, N= any base) [15]. The putative *cre* sequence of *aga* has some deviation from the known consensus sequence; i.e. the first nucleotide is A rather than T, and the 6th nucleotide is C. We tested the binding of purified CcpA to a PCR fragment encompassing *cre* of the *aga* gene. The autoradiogram clearly showed that CcpA bound to the DNA probe (Fig. 6B, lane 2). One μg of bovine serum albumin (BSA) was used as a negative control and it did not bind to the DNA probe (Fig. 6B, lane 3). The result again confirms that CcpA is a regulatory protein that interacts directly with the putative *cre* site of the *aga* gene by binding to it, and that the *cre* sequence of

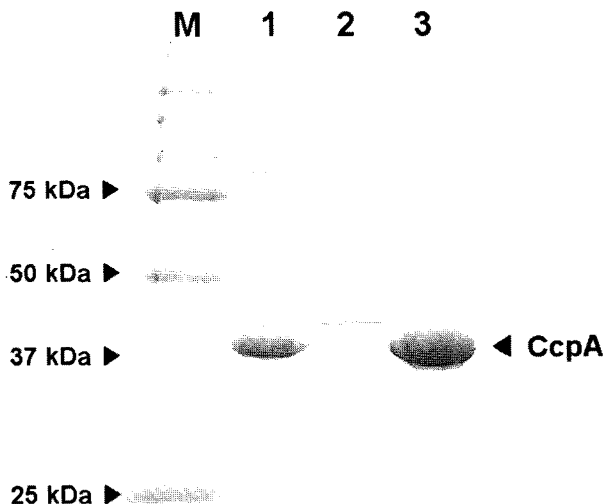

Fig. 5. SDS-PAGE of His-tagged CcpA overproduced in *E. coli* BL21(DE3).

M, Precision Plus Protein™ standard (BioRad, Hercules, U.S.A.); 1, CcpA with 1 mM IPTG induction; 2, HiTrap Chelating HP (Amersham Biosciences, Uppsala, Sweden) column unbound fraction (washing); 3, 300 mM imidazol elution fraction.

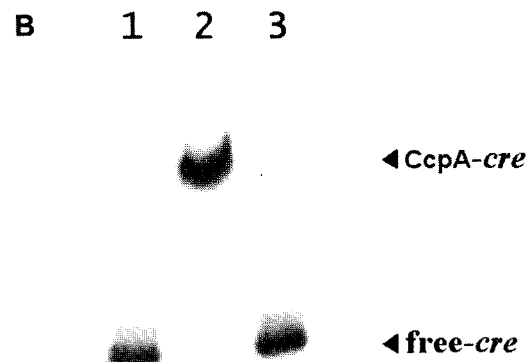

Fig. 6. Binding of CcpA to the *cre* site of *aga*.

CcpA was tested for the gel mobility shift of the 388 bp DNA fragment containing the *cre* site of *aga* using a 6% polyacrylamide gel. 1, no protein added; 2, 1 μg of CcpA added; 3, 1 μg of BSA added (-control).

L. mesenteroides is not completely identical to the known consensus *cre* sequence of *B. subtilis*. It is of interest to determine by mutagenesis experiments which base in the *cre* of *aga* is critical in the binding with CcpA.

The *ccpA* gene of *L. mesenteroides* SY1 is monocistronic and located to the opposite direction of the *pepQ* gene, and this genetic organization is common among all known *ccpA* genes from LAB. The promoter of the *ccpA* gene overlaps with a *cre* site. Therefore, it can be inferred that *ccpA* transcription in *L. mesenteroides* SY1 is likely to be repressed by the binding of CcpA to the *cre* site when glucose is present in the medium. In the present study, the regulatory role of CcpA has been proven by showing the binding of purified CcpA to the *cre* site of the *aga* gene from *L. mesenteroides* SY1, which is under CCR.

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