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Molecular Characterization of *crp*, the Cyclic AMP Receptor Protein Gene of *Serratia marcescens* KTCC 2172

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Abstract Several clones obtained from *Serratia marcescens* stimulated *E. coli* TP2139 (*Δlac*, *Δcrp*) cells to use maltose as a carbon source. The *crp* gene clone, pCKB12, was confirmed to stimulate the β-galactosidase activity, by Southern hybridization [31]. The nucleotide sequence of the *crp* region consisting of 1,979 bp was determined. The sequencing of the fragment led to the identification of two open reading frames: One of these, the *crp* gene, encoded 210 amino acids and the other encoded a truncated protein. The *S. marcescens* and *E. coli crp* genes showed a higher degree of divergence in their nucleotide sequence with 120 changes, however, the corresponding amino acid sequences showed only two amino acid differences. Yet, an analysis of the amino acid divergence revealed that the catabolite gene activator protein, the *crp* gene product, was the most conserved protein observed so far. Using a *crp-lac* protein fusion, it was demonstrated that *S. marcescens* CRP could repress its own expression, probably *via* a mechanism similar to that previously described for the *E. coli crp* gene.

Key words: *Serratia marcescens*, *crp-lacZ* fusion, *crp* gene, gene expression

S. marcescens is an enteric bacterium, closely related to *E. coli*, that secrets several enzymes through its membrane into a culture broth, including nucleases, lipase, protease, and chitinases [12].

A well-known example of cAMP-mediated regulation is catabolite repression in bacteria [10, 19, 31]. When complexed with cyclic AMP (cAMP) and a cAMP receptor protein, it binds specifically to the promoter region of the operons which it regulates. Glucose and its analogs lower the intracellular adenosine 3',5'-cyclic monophosphate (cAMP) levels, which in turn causes a reduction in the expression of a set of catabolite-sensitive operons such as *lac*, *ara*, and *mal*. In these operons, cAMP and its receptor protein (CRP) act as a positive effector for transcription [6, 10, 15, 17]. In contrast, it was recently shown that cAMP-CRP represses the transcription of another set of genes [2, 13, 18, 20]. Kawamukai *et al.* [17] reported a similar level of repression of *cya* expression by cAMP in *E. coli* using *cya-lac* fusions carried on multicopy plasmids, and concluded that the repression was physiologically significant. The observation that *crp* mutants overproduce cAMP in both *Salmonella typhimurium* and *Escherichia coli* would seem to suggest that the synthesis of cAMP was inhibited by cAMP-CRP [14].

The *crp* genes from several bacteria have previously been cloned and their structures were analyzed [1, 8, 11, 16, 23, 24, 29]. *In vivo* and *in vitro* studies have also shown that *E. coli crp* is negatively autoregulated. Reverchon *et al.* [24] demonstrated that CRP plays a crucial role in the expression of pectinolysis genes and in the pathogenicity of *E. chrysanthemi*. The direct implication of CRP in the expression of pectinolysis genes has also been suggested, because of the identification of a putative CRP binding site in the regulatory region of various pectinolysis genes [7]. However, the *crp* gene of *S. marcescens* has not previously been cloned nor sequenced. Since they are functionally the same, the CRP species from these organisms are quite likely similar, however, it is still difficult to draw a direct correlation without data on the molecular level. An interest in elucidating the structural role in the CRP function prompted us to analyze the structure of CRP in different bacteria.

Accordingly, this paper describes the characterization of the *Serratia marcescens crp* gene, the negative regulation of *crp* by cAMP-CRP, and an analysis of the structure. Furthermore, the existence and importance of *crp* in *Serratia marcescens* was also investigated.

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MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

The *S. marcescens* KCTC 2172 strain was purchased from the Korea Culture Type Collection [7]. pBluescript KS+ and pKK223-4 were used as the cloning vectors (Table 1). The bacterial strains were routinely grown at 30°C or 37°C in LB medium or M9 minimal medium, respectively. The antibiotic concentration used was 50 μg/ml ampicillin. The cultures for the enzyme assay were grown in a minimal medium supplemented with maltose as the carbon source and 0.1% concentration of casamino acids.

DNA Isolation and Manipulation

The total cellular DNA from *S. marcescens* was prepared as previously described [32]. A rapid, small-scale plasmid DNA isolation was performed according to the method of Birnbaum and Doly [4] using a Wizard kit from Promega Biotech. All the restriction enzymes, the T_4 DNA ligation kit ver. 2, and polynucleotide kinase were purchased from Takara Shuzo Co. and used according to the recommendation of the suppliers. The general recombinant DNA manipulation was carried out according to the protocol suggested by Sambrook *et al.* [27].

Cloning and Sequencing *crp* from *S. marcescens*

The pCKB series was obtained by performing hybridization using a labeled *E. coli crp* gene, whose catabolic ability to use maltose was detected with a MacConkey-supplemented carbon source using maltose in the *E. coli* TP2139(*Δlac Δcrp*) strain [32]. One of these clones, pCKB12, was inserted into the *Bam*HI site on pBluescript KS+ in TP2139 [32]. Various restriction fragments derived from the pCKB12 plasmid DNA were subcloned into the same restriction enzyme site of pBluescript KS+. Double-stranded DNA was used as the template for the sequencing reactions. Both DNA strands were sequenced by the dideoxy-chain termination method using an Autocycle DNA sequencing kit and A.L.F. DNA sequencer (Pharmacia) [28].

Table 1. Bacterial strains and plasmid.

Strains & plasmids	Genotype	Source and ref.
STRAINS		
E. coli W3110	Wild-type	Lab. stock
E. coli JM109	*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17, *sup*E44, *rel*A1, Δ(*lac-pro*AB)/F' [*tra*D36, *pro*AB$^+$, *lac*I^q,*lac*ZΔM15]	
E. coli TP2139	*crp*, *lacX74*, *xyl*, *ilvA*, *argH*, *recA1*	A. Danchin, [9]
E. coli TP2010	*xyl*, *cya*, *argH*, *lacX74*, *recA*, *ilv*, Srl::Tn10	
E. coli MK1010	W3110, *cys::km*	[12]
Serratia marceescens KTCC 2172		[7]
PLASMIDS		
pBluescript KS(+)	Amp^r	
pKK223-4	*tac*, Amp^r	
pMC1403	Amp^r *lac*$^+$	
pHA5	*crp*$^+$	[1]
pCKB12	*crp*$^+$, Amp^r	This study
pCKB12H	*crp*$^+$, Amp^r	This study
pMC1403-S6	Amp^r *crp-lacZ*	This study

Gel Retardation Assay

The 398 bp *Hinc*II-*Sac*I fragment of pCKB12 was labeled at the 5' ends using T_4 polynucleotide kinase and γ-^{32}P dATP. The labeled DNA fragments were incubated with the *E. coli* CRP protein and cAMP, as described previously [5]. CRP protein was purified by the method previously described [5].

Enzyme Assay and SDS-PAGE

The cultures for the enzyme assay were grown in a minimal medium with a carbon source and 0.1% casamino acids. The cultured cells were harvested and suspended in a cell suspension buffer [50 mM MOPS, pH 7.0, 0.5 M $(NH_4)_2SO_4$, 20% (v/v) glycerol, 1 mM EDTA]. The suspension was sonicated and any cell debris was removed by centrifugation. The protein content of the cell extracts was determined using the method of Bradford [3] with bovine serum albumin as the standard (Bio-Rad). The crude cell proteins were separated by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli [21]. The β-galactosidase activity was determined by the method of Miller [22].

Western Bloting

The protein samples were electrophoresed on 10% SDS-polyacrylamide gels and electroblotted onto Biodyne A nylon membrane filters using the method of Towbin *et al.* [30]. After blocking with 10% BSA in PBS, antiserum was added to the membrane. The antibody was detected using a second antibody conjugated with alkaline phosphatase and a chromogenic reaction through the addition of 5-bromo-4-chloro-3-indolyl phosphate disodium salt/nitroblue tetrazolium chloride.

RESULTS AND DISCUSSION

Identification of the *S. marcescens crp* Gene

The *S. marcescens crp* gene was cloned by the complementation of the maltose defect of an *E. coli crp* mutant (TP2139). Several clones were obtained from *Serratia marcescens* which stimulated the cells to use maltose as the carbon source in *E. coli* TP2139 (*Δlac*,

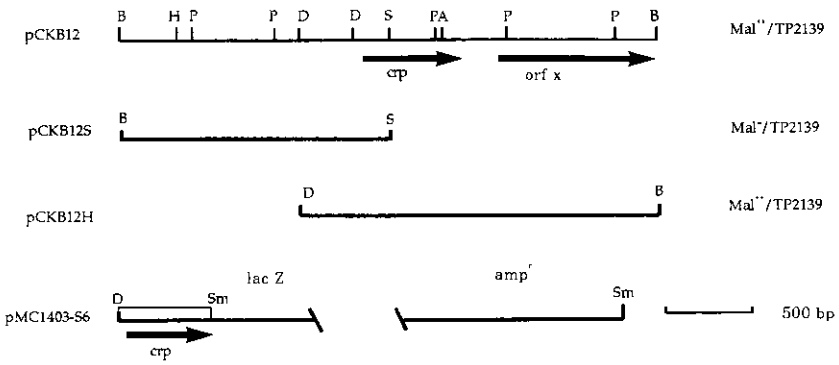

Fig. 1. Physical map of the plasmid pCKB12 of *S. marcescens* and derivative plasmids.
The transcriptional direction of the *crp* gene is shown by the thick arrow. To construct the *crp-lacZ* fused plasmid, pMC1403-S6, the 600 bp DNA fragment from the pCKB9P plasmid was inserted in the *Sma*I site of the plasmid pMC1403. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sac*I; A, *Acc*I; D, *Hin*cII; Sm, *Sma*I.

Δcrp) [32]. One of the cloned genes, pCKB12, was futher analyzed. In order to determine the expression of the gene under the direction of the cAMP-CRP complex, a restriction endonuclease cleavage map of the cloned DNA fragment and several recombinant subclones were constructed (Fig. 1). The location of the *crp* gene was determined by Southern hybridization using the labeled *E. coli crp* gene [32]. To measure the functional ability of the cloned gene from *S. marcescens*, those strains carrying the recombinant plasmids were assayed for their total β-galactosidase activity (Table 2). The results showed that the plasmids pCKB12 and pCKB12H encoded a protein that stimulated a high level of β-galactosidase, whereas pCKB12S did not encode the protein. The *crp* gene product exhibited a similar activity to that of the *E. coli crp* gene, pHA5. The cloned *S. marcescens crp* could substitute the *E. coli* CRP in the transcriptional activation of the *lacZ* gene. Therefore, it was confirmed that the clone pCKB12H encoded the *crp* gene in the upstream region.

Nucleotide Sequence of the cAMP Receptor Protein Gene

In this study, the DNA region of *Serratia marcescens* harboring *crp* was cloned and analyzed at the molecular level. The sequences of the complete structural gene as well as that of the untranslated region of the *crp* gene are shown in Fig. 2. This fragment encoded two open reading frames which included coding for 210 amino acids and a truncated protein for 357 amino acids with the same direction of transcription. The putative ribosomal binding site (GAGGA) of the *crp* was located 9 bp upstream of the ATG start codon. The putative promoter region with a −10 box (TTTTAT) and −35 box (TTAATA) was spaced by 19 nucleotides. Sequences matching with the consensus of the *E. coli* CRP binding site were found downstream of the putative promoter elements. A comparison of the regulatory regions of *S. marcescens* with the *S. typhimurium* and *E. coli crp* genes showed that the most conserved sequences were those known to be essential for the expression of *E. coli crp.* At downstream of the CRP, there was the hair-pin loop structure of the ρ-independent termination sites of *E. coli* [21] with $\Delta G = -19.2$ kcal. The structural gene sequence of the *crp* was found to be nearly identical to that of *E. coli* and *S. typhimurium.* On the nucleotide level, the difference between the *crp* genes from *S. marcescens*, *E. coli*, and *S. typhimurium* was only about 20% in each combination. Small 2-base-pair changes in the *S. marcescens* sequences at positions 586 and 740 resulted in two amino acid changes from arginine in the *E. coli* protein to serine and leucine at positions 123 and 170, respectively, in the *S. marcescens* CRP. Thus, only two amino acid differences were identified between the CRPs from *E. coli*, *S. typhimurium*, and *S. marcescens*.

The predicted amino acid sequences were then used to search through databases using the BLAST program on the University of Kyoto Genome Net WWW server. A significant homology of amino acid sequences was found between the cryptic 75 kDa protein of *E. coli* and the ORFx of *S. marcescens*. Figure 3 shows the alignment of the *crp* gene from *S. marcescens* with analogous proteins from other organisms. This protein showed a more than 98% identity with the CRPs from *E. coli*, *E. chrysanthemi*,

Table 2. β-Galactosidase activity in *E. coli* strains containing *crp* genes.

Strain	Resident plasmid	Relevant phenotype	β-Galactosidase activity[a]
TP2139		*crp*⁻	325
TP2139	pHA5	*crp*⁺	1,265
TP2139	pCKB12	*crp*⁺	1,764
TP2139	pCKB12H	*crp*⁺	1,957
TP2139	pCKB12S	*crp*⁻	395

[a]Enzyme activity determined using the method of Miller: expressed in Miller units.
[b]The cultures for the enzyme assay were grown in a minimal medium supplemented maltose with 0.2% casamino acids and 0.1 mM cAMP.

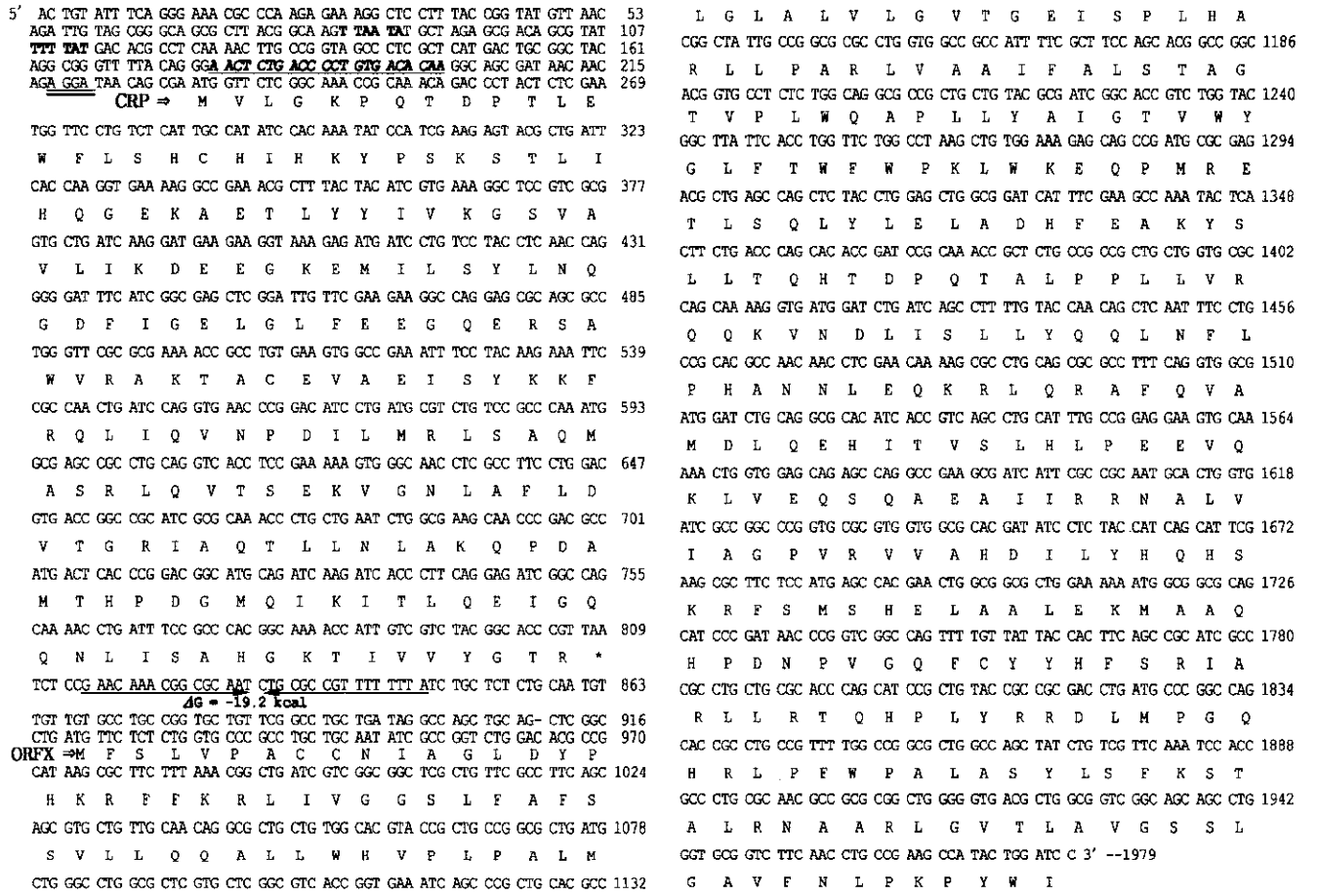

Fig. 2. Nucleotide sequence of the cAMP receptor protein gene from *S. marcescens*.
The peptide sequence corresponding to the ORFs is shown under the nucleotide sequences. The putative promoter sites (– 10 and – 35) are indicated by bold characters. The proposed CRP binding site is underlined and in bold type. The double underlined indicates the putative Shine-Dalgarno sequence. The initiation sites of the translation are indicated by the arrows. The palindromic sequence downstream of the CRP stop codon is shown by the horizontal arrows. The nucleotide sequence in this paper has been submitted to the Genebank Data Bank with accession number AF276243.

and *S. typhimurium*. The variations in the nucleotide sequence among the four genes were mostly confined to the third positions of the codon triplets. A weaker similarity was observed with other members of the CRP family [20], including the *Haemophillus influenza* CRP protein (71% identity) [24], *Pseudomonas aeruginosa*, and the *Xanthomonas campestris* catabolite activator-like protein (41% identity) [11]. However, the sequence conservation among the *crp* genes from these bacteria extends beyond the coding region and includes the regions upstream and downstream of the gene region known to be important for regulating the expression of the bacterial *crp* gene. Given the general similarity within this enteric group of bacteria, it could be argued that the similarity among the CRPs was to be expected. However, there are also numerous examples of major differences between *E. coli* and *K. aerogenes* proteins [23, 24]. The CRPs from very diverse organisms show a remarkable amino acid conservation. An analysis of the amino acid divergence, however, revealed that the catabolite gene activator protein, the *crp* gene product, is the most conserved protein observed so far.

Regulation of Expression of the *crp* Gene in *E. coli*

The regulation of the expression of the *S. marcescens crp* gene in *E. coli* was investigated with fusion plasmids. To construct a plasmid encoding a *crp-lac* fused protein, the 600 bp from the *Sma*I to the *Hin*cII fragment of the pCKB9P plasmid, which harbors the 900 bp *Pst*I fragment of pCKB12H that contains the *crp* promoter region, including the 72 codons of the *crp* gene, was inserted in the *Sma*I site of the plasmid pMC1403. The recombinant plasmid pMC1403-S6 was also isolated (Fig. 1). The nucleotide sequence of the pMC1403-S6 plasmid and the pattern of the restriction enzyme indicated that the reading frame of the *crp* and *lacZ* matched. To examine whether the *crp* expression was regulated through a cAMP-mediated process, the β-galactosidase activity was measured at various times after the addition of cAMP into a medium

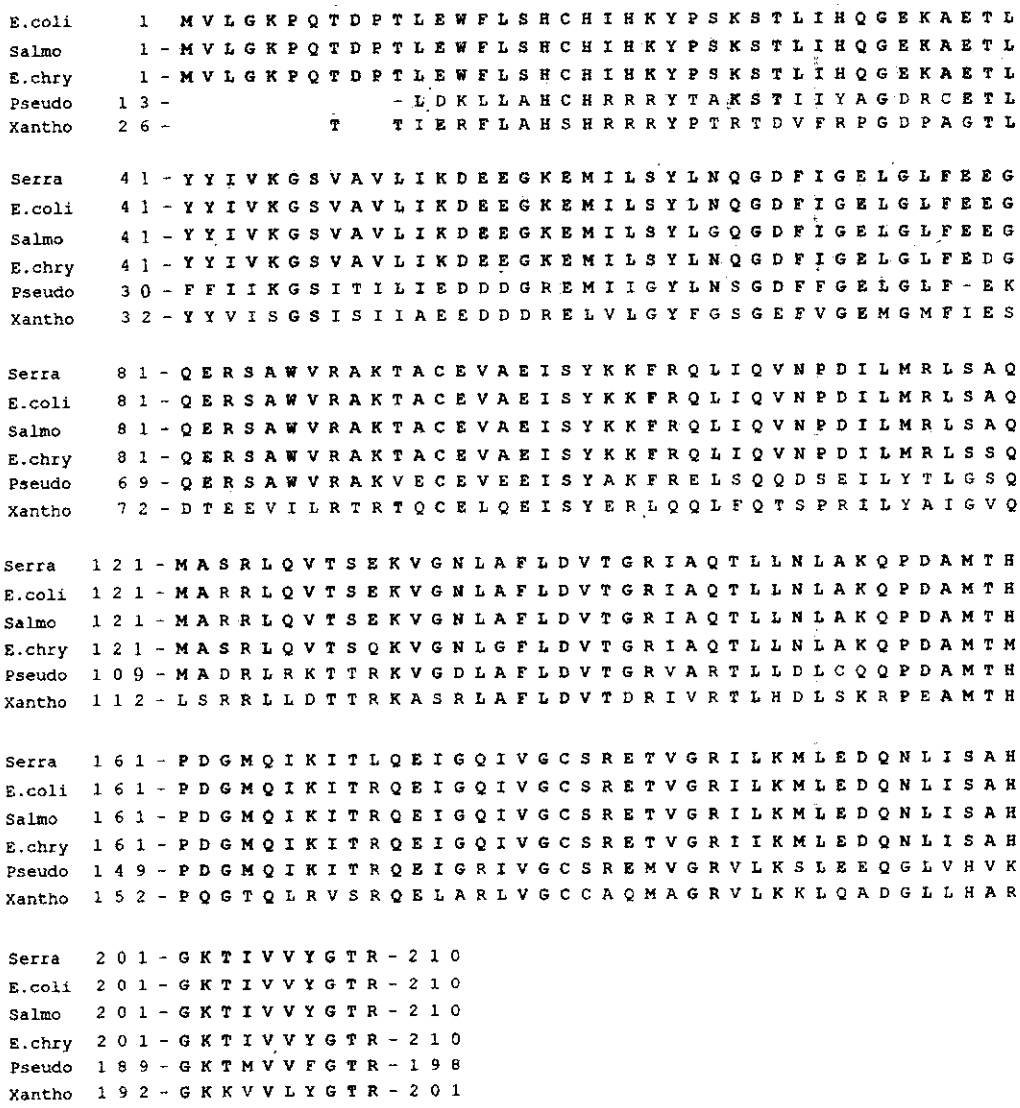

Fig. 3. Comparison of primary structure among CRPs from different organisms.
Amino acids identical to those of the CRP protein of *S. marcescens*. Lanes: Serra, *S. marcescens*; *E. coli* [1]; Salmo, *S. typhimurium* [24]; Echry, *E. chryisanthemi* [9]; Pseudo, *P. aeruginosa*; Xantho, *X. campestris* [10].

including *E. coli* cells containing the plasmid pMC1403-S6. When cyclic AMP was added to the *cya* mutant containing the fusion plasmid, the β-galactosidase activity decreased compared to the control without cAMP. This result showed that the *crp* gene could be expressed *in vivo* and negatively regulated by the cAMP-CRP binding

Table. 3. Effects of cAMP gene on the expression of the *crp-lacZ* fused gene.

Host strain	Plasmids	β-Gal activity (Unit)[a]			Efficiency[b] (%)	
		0	1 (mM cAMP)	2	1 (mM cAMP)	2
TP2010(*Δcya, Δlac*)	pMC1403	–	–	–	–	–
TP2010(*Δcya,Δlac*)	pMC1403-S6	2,260	1,430	1,360	63	60
MK1010(*cya*::Km)	pMC1403	850	1,190	1,650	140	195
MK1010(*cya*::Km)	pMC1403-S6	2,540	1,684	1,480	66	59

[a]One unit is defined as $103 \times OD_{420}/[0.5(OD_{610}) \times$reaction time (min)$\times$sample vol (ml)].

[b]Relative enzyme activity compared with that in the absence of cAMP taken as 100.

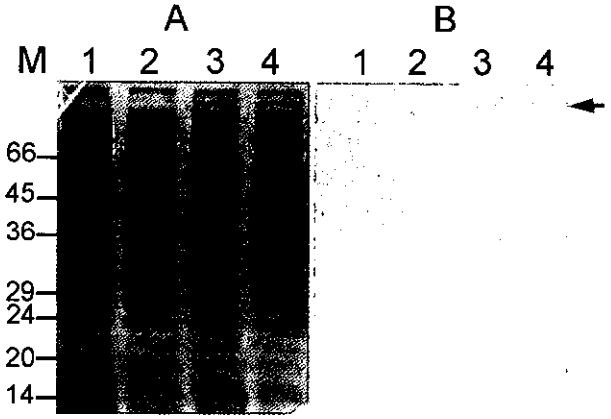

Fig. 4. Expression of the *crp-lac* protein fusion plasmid.
The same amount of protein samples were separated on a 10% SDS-PAGE and stained with Coomassie Brilliant Blue in A. A gel run in parallel to A was transferred onto a nylon membrane and stained with an antibody to the fused protein (B). Lanes: lane 1, TP2010 (pMC1403); lane 2, TP2010 (pMC1403/cAMP 1 mM); lane 3, TP2010 (pMC1403-S6); lane 4, TP2010 (pMC1403-S6/cAMP 1 mM). The arrow indicates the position of the band of *crp-lac* fusion protein.

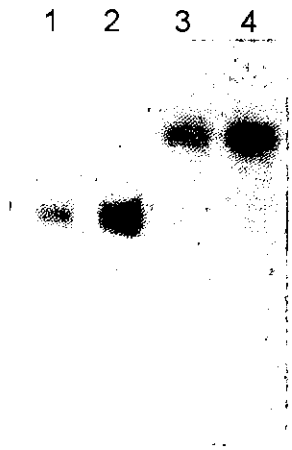

Fig. 5. Gel retardation assay for CRP binding to the *crp* regulation region.
The DNA fragment of 0.4 kb was used for the complex formation: Lane 1, DNA 0.2 pmol; 2, DNA/CRP 0.4 pmol; 3, DNA/CRP 0.4 pmol/cAMP; 4, DNA/CRP 0.6 pmol/cAMP.

complex. This result is consistent with previous results related to the *E. coli crp* promoter region [2]. Cossart and Gicquel-Sansey [9] reported that the expression of *E. coli crp* was negatively autoregulated and decreased in the presence of glucose in the growth medium.

To identify the *crp-lac*-fused protein, the plasmid pMC1403-S6 was introduced into the *E. coli* strain and an SDS-PAGE of the whole cell proteins and immunoblot analysis were performed (Fig. 4). The pMC1403-S6 plasmid did not produce a fusion protein. The corresponding band with strain TP2010 containing the same plasmid was less intense with the addition of cAMP. The expression of the gene with the plasmid encoding the fused protein was decreased about two-fold by the cAMP-CRP complex. This result indicates that *E. coli* CRP can substitute the *S. marcescens* CRP in a transcriptional expression system.

The putative CRP consensus sequence (5'AACTCTGA CCCCTG TGACACAA3') was found downstream of the putative promoter region of *S. marcescens crp*. To identify whether a binding site for CRP existed in the *crp* promoter region, a gel retardation assay for CRP was performed. Thus, purified CRP was incubated in the presence of cAMP with the fragment of pCKB12H. A specific CRP-dependent retardation in the electrophoresis of the fragment bearing the *crp* regulatory region was identified (Fig. 5). Therefore, these results indicate that the regulation of *crp* expression in *S. marcescens* is similar to that in *E. coli*.

Acknowledgments

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