# Expression of *Escherichia coli* DcuS-R Two-Component Regulatory System is Regulated by the Secondary Internal Promoter Which is Activated by CRP-cAMP

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The DcuS-R two-component system of *Escherichia coli* senses C4-dicarboxylates of the medium and regulates expression of the genes related to utilization of them. It is known that phospho-DcuR induces expression of genes such as the *dcuB-fumB* operon, the *frdABCD* operon, and the *dctA* gene. We analyzed promoters of the *dcuS-R* operon to elucidate the transcriptional regulation system. We found a novel internal promoter within the *dcuS* gene that is regulated by the transcriptional regulator, CRP-cAMP, in both aerobic and anaerobic conditions.

*Keywords*: CRP, cAMP, two-component regulatory system

*Escherichia coli* can use C4-dicarboxylates (succinate, fumarate, malate, and aspartate) as sole carbon and energy sources during both aerobic and anaerobic growth (Engel *et al.*, 1992; Golby *et al.*, 1998; Golby *et al.*, 1999; Abo-Amer *et al.*, 2004). The utilization of C4-dicarboxylates requires various enzymes associated with tricarboxylic acid (TCA) cycle as well as C4-dicarboxylate-specific transport systems (Guest, 1992). There are four known C4-dicarboxylate transporters in *E. coli*: the DctA system, which functions under aerobic growth, and the Dcu systems, DcuA, DcuB, and DcuC, which function independently during anaerobic growth (Kay and Kornberg 1971; Engel *et al.*, 1992; Six *et al.*, 1994; Zientz *et al.*, 1996; Davies *et al.*, 1999; Janausch *et al.*, 2002a). Some of the genes encoding the C4-dicarboxylate utilization systems are transcriptionally regulated in response to the C4-dicarboxylates in the medium. This regulation is mediated by a two-component regulatory system designated as DcuS-R (Zientz *et al.*, 1998; Golby *et al.*, 1999). The DcuS-R system induces transcription of the *dcuB-fumB* operon (encoding DcuB and the anaerobic fumarase B), the *frdABCD* operon (encoding the anaerobic fumarate reductase complex), and the *dctA* gene (encoding DctA) in response to external C4-dicarboxylates (Zientz *et al.*, 1998; Golby *et al.*, 1999).

Recent studies have shown that DcuS autophosphorylates *in vitro*, using ATP as a substrate, and this activity is stimulated by C4-dicarboxylates (Janausch *et al.*, 2002b). In addition, phospho-DcuS converted DcuR into phospho-DcuR that is a high-affinity DNA-binding protein with specificity to the *dcuB* fragment (Janausch *et al.*, 2002b). Thus, the available evidence suggests that the DcuS-R system of *E. coli* functions as a classical two-component sensor-regulator in controlling gene expression in response to

external C4-dicarboxylates (Janausch *et al.*, 2002a).

In *E. coli*, aerobic and anaerobic respirations are intertwined with the TCA cycle, and enzyme synthesis of the cycle is regulated by both carbon source and respiratory oxidant. Enzyme synthesis of the cycle is activated by cyclic AMP (cAMP) and cAMP receptor protein (CRP) in the absence of glucose. On the other hand, *E. coli* cells cultured in glucose synthesize TCA cycle enzymes at low levels. Enzyme synthesis relating to the TCA cycle is also decreased during anaerobic growth (Iuchi and Lin, 1995). In particular, the pathway from 2-oxoglutarate to fumarate (clockwise direction of the TCA cycle) is sharply decreased in anaerobic condition
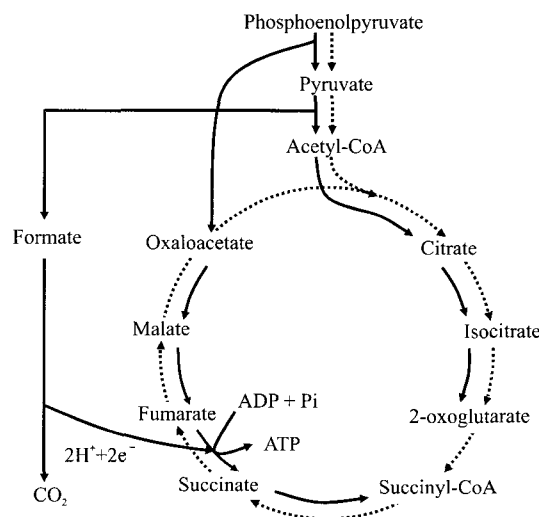


Fig. 1. Metabolic pathways of respiration in *E. coli*. Dotted lines indicate the TCA cycle under aerobic condition. Solid lines indicate branched noncyclic and fumarate respiration pathways under anaerobic condition.

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**Table 1.** Bacterial strains and plasmids used in this study

| Strain or plasmid | Genotype or description | Reference |
|---|---|---|
| **Strains** | | |
| KM1003 | *proAB/Tn10, Δ(argF-lac)U169, xyl, argH, his* | Yokoyama *et al.*, 2005 |
| KM1005 | *proAB/Tn10, Δ(argF-lac)U169, xyl, argH, his, Δcrp-39* | Yokoyama *et al.*, 2005 |
| KM1007 | *proAB/Tn10, Δ(argF-lac)U169, xyl, argH, his, Δcya* | Yokoyama *et al.*, 2005 |
| **Plasmids** | | |
| pMW222 | low copy promoter cloning vector | Yokoyama *et al.*, 2005 |
| pUC19 | cloning vector | Yanisch-Perron *et al.*, 1985 |
| pUC1906 | pUC19 carrying fragment 6 | this study |
| pMW22201 | pMW222 carrying fragment 1 | this study |
| pMW22202 | pMW222 carrying fragment 2 | this study |
| pMW22203 | pMW222 carrying fragment 3 | this study |
| pMW22204 | pMW222 carrying fragment 4 | this study |
| pMW22205 | pMW222 carrying fragment 5 | this study |
| pMW22206 | pMW222 carrying fragment 6 | this study |

(Fig. 1). Instead, the cycle is reconfigured to form the reductive branch operating from oxaloacetate to succinyl-CoA, which is the counter-clockwise pathway of the TCA cycle. Fumarate reduction to succinate functions in anaerobic respiration, namely fumarate respiration (Spiro *et al.*, 1990; Tran *et al.*, 1997; Tran and Unden, 1998). Therefore, an additional task of the TCA cycle to the reductive branch is to produce fumarate as a respiratory acceptor. The respiration is an ATP-generating process in which formate acts as an electron donor through a chain of electron transfer to the electron acceptor, fumarate, as described in the reviews (Cecchini *et al.*, 2002; Lancaster, 2002).

CRP-cAMP modulates the transcription activities of many genes in response to glucose levels in culture medium. High levels of glucose reduce the levels of cAMP within cells. Conversely, glucose starvation leads to an increase in cAMP levels allowing a molecule of cAMP to bind CRP. The increased CRP-cAMP complex activates or inactivates transcription from many specific gene-promoters scattered in the genome.

In this paper, we analyzed promoters of the *dcuS-R* operon that regulates $C_4$-dicarboxylate metabolism genes, and found a novel internal promoter, P2$_{dcuR}$, within the *dcuS* gene that was regulated by CRP-cAMP aerobically and anaerobically.

## Materials and Methods

### Bacterial strains and plasmids
The bacterial strains of *E. coli* and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) medium was used for the growth of bacteria under aerobic or anaerobic condition at 37°C. For anaerobic growth, cells were grown in LB medium using BBL GasPak Pouch Anaerobic System (Becton, Dickinson and Company). To know β-galactosidase (β-Gal) activities of transformants roughly, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was added into the LB agar medium at concentration with 132 μg/ml. If necessary, antibiotics were used at the following concentrations:

**Table 2.** Primers used in this study

| Primer | Sequence (5' to 3') |
|---|---|
| -21M13 | GTGCTGCAAGGCGATTAAGTTGG |
| M13RV | TCCGGCTCGTATGTTGTGTGGA |
| B1 | CCCGGATCCAATGAATGTCTCATCAGC |
| B2 | CCCGGATCCGAGACATCTACCACCTG |
| B3 | CCCGGATCCCAGCAGAGAGCCAATCTC |
| B4 | CCCGGATCCGCCAGCCGGTGAGCGC |
| E1 | GCGGAATTCACTATAAGCATAGCCCTG |
| E2 | GCGGAATTCCCATCTACGATGAAAATC |
| E3 | GCGGAATTCTGATGCAGCGACTCGACG |

ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; tetracycline, 20 μg/ml. Oligo DNAs synthetically made were purchased from Invitrogen (Table 2). The various kinds of DNA fragments (Fig. 2) were amplified by PCR with *E. coli* K12 genomic DNA and a pair of upstream and downstream primers (Table 2 and Fig. 2). PCR reactions were performed using the ABI GeneAmp PCR System 9600 in the following conditions: genomic DNA, 200 ng; primers, 1.0 μM each; dNTPs, 200 μM and *Ex Taq* polymerase (TaKaRa), 2.5 units in a 50 μl reaction mixture. Thirty cycles of a reaction were run as follows: 95°C, 20 sec; 55°C, 1 min and 70°C, 2 min. Amplified DNA fragments were purified by PCR purification kit (Qiagen) and digested with the restriction enzymes, *Bam*HI and *Eco*RI. The DNA fragments were recovered by polyacrylamide gel electrophoresis and ethanol precipitation. The resultant products were ligated with a *lacZ* operon fusion vector, pMW222 (for β-Gal assay) and pUC19 vector (for footprinting experiment and primer extension assay) which were previously digested with *Bam*HI and *Eco*RI restriction enzymes, respectively. The low-copy plasmid for promoter cloning, pMW222, possesses a promoter-less *lacZ* gene and *Eco*RI and *Bam*HI sites in front of the gene
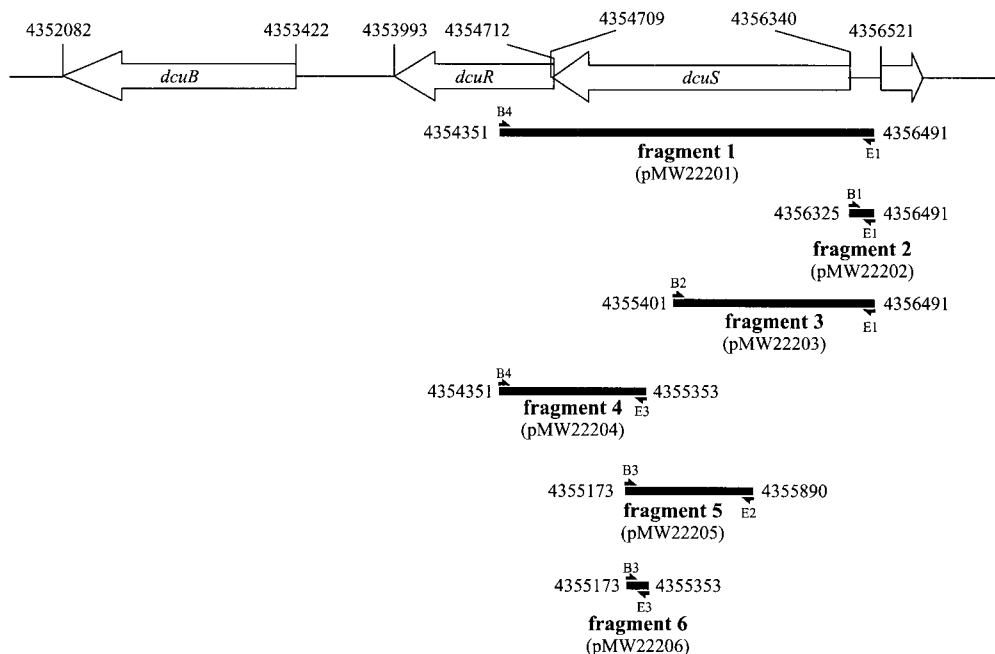
**Fig. 2.** Gene organization in the *dcuS-R* region on the *E. coli* chromosome and DNA fragments used in this study. The positions for genes and fragments are given by the numbers with respect to the *E. coli* K12 chromosome map (GenoBase reference search; NAIST [http://ecoli.naist.jp/GB6/search.jsp]). The solid bars show various kinds of DNA fragments amplified by PCR using primers indicated by the arrows accompanied with names (see Table 2). The pMW222 derivatives carrying each fragment are shown in parentheses.

(Yokoyama *et al.*, 2005). The *Eco*RI site is more upstream than the *Bam*HI site from the *lacZ* gene. The constructed plasmids were introduced into *E. coli* strains.

### *β-Gal assay*
The plasmids, pMW22201 to pMW22206, were introduced into *lac*-deletion strains, KM1003 (wild-type), KM1005 (Δ*crp*), and/or KM1007 (Δ*cya*) (Table 1). The transformants were grown aerobically and anaerobically in the LB medium. If necessary, cAMP was added at the final concentration of 0.1 mM. Cells were grown exponentially, and β-gal activities were measured by the method as described by Miller (1992).

### *Primer extension assay*
The plasmid pUC1906 was introduced into the KM1007 strain and the transformant was cultured with or without cAMP in LB medium at 37°C. *E. coli* RNAs were purified by using RNeasy Mini Kit (Qiagen) and resuspended in RNase free water. Purified RNAs (10 μg each) were mixed with the universal M13RV sequencing primer (0.1 pmol) labeled with one of fluorescent dyes, 6-FAM, at the 5'-end, and 10× Reverse transcriptase buffer (250 mM Tris-HCl; pH 8.3, 500 mM KCl, 20 mM DTT, and 50 mM MgCl₂) in the total volume of 45 μl. The mixtures were heated at 80°C for 5 min and allowed to cool to 37°C gradually. After annealing, 4 μl of 2.5 mM dNTP solution and 1 μl of AMV reverse transcriptase XL (TaKaRa) were added to each mixture and incubated at 37°C for 30 min. Each DNA was precipitated by ethanol precipitation and suspended in 10 μl loading buffer (8.3 mM EDTA, 5 mg/ml blue dextran, and 83% formamide). The samples were run on the gel with G, A, T, and C sequence samples by the ABI DNA sequencer. G,

A, T, and C sequence ladders of plasmid pUC1906 were obtained using the same primer by BcaBEST Dideoxy sequencing Kit (TaKaRa).

### *Footprinting experiment*
The DNA region containing the fragment 6 was amplified using pUC1906 (Table 1) by PCR with universal primers, -21M13 and M13RV labeled with 6-FAM at the 5'-end (Table 2), and was gel purified. The resultant DNA fragment (1.8 pmol), CRP (18 pmol), and cAMP (1 mM) were mixed in the buffer (20mM Tris-HCl; pH 7.9, 3 mM MgCl₂, 5 mM CaCl₂, 0.1 M NaCl, 0.1 mM EDTA, 0.1 mM DTT, and 50 μg/ml bovine serum albumin) in a total volume of 100 μl. No CRP-cAMP sample was also prepared without CRP and cAMP. Reaction mixtures were incubated at 37°C for 10 min, then at 25°C for 5 min. After addition of 1.5 μl of 20 μg/ml DNase I, the mixture was mixed immediately, and incubated at 25°C for 1 min, then 25 μl of stop solution (1.5 M CH₃COONa; pH 5.2, 20 mM EDTA, and 100 μg/ml tRNA), and 100 μl of TE (10 mM Tris-HCl; pH 8.0 and 1 mM EDTA) saturated phenol were added and mixed thoroughly and centrifuged at for 8,000×g 5 min. The DNAs were precipitated and resolved in 10 μl of electrophoresis buffer (8 M urea, 50 mM Tris-borate; pH 8.3, 1 mM EDTA, 0.025% bromophenol blue, and 0.025% xylene cyanol). The DNA samples were analyzed with G, A, T, and C sequence ladders by ABI PRISM 377 DNA sequencer (Perkin-Elmer). G, A, T, and C sequences were done using BcaBEST Dideoxy Sequencing Kit (TaKaRa) with pUC1906 and the universal M13RV sequencing primer labeled with 6-FAM at the 5'-end.
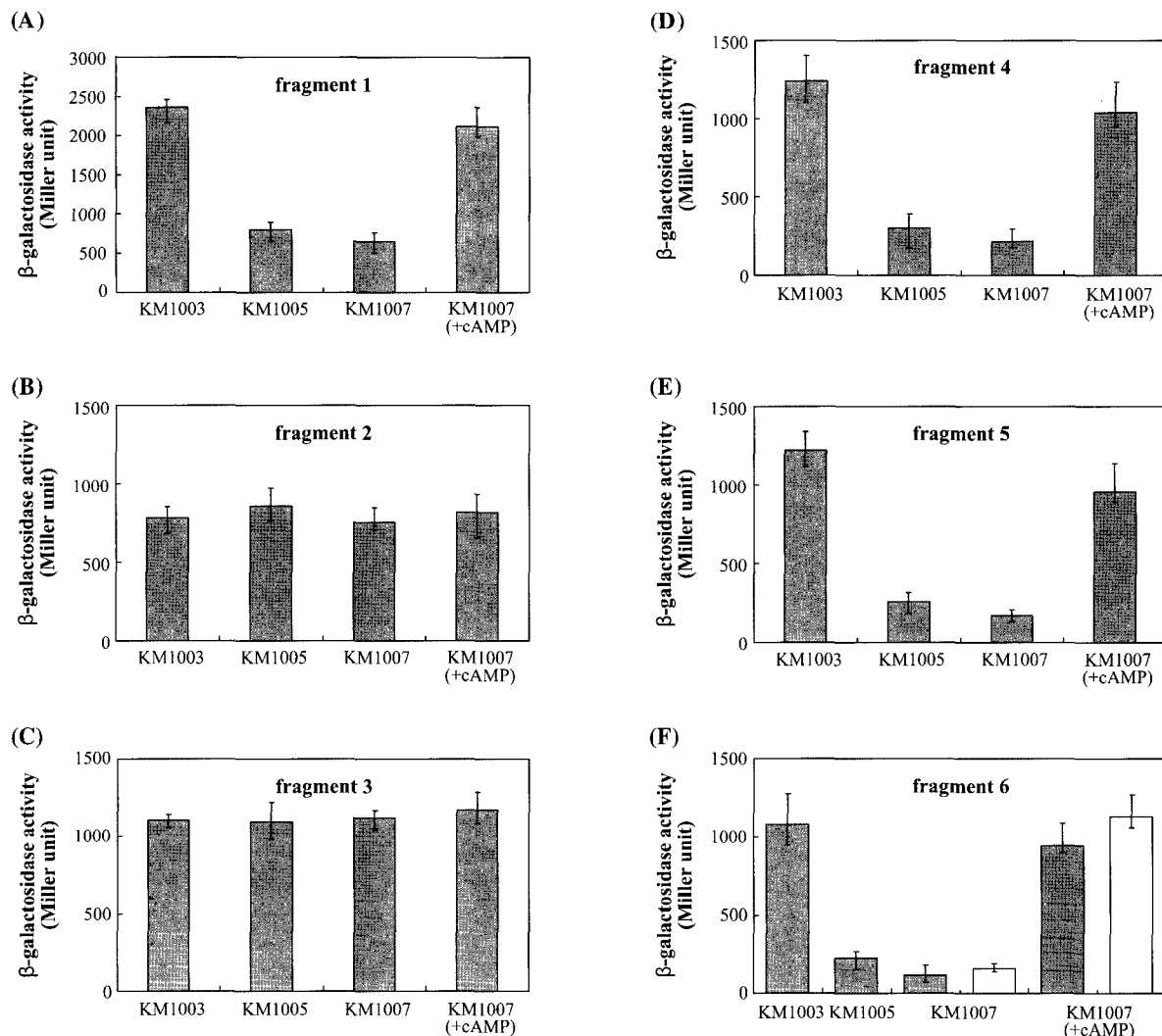
**Fig. 3.** Effects of CRP-cAMP complex on *lacZ* expression in *lac*-deletion strains [KM1003 (wild-type), KM1005 (Δ*crp*), and KM1007 (Δ*cya*)], containing pMW22201 (A), pMW22202 (B), pMW22203 (C), pMW22204 (D), pMW22205 (E), or pMW22206 (F) as shown in Fig. 2. The fragment carried by the vector pMW222 is shown at the top in each panel (see Fig. 2). Cultures were grown aerobically (gray bars) or anaerobically (open bars) in LB medium without cAMP (KM1003, KM1005, and KM1007) or with 0.1 mM cAMP [KM1007 (+cAMP)]. The β-gal activities are the means of the three experiments with the standard deviations by the protocol of Miller (1992). The activities of each strain carrying the vector plasmid pMW222 were about 10 units.

## Results and Discussion

### Identification of the internal CRP-cAMP dependent promoter within the dcuS-R operon

It is known that expression of the *dcuS-R* operon is constitutive, but negatively regulated by the NarX-L two-component system in the presence of nitrate (Goh *et al.*, 2005). We have previously constructed a cloning system for CRP-cAMP dependent promoters from *E. coli* genomes (Yokoyama *et al.*, 2005). In this system, the DNA fragments carrying a CRP-cAMP dependent promoter can be easily cloned by comparing the colony colors of KM1007 (Δ*cya*) containing a pMW222 derivative on the X-Gal plates with and without cAMP. We analyzed various DNA fragments of the *dcuS-R* region to elucidate the transcriptional system in detail. For this purpose, we cloned DNA fragments amplified by PCR

into the *Eco*RI and *Bam*HI sites of pMW222, and the resultant plasmids were introduced into the KM1007 cells.

The colony colors of KM1007 cells carrying pMW22201 (containing the fragment 1), pMW22202 (the fragment 2), or pMW22203 (the fragment 3) (Fig. 2) were compared on the LB-X-Gal plates with and without cAMP. The colony color of KM1007 cells carrying pMW22201 with cAMP was bluer than that without it, whereas colony colors of the cells carrying pMW22202 or pMW22203 were almost same on the plates with and without cAMP. We measured β-gal activities of the cells carrying these plasmids. As shown in Fig. 3A, KM1007 cells carrying pMW22201 with cAMP [KM1007 (+cAMP)] caused about 3-fold increase in β-gal activity as compared with those without cAMP. The β-gal activity of the wild-type strain KM1003 carrying pMW22201 was almost same as that of KM1007 with cAMP. The activity of a *crp*

mutant strain KM1005 carrying the plasmid was lower than that of the wild-type strain. The activities of KM1007 cells carrying pMW22202 and pMW22203 were not affected by the cAMP addition (Fig. 3B and C). No effects of CRP-cAMP complex were also shown in the activities of KM1003 and KM1005 carrying these plasmids. These results suggest that the specific region of the fragment 1, which is neither covered by the fragment 2 nor the fragment 3, is responsible for the CRP-cAMP dependency (Fig. 2). Slightly high activities of the strains harboring pMW22203 (the fragment 3) as compared with those of pMW22202 (the fragment 2) may be caused by stability of mRNA. To determine the CRP-cAMP dependent promoter region of the fragment 1, the fragments 4 and 5 (Fig. 2) were introduced into pMW222 and the activities of the cells carrying these plasmids (pMW22204 and pMW22205) were measured (Fig. 3D and E). The results showed that both of the fragments 4 and 5 contained the CRP-cAMP dependent promoter region, indicating that the CRP-cAMP dependent promoter existed in the overlap region between the fragments 4 and 5. The 180 bp overlap region (the fragment 6, Fig. 2) was cloned into pMW222 and the resultant plasmid was named as pMW22206. The strains (KM1003, KM1005, and KM1007) were transformed by the plasmid and their β-gal activities were measured. The activity of KM1007 in the presence of cAMP was increased to about 8-fold as compared with that in the absence of cAMP (Fig. 3F). The β-gal activity of the wild-type strain KM1003 carrying pMW22206 was almost same as that of the KM1007 with cAMP. The activity of the *crp* mutant strain KM1005 carrying the plasmid was also lower than that of the wild-type strain. We also measured β-gal activities of the KM1007 carrying pMW22206 in the anaerobic condition with and without cAMP (Fig. 3F). The activity in the presence of cAMP in the anaerobic condition was also increased as same as the activity in the aerobic condition. The activities in the anaerobic condition were slightly higher than those in the aerobic condition. We supposed that FNR was responsible for the effect in the anaerobic condition, because FNR was the global transcriptional regulator that controlled gene expression in response to the transition between aerobic and anaerobic growth (Green *et al.*, 2001). To figure out the effect of FNR in the anaerobic condition, we made a *fnr*⁻ strain isogenic to KM1007, and pMW22206 was introduced into the strain. In the anaerobic condition, the β-gal activities of the *fnr*⁻ strain carrying pMW22206 were almost same as those of KM1007 carrying pMW22206 (data not shown), indicating that the fragment 6 did not contain any DNA sequence related to the regulation by FNR.

These results described above indicated that in addition to the *dcuS-R* transcriptional system that was previously reported (Goh *et al.*, 2005), expression of *dcuR*, not of *dcuS*, was also regulated by the novel internal CRP-cAMP dependent promoter in aerobic and anaerobic conditions, which was located within the *dcuS* coding region. We designated the previously reported promoter of *dcuS-R* as P1$_{dcuSR}$ and the novel internal promoter as P2$_{dcuR}$ (see Fig. 7).

***Determination of the transcriptional initiation site from the internal promoter (P2$_{dcuR}$) of the dcuS-R operon***
To elucidate whether CRP-cAMP regulation occurs at the

transcriptional level, we performed primer extension assay using the total RNAs extracted from KM1007 cells carrying pUC1906 (pUC19 carrying the fragment 6, Fig. 2 and Table 1) grown with or without cAMP in the aerobic condition. In the absence of cAMP, no primer extension product was found (Fig. 4; lane 1), and the product was detected in the presence of cAMP (Fig. 4; lane 2), indicating that expression from the promoter occurs in the presence of CRP-cAMP complex. These results are consistent with the promoter activities of the KM1007 carrying pMW22206 as shown in *in vivo* data (Fig. 3F). The initiation site is located at the T residue that is 530 bp upstream from the translation initiation site of the *dcuR* gene. At the 9 bp upstream from the initiation site, there is a DNA sequence (TTGCAT) slightly similar to the consensus sequence for the -10 regions of *E. coli* promoters (Fig. 4). Furthermore, 16 bp upstream from the -10 region, a putative -35 sequence (ATTGCA) exists (see Fig. 6).

***Determination of the CRP-cAMP binding site in the internal promoter (P2$_{dcuR}$) of dcuS-R***
To know whether the CRP-cAMP complex directly binds to DNA *in vitro*, DNase I footprinting experiment was performed. The CRP-cAMP complex protected the DNA segment spanning nucleotides -46 to -69 (from the transcription initiation site) from DNase I digestion in the non-coding strand (Fig. 5). Moreover, the CRP-cAMP binding enhanced DNase I di-
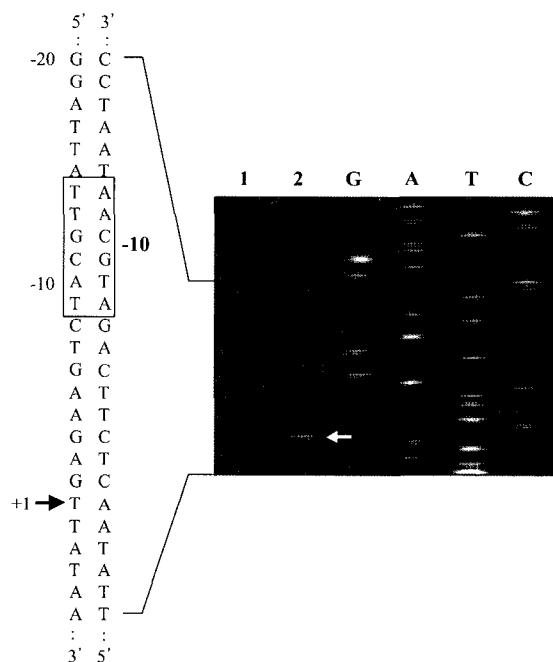


**Fig. 4.** Transcriptional initiation site from the internal promoter P2$_{dcuR}$ in the *dcuS-R* operon. Total RNAs were isolated from KM1007 cells containing pUC1906 grown in LB medium without (lane 1) or with 0.1 mM cAMP (lane 2). Primer extension analysis was carried out as described in the Materials and Methods. Lanes G, A, T, and C show the DNA sequencing reaction ladders of pUC1906 using the same primer. The nucleotide sequences around the initiation site are shown in the left. The box indicates the -10 region. The primer extension product and corresponding sequence (T) are indicated by arrows.
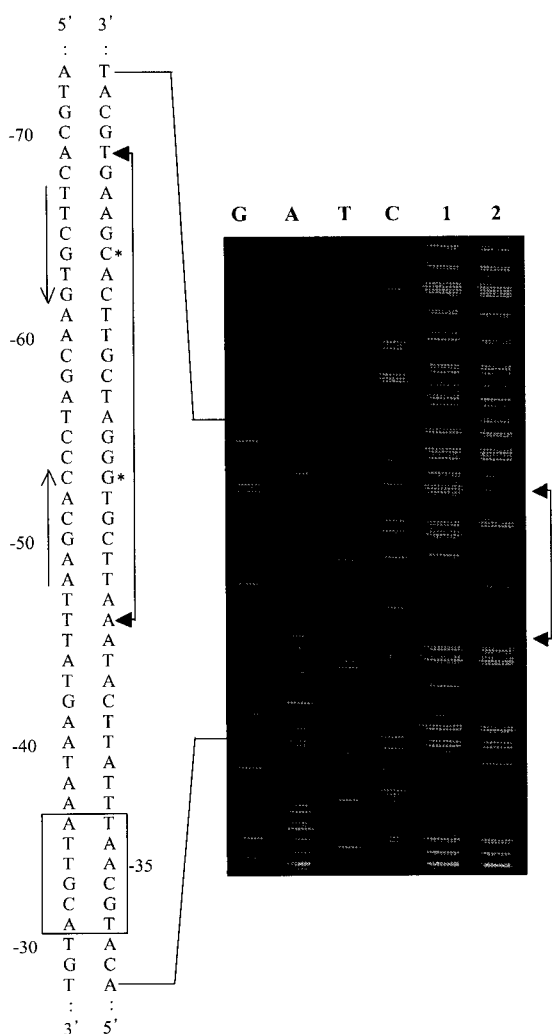
Fig. 5. DNase I footprinting analysis of the P2dcuR promoter region. Templates were labeled on the bottom strand and subjected to DNase I protection as described in the Materials and Methods. Lanes G, A, T, and C; the DNA sequencing reaction products. Lane 1; without CRP-cAMP. Lanes 2; with CRP-cAMP. The corresponding nucleotide sequences are shown in the left. The box indicates the -35 region. The brackets with arrows show regions protected from DNase I digestion in the presence of CRP-cAMP. The asterisks show the nucleotides enhanced from DNase I digestion. The arrows indicate inverted repeat sequences within the CRP-cAMP binding site.
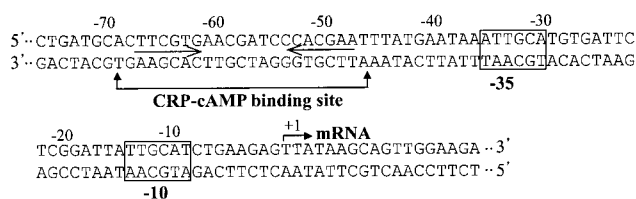


Fig. 6. Schematic presentation of the CRP-cAMP binding site in the P2dcuR promoter. Boxes; the putative -10 region and -35 region. Bracket with arrows; the region protected from DNase I digestion in the presence of CRP-cAMP. Arrows; positions of inverted repeat sequences for the CRP-cAMP binding site. The transcription initiation site is indicated as +1.
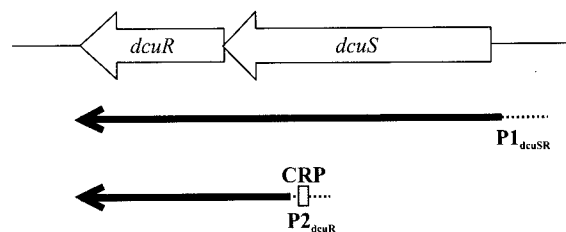


Fig. 7. Schematic presentation of transcriptional regulations of the dcuS-R operon. Bold arrows indicate transcripts from the P1dcuSR or the P2dcuR promoters. The CRP-cAMP biding site is indicated by the gray box. Expression from the P1dcuSR promoter is constitutive as described previously (Goh et al., 2005). Expression from the P2dcuR promoter is activated in the presence of CRP-cAMP, which results in increased expression of dcuR.

gestion at positions -53G and -64C from the transcription initiation site. We found a CRP-cAMP binding sequence (TTCGTGAACGATCCCACGAA) within the protected region, which was very similar to the CRP-cAMP binding consensus sequence (ttTGTGAnnnnnnTCACAaa) (Spiro et al., 1990). The CRP-cAMP binding site also contains inverted repeat sequences (TTCGTG) (Fig. 5). These data described above are summarized in Fig. 6. CRP-cAMP is known to activate transcription by binding to sites located at various distances from the transcription initiation sites (-41.5 to -103). CRP-cAMP dependent promoters can be grouped into three classes: class I; class II; and class III (Ishihama, 2000). It is suggested that the P2dcuR promoter is classified as a class

I CRP-cAMP dependent promoter that has the CRP-cAMP binding site located upstream from the -35 sequence, and CRP-cAMP bound to the promoter interacts with the carboxy-terminal domain of the α-subunit of RNA polymerase. Our data together with the previously reported results (Goh et al., 2005) have shown that the P1dcuSR promoter located at upstream of the dcuS-R operon is constitutive in the absence of nitrate and the internal promoter (P2dcuR) located within the dcuS coding region is positively regulated by CRP-cAMP in aerobic and anaerobic conditions (Fig. 7). Though the transcriptional initiation site from the P2dcuR promoter is over half a kb upstream from the translation start site of dcuR, there is no open reading frame (>100 amino acids) except dcuR in the region from the P2dcuR promoter to dcuR. Therefore, expression of DcuR is activated by the transcription from the P2dcuR promoter in the presence of CRP-cAMP. Based on these results, we suggest that in addition to the qualitative change of DcuS by $C_4$-dicarboxylates such as fumarate, the quantitative increase of DcuR by CRP-cAMP is required for high activation of signal transduction by DcuS-R, and also suggest that transcriptional regulation of dcuR by CRP-cAMP affects expression of the genes under control of the DcuS-R two-component system.

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