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Regulatory Role of cAMP Receptor Protein over *Escherichia coli* Fumarase Genes

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***Escherichia coli* expresses three fumarase genes, namely, *fumA*, *fumB*, and *fumC*. In the present study, catabolite repression was observed in the *fumA-lacZ* and *fumC-lacZ* fusion strains, but not in the *fumB-lacZ* fusion strain. The Crp-binding sites in *fumA* and *fumC* were identified using an electrophoretic mobility shift assay and footprint analysis. However, the electrophoretic mobility shift assay did not detect band shifts in *fumB*. Fnr and ArcA serve as transcription regulators of fumarase gene expression. In relation to this, different mutants, including Δ*cya*, Δ*crp*, Δ*fnr*, and Δ*arcA*, were used to explore the regulatory role of Crp over *fumA* and *fumC*. The results show that Crp is an activator of *fumA* and *fumC* gene expression under various oxygen conditions and growth rates. ArcA was identified as the dominant repressor, with the major repression occurring at 0–4% oxygen. In addition, Fnr was confirmed as a repressor of *fumC* for the first time. This study elucidates the effects of Crp on fumarase gene expression.**

***Keywords*:** fumarase, cyclic AMP receptor protein, electrophoretic mobility shift assay, footprint assay

Introduction

Malic acid can be derived from the aerobic enzymatic conversion of fumaric acid using fumarase in *Escherichia coli* (Moon *et al.*, 2008). FumA and FumC appear to be components of the tricarboxylic acid (TCA) cycle because they are predominantly expressed under aerobic conditions. Research has shown that FumA and FumC activities decline and become growth-rate independent in the mutation *cya*, which is responsible for cAMP synthesis and results in an active cAMP-Crp complex (Botsford and Harman, 1992; Tseng *et al.*, 2001). Microarray results have demonstrated that Crp activates *fumA* gene expression (Gosset *et al.*, 2004). Although glucose catabolite repression is observed for *fumA* and *fumC* gene expression, data on Crp regulation of the *E. coli fumC* gene are limited (Park and Gunsalus, 1995; Gama-Castro *et al.*, 2011; Keseler *et al.*, 2011). The difference in regulation between these two genes is not readily clear. In contrast to the aerobic increase in FumA and FumC activities, FumB is more abundant under anaerobic conditions and *fumB* gene expression requires ArcA and Fnr as transcriptional activators (Tseng, 1997). Golby *et al.* (1998) reported that a *dcuB-fumB* cotranscript can be driven by the *dcuB* promoter, which is activated by Crp. However, the monocistronic transcript of *fumB* is two times higher than that from the *dcuB-fumB* operon (Golby *et al.*, 1998). This finding suggests that the independent promoter of the *fumB* gene is dominant in its transcriptional expression. Nevertheless, no study has focused on Crp regulation of the independent promoter of the *fumB* gene.

The Crp-DNA binding site of target promoters includes palindromic TGTGAN$_6$TCACA sequences. More than 200 genes are modulated by Crp, which plays a critical role in gene expression (Ishihama, 2010). Recently, Shimada *et al.* (2011) identified several novel regulation targets in the transport and carbon metabolizing genes of *E. coli* using genomic SELEX screening, and found that cAMP-Crp is essential in the transcriptional regulation of central metabolism. The present study elucidates the effects of Crp regulation on the *fumA*, *fumB*, and *fumC* genes with independent promoters, using *in vivo* and *in vitro* experiments. Crp-DNA binding assays were performed using an electrophoretic mobility shift assay (EMSA) and a footprint analysis. Two regulatory systems – Fnr and ArcA – were investigated to explore the regulatory role of Crp over *fumA* and *fumC* because fumarase genes are involved in the aerobic TCA cycle and anaerobic respiratory pathways. The current study provides evidence for the transcriptional activation of *fumA* and *fumC* by Crp, and reveals that the independent promoter of *fumB* is not controlled by Crp. Moreover, ArcA repression of *fumA* and *fumC* at various oxygen concentrations was found to be stronger than that of Fnr. These findings help clarify an *E. coli* regulatory network, consisting of Crp, ArcA, and Fnr, that regulates the fumarase genes.

Materials and Methods

Bacterial strains and cell growth

Wild-type *E. coli* MC4100 was used, from which the Δ*crp*, Δ*cya*, Δ*fnr*, Δ*arcA*, Δ*fnr* Δ*arcA* double-mutant, and Δ*crp* Δ*fnr* Δ*arcA* triple-mutant strains were derived (Table 1). The P1 phages containing Δ*crp*, Δ*fnr*, and Δ*arcA* deletions were introduced into the *fumA-lacZ*, *fumB-lacZ*, and *fumC-*

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Table 1. Strains used in the present study

Name	Characteristics	References
Strains		
MC4100	F⁻ *araD139 Δ(argF-lac)U169 rpsL 150 relA1 flb-5301 deoC1 ptsF25 rbsR*	Park and Gunsalus (1995)
HT28	W3110 Δ*cya::kan*	Kimata *et al.* (1997)
IT1409	W3110 Δ*crp::tet*	Kimata *et al.* (1997)
fumA-lacZ	MC4100 Φ(*fumA-lacZ*)	Park and Gunsalus (1995)
fumB-lacZ	MC4100 Φ(*fumB-lacZ*)	Tseng (1997)
fumC-lacZ	MC4100 Φ(*fumC-lacZ*)	Park and Gunsalus (1995)
fumA-lacZ ΔarcA	MC4100 Φ(*fumA-lacZ*) Δ*arcA::kan*	Park and Gunsalus (1995)
fumC-lacZ ΔarcA	MC4100 Φ(*fumC-lacZ*) Δ*arcA::kan*	Park and Gunsalus (1995)
fumA-lacZ Δfnr	MC4100 Φ(*fumA-lacZ*) Δ*fnr*	Park and Gunsalus (1995)
fumC-lacZ Δfnr	MC4100 Φ(*fumC-lacZ*) Δ*fnr*	Park and Gunsalus (1995)
fumA-lacZ Δfnr, ΔarcA	MC4100 Φ(*fumA-lacZ*) Δ*fnr*, Δ*arcA::kan*	Park and Gunsalus (1995)
fumC-lacZ Δfnr, ΔarcA	MC4100 Φ(*fumC-lacZ*) Δ*fnr*, Δ*arcA::kan*	Park and Gunsalus (1995)
fumA-lacZ Δcya	MC4100 Φ(*fumA-lacZ*) Δ*cya::kan*	This study
fumC-lacZ Δcya	MC4100 Φ(*fumC-lacZ*) Δ*cya::kan*	This study
fumA-lacZ Δcrp	MC4100 Φ(*fumA-lacZ*) Δ*crp::tet*	This study
fumC-lacZ Δcrp	MC4100 Φ(*fumC-lacZ*) Δ*crp::tet*	This study
fumA-lacZ Δcrp, Δfnr, ΔarcA	MC4100 Φ(*fumA-lacZ*) Δ*fnr*, Δ*arcA::kan*, Δ*crp::tet*	This study
fumC-lacZ Δcrp, Δfnr, ΔarcA	MC4100 Φ(*fumA-lacZ*) Δ*fnr*, Δ*arcA::kan*, Δ*crp::tet*	This study

lacZ fusion strains (Park and Gunsalus, 1995; Tseng, 1997). The expression of β-galactosidase was driven by the independent promoters of *fumA*, *fumB*, and *fumC*. A 1.5-L fermentor was operated with a 1 L working volume of Vogel-Bonner medium supplemented with casamino acids (0.25 mg/L) and glucose (2.25 mM). The air saturation of the medium was controlled by sparging the vessel with a stream of premixed air (21% O_2) and nitrogen (99.8%) before determiningthe effect of oxygen level on the *E. coli* strains. The oxygen in the fermentor was monitored with an Ingold oxygen probe (model 1046) and calibrated with 100% air and 99.8% nitrogen. An aerobic, continuous culture was maintained at 300 ml/min with sterile air during experiments determining *fumA-lacZ* and *fumC-lacZ* expression under different growth rates (Tseng, 1997).

β-Galactosidase and northern blot assays

β-Galactosidase was determined by hydrolysis of *o*-nitrophenyl-β-d-galactopyranoside as previously described (Cotter and Gunsalus, 1989). The total RNA from *E. coli* was extracted using TRIzol Reagent (Invitrogen, USA) and treated with 2 U of RNase-free DNase for 1 h. Northern hybridization was performed using a digoxigenin (DIG) system (DIG Wash and Block Buffer Set; Roche Diagnostics, Germany). RNA transfer, immobilization, and hybridization were conducted as described by Sambrook *et al.* (1989). The *fumA* and *fumC* probes were DIG-labeled via polymerase chain reaction (PCR) amplification using a PCR DIG Probe Synthesis Kit (Roche Diagnostics). The primers used for *fumA* were fumA-f GTTACTGACGCCGGGGAAACTG and fumA-r CCAAG ATTTTGCGCTTCGATCA, whereas those used for *fumC* were fumC-f GATTATGCTCGAGCATCGCTACCCA and fumC-r CGACGAATTCCCGCTGGCTATCTGGCA.

Expression and purification of Crp protein

The recombinant Crp protein was constructed according to the method described by Peekhaus and Conway (1998) 1998) and purified using a nickel column. The *crp* gene was introduced into the pQE30 plasmid to obtain the plasmid pCRP. *E. coli* BL21(DE3) was transformed with pCRP and inoculated using a single colony for overnight culture. The *E. coli* harboring pCRP was transferred into LB medium and grown until an OD_{600} of 0.4–0.6 was obtained at 37°C. The culture was induced by the addition of 1 mM isopropyl β-d-thiogalactoside and incubated for 3 h at 37°C. Then, the cells were harvested and disrupted using a French press. Protein expression was monitored via SDS-PAGE (10% gel) of the total cellular and soluble proteins, followed by Coomassie Blue staining. A nickel column and an Invitrogen Ni-NTA Purification System were used according to the manufacturer's instructions for the purification of the Crp protein from the *E. coli* culture.

EMSA of Crp-DNA binding sites

A similar palindromic sequence, 5′-TGTGAN$_6$TCACA-3′, was found at the Crp binding site for both *fumA* and *fumC*. To confirm Crp binding to palindromic sequences, oligonucleotides (50 mer) were designed as follows: positive control, GTAAGCGATTACACTGATGTGATTTGCTTCA CATCTTTTTACGTCGTACT; fumA+56, GATAATGAA AGGGTTTGTTTGACATTGTTCTCTCACTTACTGCCT GGTTT; fumA*+56, GATAATGAAAGGGTTTGTCTCA CATTGTTGTGTCACTTACTGCCTGGTTT; fumC-61, G GAAATGACTTCTTCCAGCAGATACAACTCACACAA TGCACCCGCTGTGT; fumC*-61, GGAAATGACTTCTT CCAGCACATACAACTGAGACAATGCACCCGCTGTG T; fumC-492, GCACGATTATCGTCGGTCGTGATATTG CTCACGCCAAACTGAAAGAGCGG; and fumC*-492, G CACGATTATCGTCGGTCCTCATATTGCTGAGGCCA AACTGAAAGAGCGG. The Crp binding sites at positions +56, -61, and -492 were numbered relative to the transcription start site of *fumA* and *fumC*. The EMSA assays were con-

ducted by annealing commercially synthesized complementary 50 mer oligonucleotides to DNA fragments, from 65°C to room temperature. The EMSA reaction mixtures contained DNA oligonucleotide (20 nM), cAMP (100 μM), and Crp at various concentrations (0–200 nM) in buffer solution consisting of 20 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EDTA, and 100 mM KCl. The reaction mixtures were gently mixed and incubated for 15 min at 37°C to allow binding. The loading dye containing Novel Juice (GeneDireX, USA) was subsequently added to the reaction mixtures, which were then separated using 6% non-denaturing polyacrylamide gels.

Footprint analysis of Crp-DNA binding sites

The footprint assay for DNA binding was conducted using a nonradioactive dye primer sequenced as previously described (Zianni *et al.*, 2006). The probes were amplified by PCR with the following primers: fumA-1F (labeled with FAM), CGCCACATATTTATCATTTTCGC TG; fumA-1R, TTCAGTCAACGCCGTTTTGCTG; fumC-1F (labeled with FAM), AAGCGCAGGGCGGAAGTATG; fumC-1R, CGA GCGTTGAGTTTGTGCGC; fumC-2F (labeled with FAM), ATCCTGCCCGGTCGGTATG; and fumC-2R, AGGTAG AAGCCGCCGTGTTTTTTA. The DNA binding protein mixtures contained a labeled probe, cAMP (100 μM), and one of two Crp concentrations (0 or 400 nM) in a buffer solution consisting of 20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EDTA, and 100 mM KCl. The reaction mixtures were incubated for 15 min at 37°C to allow binding. Then, DNaseI (0.05 U; New England Biolabs, USA) was added and the mixture was incubated for 2 min at room temperature. The reaction was immediately deactivated at 75°C for 10 min, and a QIAquick Nucleotide Removal Kit (QIAGEN, USA) was used to purify the DNA fragments. The final product was analyzed using a 3730 DNA Analyzer (Applied Biosystems, USA).

Analysis of *fumA* and *fumC* promoters

The promoters for *fumA* and *fumC* (347 bp and 677 bp) were amplified by PCR with the following primers, respectively: pfumA-1F, CCGGAATTCAACTGCTTTTCGGAT GTGCG; pfumA-1R, GGGAAGCTTGTTTGTTTGACATT GTTCTCTCAC; pfumC-1F, CCGGAATTCCAGTATCCC GTTTCTACACGCT and pfumC-1R, CGGGAAGCTTGT ACTGTATTCATGACCTGCTCCT. The *fumA* mutation at position +56 was obtained using PCR with the primers, pfumA-1F and pfumA-2R, GGGAAGCTTGTTTGTCTCAC ATTGTTGTGTCAC. The *fumC* deletion at position -492 for 576 bp was obtained by PCR with the primers, pfumC-2F, CCGGAATTCGCTGCCGCAGTACATCAAAG, and pfumC-1R. The amplified PCR products with *Eco*RI and *Hin*dIII restriction sites were introduced into pRW50 for β-galactosidase analysis (Hollands *et al.*, 2007). The *crp* gene with its original promoter was cloned into the plasmid pDU9 to obtain the plasmid pDCRP, which was used for complementation tests.

Results

Effects of catabolite repression and cAMP on *fumA-lacZ*, *fumB-lacZ*, and *fumC-lacZ* expression

The addition of glucose results in repression of the cAMP-Crp levels in *E. coli* (Gosset *et al.*, 2004). Previous research detected catabolite repression in the *fumA* gene (Park and Gunsalus, 1995). The current study further examined whether the *fumB* and *fumC* genes were controlled by glucose. We used *lacZ* coupled with the independent promoter corresponding to *fumA*, *fumB*, and *fumC* genes to investigate the gene expression (Table 1). Subsequently, the catabolite repression of *fumA-lacZ* and *fumC-lacZ* was observed when the cellular cAMP levels were reduced by increasing the glucose concentration in the culture medium. This process reduced the expression of both genes. However, the presence of glucose did not significantly affect *fumB-lacZ* expression (Fig. 1A). These results indicate that the independent promoter of *fumB* is not subject to cAMP-Crp regulation, whereas *fumC-lacZ* is regulated.

It has been suggested that glucose attenuates *fumA* gene

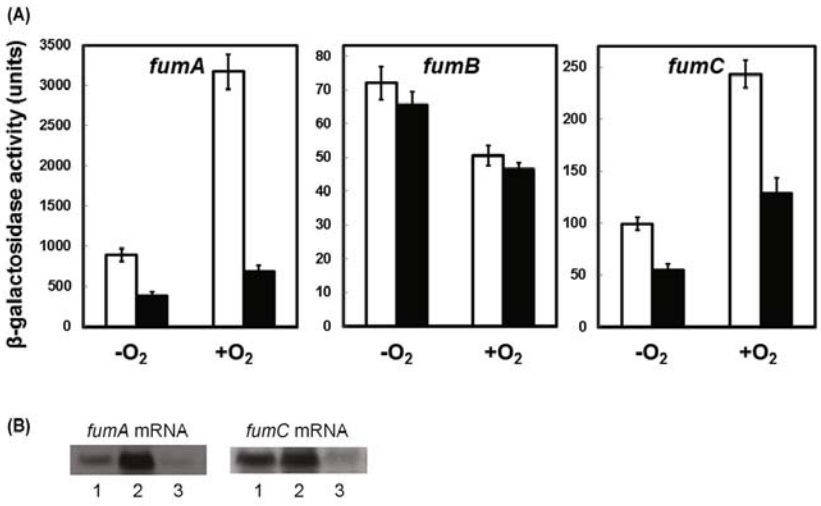

Fig. 1. Identification of Crp regulation of the fumarase genes. (A) Effects of glucose and oxygen on the expression of *fumA-lacZ*, *fumB-lacZ*, and *fumC-lacZ* in LB medium with and without glucose and oxygen. The open bars represent standard LB medium, and the solid bars represent the LB medium with 5 mM glucose. (B) The effects of different carbon sources on *fumA* and *fumC* gene expression using northern blot analysis. *E. coli* MC4100 was grown in LB medium supplemented with glucose (5 mM) (lane 1) or with sodium acetate (5 mM) (lane 2). Lane 3 *fumA* and *fumC* gene expression in the Δ*cya* strain with sodium acetate (5 mM).

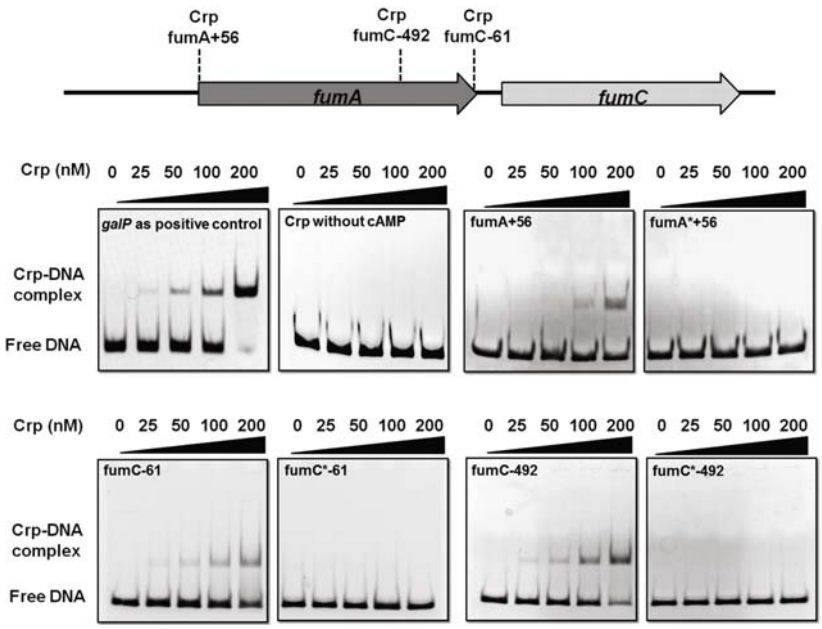

Fig. 2. EMSA of oligonucleotides overlapping with the putative Crp-binding sites in the different promoters of *fumA* and *fumC*. Oligonucleotides binding to *galP*, as the positive control, contained the 5′-TGTGAN$_6$TCACA-3′ palindrome. The binding reaction without cAMP was used as the negative control. Also shown are the Crp binding assays with fumA+56 (TTTGACATTGTTCTCT) and fumA*+56 (TCTCACATTGTTGTGT), fumC-61 (GCAGATACAACTCACA) and fumC*-61 (GCACATACAACTGAGA), as well as fumC-492 (CGTGATATTGCTCACG) and fumC*-492 (CCTCATATTGCTGAGG). The oligonucleotides labeled with a star have the same sequences among fumA+56, fumC-61, and fumC-492, except for the underlined Crp binding sequences.

expression through the reduction of cAMP levels (Park and Gunsalus, 1995; Zhang *et al.*, 2005). Thus, different carbon sources were used to determine the effects of glucose catabolite repression on the *fumC* gene, using northern blot analysis of the aerobic batch culture (Fig. 1B). As expected, the expression of *fumA* grown with acetate was fivefold higher than that of *fumA* grown with glucose. Likewise, the substitution of acetate for glucose resulted in an approximately twofold increase in *fumC* transcripts. These findings correspond to those of the β-galactosidase assays of *fumA-lacZ* and *fumC-lacZ*. The effect of catabolite repression on *fumA* was stronger than that on *fumC*. Moreover, cAMP deficient *E. coli* (Δ*cya* strain), grown aerobically, resulted in a drastic reduction in the expression of both *fumA-lacZ* and *fumC-lacZ*. This result suggests that the expression of these genes is dependent on cAMP and that cAMP-Crp is potentially an activator for both genes.

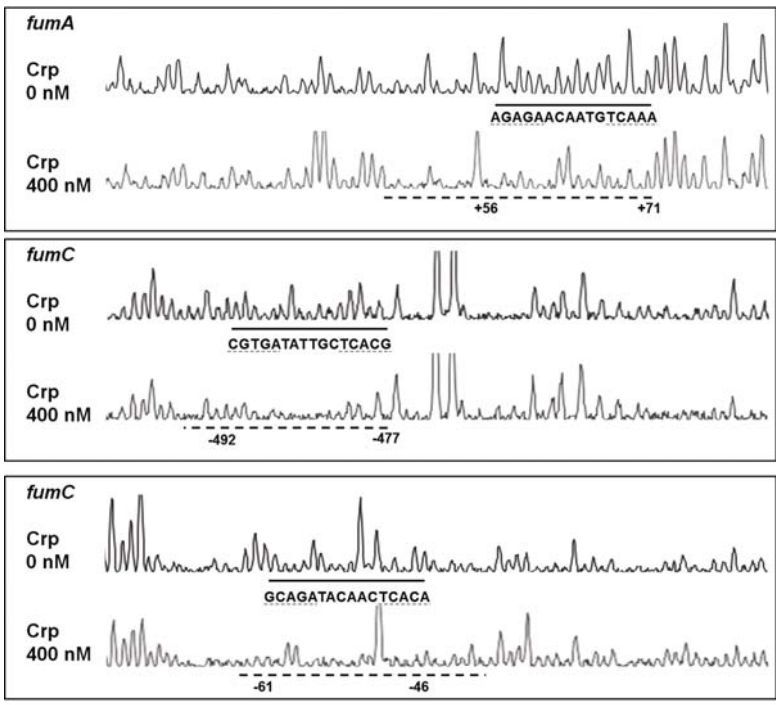

Fig. 3. Footprint assay of the *fumA* and *fumC* promoter regions with and without Crp. The promoter region was amplified via PCR and fluorescently labeled with FAM. The PCR products corresponded with the -198 to +324 region of *fumA* as well as to the -314 to +208 and -745 to -224 regions of *fumC*. The dashed lines indicate the protected region of the Crp-binding sites.

EMSA and footprint analysis of Crp binding sites in the *fumA*, *fumB*, and *fumC* genes

TGTGAN$_6$TCACA, a well-known palindromic sequence, is bound by the Crp transcription factor (Gunasekera *et al.*, 1992). However, a non-consensus base sequence was also found to exhibit affinity for, and control by, Crp (Desai *et al.*, 2009). *In vitro* EMSA was performed to determine the Crp binding sites. The promoter region was scanned systematically to search for the putative Crp binding site, and 50-bp oligonucleotides were used to confirm its existence. The results indicate that Crp clearly retarded the migration of the *fumA* and *fumC* oligonucleotides (Fig. 2). One site for *fumA* was detected at +56 from the transcription start site, and two sites for *fumC* were located at positions -61 and -492. In contrast, no retardation was observed when the consensus sequences of fumA+56, fumC-61, and fumC-492 were replaced by the mutations at fumA*+56, fumC*-61, and fumC*-492, respectively. These results clearly demonstrate that Crp was responsible for regulating the *fumA* and *fumC* promoters. In addition, several DNA fragments were designed to authenticate the Crp binding site on the independent promoter of *fumB*. However, no band shift ranging from +170 to -470 of the *fumB* transcription start site was found. These results suggest that Crp directly regulates *fumA* and *fumC* but not *fumB*.

DNaseI footprint analysis was used to assess further the bases in the *fumA* and *fumC* genes involved in Crp binding. The primers fluorescently labeled with FAM were designed to amplify the 522-bp DNA fragments for the *fumA* and *fumC* promoters (Zianni *et al.*, 2006). The DNA patterns with and without Crp were compared (Fig. 3). The protected regions of *fumA* and *fumC* were determined to be 20–30 bases long. The palindromic sequences of *fumA* from base +56 to +71 region and those of *fumC* from the base -61 to -46, and -492 to -477 regions were included in the protected regions. A hypersensitive site located at base -50 of *fumC* was also observed. These findings are consistent with those of the *in vitro* EMSA.

Given that the Crp-binding sites to +56 of the *fumA* promoter and -492 of the *fumC* promoter were remote from -10 to -35 regions; the effect of Crp regulation on these two positions was determined further. The expression constructs, *fumA-lacZ* and *fumC-lacZ*, consisting of the *fumA* mutation at positions +56 to +71 and the *fumC* deletion at Crp-binding site -492 were obtained using a *lacZ* fusion expression vector, pRW50 (Hollands *et al.*, 2007). Expression activities of *fumA-lacZ* and *fumC-lacZ*, driven by the *fumA* and *fumC* promoters, were detected in the wild-type strain, whereas they were almost undetectable in the Δ*crp* strain (Fig. 4). When Crp was present to complement the Δ*crp* strain after transformation with the pDCRP expression vector containing the native *crp* gene, the *fumA-lacZ* and *fumC-lacZ* expressions returned to that of the wild type. In contrast, the *fumA-lacZ* expression, driven by the *fumA* mutation at positions +56 to +71, remained low in the wild type, Δ*crp*, and complemented strain. The *fumA-lacZ* expression suggested that the *fumA* promoter was regulated by Crp at positions +56 to +71. However, the *fumC-lacZ* expression driven by the *fumC* deletion at position -492 was not regulated in wild type and complemented strains. This result indicated that position -492 of the *fumC* promoter was not involved in the Crp regulation.

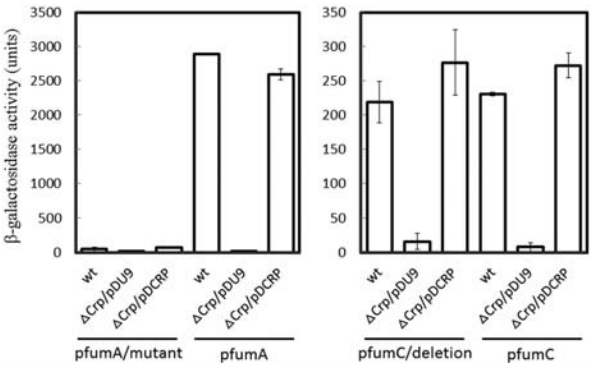

Fig. 4. Analysis of the Crp-binding sites at +56 of the *fumA* promoter and -492 of the *fumC* promoter. The pfumA and pfumC indicate that the expression vectors contained the native promoters of *fumA* and *fumC* for 347 and 677 bp. The pfumA/mutant and pfumC/deletion were expression vectors harboring the mutant *fumA* promoter at +56 to +71 and *fumC* deletion at position -492 for 576 bp. The pDCRP with the native *crp* gene was used to conduct complementation tests, while pDU9 was used as the negative control.

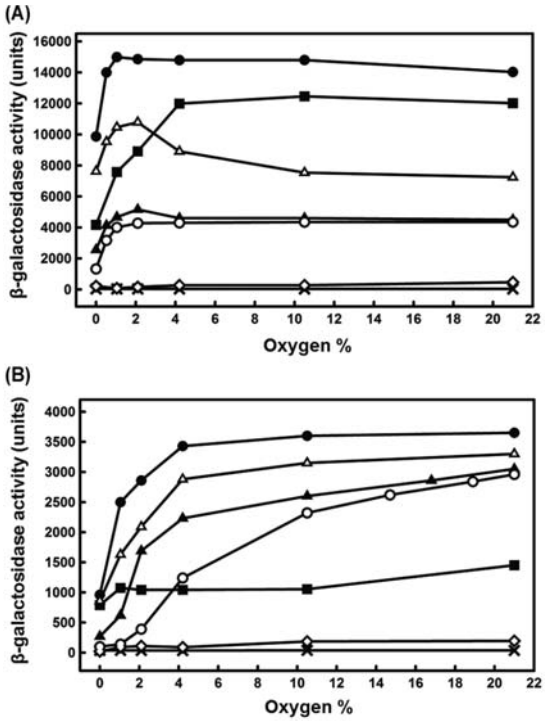

Fig. 5. Effects of various oxygen levels on *fumA-lacZ* and *fumC-lacZ* expressions in continuous culture. The cells were grown in Vogel–Bonner medium supplemented with casamino acids (0.25 mg/L) and glucose (2.25 mM), with *k* equal to 0.6/h. (A) *fumA-lacZ* expression at various oxygen levels. (B) *fumC-lacZ* expression at various oxygen levels. Also shown are the β-galactosidase activities in the wild-type (○), Δ*fnr* (▲), Δ*arcA* (△), Δ*crp* (x), Δ*cya* (◇), Δ*fnr* Δ*arcA* double-mutant (●), and the Δ*crp* Δ*fnr* Δ*arcA* triple-mutant (■) strains. All values are the averages of three determinations.

Effects of Crp, ArcA, and Fnr on *fumA-lacZ* and *fumC-lacZ* expression at different oxygen levels and cell growth rates

To explore the regulatory role of three transcriptional regulators on the *fumA* and *fumC* genes, the reporter strains were grown in continuous culture at various degrees of oxygen saturation (0–21%) to determine the expression levels of *fumA-lacZ* and *fumC-lacZ* at different levels of oxygen, when present in the wild-type, the Δ*crp*, Δ*cya*, Δ*fnr*, Δ*arcA*, Δ*fnr* Δ*arcA* double-mutant, and the Δ*crp* Δ*fnr* Δ*arcA* triple-mutant strains (Figs. 5A and 5B). The results showed that the expression pattern of *fumA-lacZ* differed significantly from that of *fumC-lacZ*. The expression of *fumA-lacZ* increased fourfold when the wild-type strain was transferred from 0% to 2% oxygen saturation, and maintained this level from 2% to 21% (Fig. 5A). In contrast, the *fumC-lacZ* expression continuously increased as the oxygen saturation level increased from 0% to 21% (Fig. 5B). The maximum level of *fumC-lacZ* expression was observed under completely aerobic conditions (i.e., 21% oxygen). These results suggest that *fumA* is more sensitive to oxygen induction than *fumC* under microaerobic conditions.

The *fumA-lacZ* expression in the Δ*arcA* strain was higher than that in the wild-type strain at each corresponding oxygen concentration (0–21%), but it was repressed by Fnr between 0% and 4% oxygen. A similar result was observed for *fumC-lacZ* expression. In general, the *fumA-lacZ* and *fumC-lacZ* expressions were higher in the Δ*arcA* strain compared with the wild-type and Δ*fnr* strains. Significant de-repression of *fumA-lacZ* and *fumC-lacZ* occurred when air saturation was between anaerobiosis (0%) and microaerobiosis (4%). Interestingly, the accumulative de-repression of both fusions was detected in the Δ*arcA* Δ*fnr* double-mutant strain. The maximal expression of *fumA-lacZ* in the Δ*fnr* Δ*arcA* double-mutant strain was fourfold higher than that in the wild type at 1% oxygen. Moreover, there was a 17-fold de-repression of *fumC-lacZ* in the Δ*fnr* Δ*arcA* double-mutant strain at 1% oxygen, which suggests that ArcA and Fnr were responsible for the repression. In contrast, the *fumA-lacZ* and *fumC-lacZ* expressions in the Δ*crp* strain remained constantly low over the different oxygen levels. This result is congruent with the expression in the Δ*cya* strain, which indicates that the *fumA* and *fumC* gene expression was strongly activated by Crp and cAMP. The Δ*fnr* Δ*arcA* Δ*crp* triple-mutant strain was used to interpret the effects of the three transcription regulators on *fumA-lacZ* and *fumC-lacZ* expression. The de-repressive effect in the triple mutants decreased for *fumA-lacZ* (at 0–4% oxygen) and *fumC-lacZ* (at 0–21% oxygen) expression coordinated by Fnr and ArcA double mutants.

The responses of *fumA-lacZ* and *fumC-lacZ* to different growth rates were parallel and dependent on Crp and Cya, as shown by the analysis of *fumA* and *fumC* gene expressions at various growth rates. When the cell growth rate increased from a *k* value of 0.24/h to 0.96/h, the *fumA-lacZ* and *fumC-lacZ* expressions diminished significantly (Fig. 6). However, gene expression was not affected by growth rate in the Δ*crp* strain. The same results were also obtained for the *fumA-lacZ* and *fumC-lacZ* expressions in the Δ*cya* strain, which is consistent with the expression at various oxygen levels. This study has clearly confirmed the importance of Crp and Cya in activating *fumA* and *fumC*.

Discussion

One of the three fumarase genes in *E. coli*, *fumA* is well known to be Crp-regulated, and subject to catabolite repression, which has been examined through transcriptome studies using Crp mutants and microarray analysis (Park and Gunsalus, 1995; Gosset *et al.*, 2004; Zhang *et al.*, 2005). However, data on the Crp-controlled regulation of *fumB* and *fumC* remain limited. The current study demonstrated the *fumA*, *fumB*, and *fumC* expression associated with Crp by analyzing the *fumA-lacZ*, *fumB-lacZ*, and *fumC-lacZ* expressions under the control of their independent promoters (Fig. 7). Evidently, *fumB-lacZ* was not affected by catabo-

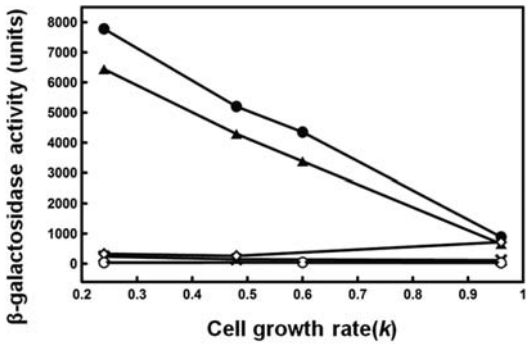

Fig. 6. Effects of cell growth rate on the *fumA-lacZ* and *fumC-lacZ* expression in continuous culture. The β-galactosidase activities of *fumA-lacZ* (●) and *fumC-lacZ* (▲) in the wild-type strain, *fumA-lacZ* (○), and *fumC-lacZ* (△) in the Δ*crp* strain, and *fumA-lacZ* (◇) and *fumC-lacZ* (x) in the Δ*cya* strain are shown. All values are the averages of three determinations.

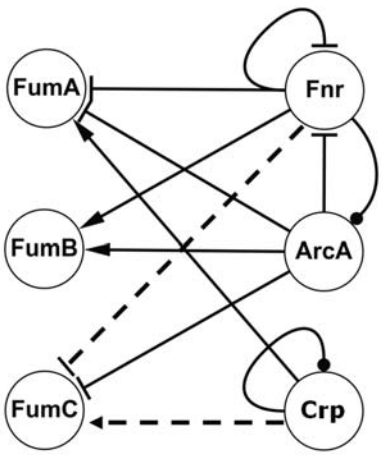

Fig. 7. The fumarase gene regulatory network involving Crp, ArcA, and Fnr. The continuous line represents the results of previous studies according to the RegulonDB and Ecocyc databases, whereas the dashed line denotes the results of the present study. The arrow indicates activation; T, repression; and the circle, both activation and repression.

lite repression under either aerobiosis or anaerobiosis. *In vitro* EMSA further supported that Crp did not regulate the independent promoter of *fumB*. Although part of *fumB* expression was regulated by Crp through the expression of the *dcuB-fumB* co-transcript, the *fumB* gene expression was predominantly driven by the independent *fumB* promoter rather than by the *dcuB* promoter (Golby *et al.*, 1998).

According to the previous study, *fumA* and *fumC* are cAMP dependent (Tseng *et al.*, 2001). The results obtained in the present study provide direct evidence of the Crp regulation of *fumC* gene expression. The expression of both *fumA* and *fumC* responded well to the presence of oxygen. In contrast to *fumA* (where expression immediately reached the maximum level at 2% oxygen), *fumC* expression started at 2% and continued to increase over the range of 2% to 21% oxygen. The *fumC-lacZ* expression in the Δ*crp* and Δ*cya* mutants was constantly low at various growth rates and oxygen levels (Figs. 5B and 6). Under microaerobiosis, the *fumA-lacZ* expression was significantly more sensitive to oxygen as compared with *fumC-lacZ* expression. This result indicates that the catabolite repression of the *fumA* gene was more evident than that of the *fumC* gene when the oxygen level was very low. Nevertheless, when the oxygen level was increased, the *fumC-lacZ* expression was discernible in the wild-type, Δ*crp*, and Δ*cya* strains. The expression was consistent with the increase in fumarase activities when the oxygen level was elevated. FumC activity can be further induced by superoxide (Tseng *et al.*, 2001). ArcA, as a two-component regulatory system, and Fnr, as a redox sensor in *E. coli*, together coordinate the adaptive responses to the availability of oxygen and presence of electron acceptors (Salmon *et al.*, 2003, 2005). Thus, the present study explored further the regulation of *fumA* and *fumC* at various oxygen levels. Previously, ArcA and Fnr have been reported to suppress *fumA* expression under anaerobiosis, whereas *fumC* was repressed only by ArcA (Fig. 7) (Park and Gunsalus, 1995). In the current study, the effect of Fnr on the *fumA* expression was weak in the presence of oxygen (>4%). This may result from ArcA repression of *fnr* (Fig. 7). When *arcA* and *fnr* mutants were present simultaneously, the de-repression was higher in the Δ*arcA* Δ*fnr* double-mutant or Δ*fnr* Δ*arcA* Δ*crp* triple-mutant strains than that of the single mutant Δ*arcA* or Δ*fnr*. Moreover, *fumC-lacZ* de-repression was found in the Δ*fnr* strain, which suggests that Fnr is also a repressor of *fumC* gene expression (Fig. 5B). These findings reveal that ArcA and Fnr exhibit suppressive effects, and that ArcA is the dominant regulator of *fumA* and *fumC* during TCA cycle gene expression (Shalel-Levanon *et al.*, 2005a, 2005b). In addition, the results for *fumA-lacZ* expression show that *fumA* was the most active gene under anaerobic conditions (Fig. 5A), whereas *fumC* was activated under highly oxidative conditions (Fig. 5B). These results agree with the analysis of fumarase activity using various fumarase gene deletion strains (Tseng *et al.*, 2001). Interestingly, similar gene regulation mechanisms for the transcriptional factors ArcA, Fnr, and Crp were observed in the TCA cycle and the respiratory pathway (Gama-Castro *et al.*, 2011; Keseler *et al.*, 2011).

The putative Crp binding site of *fumA* has been predicted to be at the -410 to -395 region upstream of *fumA*, having the sequence TGTGCTGCGCATAATA (Gosset *et al.*, 2004). However, although this region is not included in the *fumA-lacZ* fusion, the expression of *fumA-lacZ* was still regulated by Crp (Figs. 5A and 6) (Park and Gunsalus, 1995). Hence, an additional Crp-binding site for *fumA* was identified at the +56 to +71 region downstream of *fumA* with the sequence AGAGAACAATGTCAAA, while the Crp-binding sites of *fumC* were recognized at the -61 to -46, and -492 to -477 regions upstream of *fumC* with the sequences GCAGATA CAACTCACA and CGTGATATTGCTCACG, respectively, using EMSA and footprint assays (Figs. 2 and 3). The Crp-binding site at +56 of the *fumA* promoter was further confirmed by the *fumA-lacZ* expression, when compared with the *fumA* mutation at position +56 (Fig. 4). The long distance between the Crp-binding site and the -10 to -35 position is known to reduce promoter activity significantly because of the weak interaction between Crp and RNA polymerase (Ushida and Aiba, 1990; van Hijum *et al.*, 2009). Thus, the effect of the *fumC* sequence at the -492 to -477 region on transcription expression is likely lower than that of the *fumC* sequence at the -61 to -46 region. Based on the *fumC-lacZ* expression allowed by the *fumC* deletion at position -492, this position of the *fumC* promoter is independent of Crp.

In conclusion, Crp directly activates the expression of *fumC-lacZ*, whereas Fnr represses *fumC*. ArcA regulates *fumA* and *fumC* at 0% to 21% oxygen concentration, and strongly represses the genes at 0–4%. Nevertheless, Fnr narrowly regulates the *fumA* and *fumC* at 0–4% oxygen concentration. Overall, the data show that *fumA* gene expression is mainly stimulated under anaerobiosis to microaerobiosis, while *fumC* gene expression is activated under aerobiosis. These findings also suggest that physiologically, *fumA* is a "house-keeping" gene that is highly expressed during anaerobic growth, and reaches its limit at 4% oxygen. On the other hand, the *fumC* gene is "aerobic" by nature, and is expressed continuously and dramatically to facilitate the TCA cycle when oxygen content is progressively increased. Furthermore, the EMSA and footprint assay identified novel Crp-binding sites in *fumA* and *fumC* but not in *fumB*, which suggests that the independent promoter of *fumB* is not regulated by Crp.

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