Glucose repression of the *Escherichia coli* sdhCDAB operon, revisited: regulation by the CRP·cAMP complex

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Received October 24, 2005; Revised and Accepted November 8, 2005

ABSTRACT

Expression of the *Escherichia coli* sdhCDAB operon encoding the succinate dehydrogenase complex is regulated in response to growth conditions, such as anaerobiosis and carbon sources. An anaerobic repression of sdhCDAB is known to be mediated by the ArcB/A two-component system and the global Fnr anaerobic regulator. While the cAMP receptor protein (CRP) and Cra (formerly FruR) are known as key mediators of catabolite repression, they have been excluded from the glucose repression of the sdhCDAB operon. Although the glucose repression of sdhCDAB was reported to involve a mechanism dependent on the ptsG expression, the molecular mechanism underlying the glucose repression has never been clarified. In this study, we re-examined the mechanism of the sdhCDAB repression by glucose and found that CRP directly regulates expression of the sdhCDAB operon and that the glucose repression of this operon occurs in a cAMP-dependent manner. The levels of phosphorylated enzyme IIA^Glc^ and intracellular cAMP on various carbon sources were proportional to the expression levels of *sdhC-lacZ*. Disruption of *crp* or *cya* completely abolished the glucose repression of *sdhC-lacZ* expression. Together with data showing correlation between the intracellular cAMP concentrations and the *sdhC-lacZ* expression levels in several mutants and wild type, in vitro transcription assays suggest that the decrease in the CRP·cAMP level in the presence of glucose is the major determinant of the glucose repression of the sdhCDAB operon.

INTRODUCTION

The term carbon catabolite repression is currently in use to describe the general phenomenon in microorganisms whereby the presence of a carbon source in the medium can repress expression of certain genes and operons, whose gene products are often concerned with catabolism of alternative carbon sources. The mechanisms of carbon catabolite repression in response to rapidly metabolizable carbon sources have been extensively examined in *Escherichia coli* (1,2). In the vast majority of documented cases, the preferred carbon source is glucose with the famous *E.coli* glucose–lactose diauxie as the classical example. The glucose-mediated catabolite repression, termed glucose repression, is mainly mediated by the proteins of the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS). This system consists of sugar-specific PTS permeases, also referred to as enzymes II, and two general PTS proteins, enzyme I and histidine-containing protein (HPr), that participate in the phosphorylation of all PTS-transported carbohydrates. The glucose-specific PTS proteins consist of the soluble enzyme IIA^Glc^ (EI^A^Glc) and the membrane-bound enzyme II^CB^Glc^ (EI^CB^Glc). During translocation of glucose, a phosphoryl group derived from PEP is transferred sequentially along a series of proteins (enzyme I, HPr, EII^A^Glc^ and EI^CB^Glc^) to the transported glucose molecule.

Central to carbon catabolite repression is the phosphorylation state of EII^A^Glc^. In the presence of glucose, unphosphorylated EII^A^Glc^ binds and inhibits various proteins involved in uptake and metabolism of non-PTS carbohydrates by a mechanism termed inducer exclusion (1,3). However, in the absence of glucose, adenylate cyclase is known to be activated to increase the intracellular amount of cAMP, the allosteric effector necessary for the cAMP receptor protein (CRP) to bind efficiently to DNA and activate transcription at more than 100 promoters (4). A popular model for the
regulation of adenylate cyclase activity is that the phosphorylated form of EIIAGlc generated in the absence of glucose stimulates adenylate cyclase activity; thus, glucose transport is presumed to lead to dephosphorylation of IIAGlc, resulting in a de-activation of adenylate cyclase and the glucose repression of many genes (1). Recently, the dephospho-form of EIIAGlc was also shown to interact with FrsA to regulate the flux between respiration and fermentation pathways (5), supporting importance of EIIAGlc in catabolic regulations.

Expression of the E.coli sdhCDAB operon encoding the succinate dehydrogenase complex, the sole membrane-bound enzyme of the tricarboxylic acid (TCA) cycle, has also been known to be regulated in response to carbon supply as well as anaerobiosis (6,7). Recent study also revealed that a small RNA, RyhB, down-regulates the mRNA level for sdhCDAB operon at the post-transcriptional initiation level in response to iron availability (8). For the anaerobic repression of sdhCDAB, two global regulatory circuits were shown to be involved: the ArcB/A two-component system and the Fnr anaerobic regulator which modulated sdhCDAB expression over a 70-fold range to provide different amounts of enzyme depending on the cells’ needs for energy and carbon intermediates (7,9). While the molecular mechanisms underlying the anaerobic repression and the iron availability-dependent regulation have been well documented, the mechanism underlying the glucose repression is still not clear. Although CRP and Cra have been known to be the key regulators of catabolite repression, they had been dismissed from the glucose repression of the sdhCDAB operon, although a putative CRP-binding site was previously described for the Mlc regulon including the genes encoding PTS proteins (5), supporting importance of EIIAGlc in catabolic regulations.

This study, we re-investigated regulation of the sdhCDAB expression by glucose and the PTS to elucidate the mechanism underlying the glucose repression. We conclude that the general carbon catabolite repression regulator CRP directly mediates the glucose repression of the sdhCDAB operon in a cAMP-dependent manner.

MATERIALS AND METHODS

Materials

Cyclic AMP and orthonitrophenyl-β-d-galactopyranoside (ONPG) were obtained from Sigma. RNA polymerase saturated with σ70, [γ-32P]ATP and [α-32P]CTP were purchased from Amersham Biosciences. Nucleotide triphosphates were from MBI Fermentas. The cycle sequencing kit was from Epicentre Technologies (Madison, WI).

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. To generate the isogenic arcA, crr, ptsG, mlc, crp and cyr deletion mutants, the indicated alleles were introduced into parental strain TSDH00 by P1 transduction (14). Luria–Bertani broth (LB) medium was used for the routine growth of bacteria unless otherwise indicated. If necessary, media were supplemented with sugars (40 mM). Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 20 μg/ml; chloramphenicol, 30 μg/ml and tetracycline, 25 μg/ml.

To construct pHisEIIIB, in which expression of the EIIIB domain (the cytosolic domain of EIIICB(Glc)) tagged with 6 histidines at its N-terminus (His-EIIIB) is under the control of the pRE1-vector system (15), the pHK plasmid (12) was digested with Ndel and BamHI, and the fragment encoding the EIIIB domain was cloned into pRE-His-Tag (16).

To construct pBRcrp, in which expression is under the control of its own promoter, the sequence covering the crp promoter and coding regions was amplified by PCR using a mutagenic primer to create a PstI site (underlined) 316 nt upstream of the crp start codon (5'-CCC TTC GAC CCA CTG CAG TCG CGC TTG CAT-3') and a reverse primer located 256 nt downstream of the TAA stop codon (5'-GCC ACG CAC CAA TGA TTA AGC GTT TGA TGA AAA-3'). An SspI site is located 194 nt downstream of the stop codon in this PCR product and the 1137 bp PCR product digested with PstI and SspI was cloned into vector pBR322. Primer extension assay

Primer extension reactions were carried out as described previously (18). Cells were grown to A600 of 0.5, and total E.coli RNA was purified using RNaseasy mini kit (Qiagen) and resuspended in sterile distilled water. Purified [γ-32P]end-labeled primer SdhPex (Figure 1) was mixed with 30 μg of total cell RNA. The mixture was heated to 60°C and then allowed to cool to room temperature over a period of 1 h. After annealing, 50 μl of reaction solution was added, which contained 700 μM dNTPs, 10 mM MgCl2, 5 mM DTT, 20 mM Tris–HCl, pH 8.3 and 100 U of SuperscriptII reverse transcriptase (Invitrogen). After the mixture was incubated at 40°C for 70 min, 2 μl of 0.5 M EDTA was added into the reaction mixture and incubated at 37°C for 30 min. The DNA was precipitated and resolved on an 8 M urea, 5% polyacylamide gel and visualized by autoradiography. The same primer was also used for sequencing the sdhC promoter region.
Table 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Genotype or description | Source or reference |
|-------------------|-------------------------|---------------------|
| Strains           |                         |                     |
| MG1655            | Wild-type E.coli        | (44)                |
| MC4100            | pRE-His-Tag             | (16)                |
| ECL618            | MG1655 Δmlc             | (21)                |
| TP2865            | MG1655 mlc::Tet         | (21)                |
| SR702             | MG1655 mlc::Tet         | (21)                |
| Y12004            | MG1655 Δmlc             | (21)                |
| SA277             | MG1655 Δmlc             | (21)                |
| CA8000            | MG1655 Δmlc             | (21)                |
| TSDH00            | MG1655 Δmlc             | (21)                |
| TSDH01            | MG1655 Δmlc             | (21)                |
| TSDH02            | MG1655 Δmlc             | (21)                |
| TSDH03            | MG1655 Δmlc             | (21)                |
| TSDH04            | MG1655 Δmlc             | (21)                |
| TSDH05            | MG1655 Δmlc             | (21)                |
| TSDH06            | MG1655 Δmlc             | (21)                |
| TSDH10            | MG1655 Δmlc             | (21)                |
| TSDH20            | MG1655 Δmlc             | (21)                |
| TSDH30            | MG1655 Δmlc             | (21)                |
| TSDH40            | MG1655 Δmlc             | (21)                |
| TSDH50            | MG1655 Δmlc             | (21)                |
| Plasmids          |                         |                     |
| pRE-His-Tag       | pRE-His-Tag             | (16)                |
| pBR322            | pBR322                  | (48)                |
| PJHK              | pRE1-based expression vector for EIEB | (12) |
| pHiEIIB          | pRE1-based expression vector for His-EIEB | This work |
| pSA600           | pSA600                  | (17)                |
| PTSDH10          | pSA600                  | (17)                |
| pRS415           | pRS415                  | (19)                |
| pRS-sdh0         | pRS415                  | (19)                |
| pRS-sdh1         | pRS415                  | (19)                |
| pRS-sdh2         | pRS415                  | (19)                |
| pRS-sdh3         | pRS415                  | (19)                |
| pRS-sdh4         | pRS415                  | (19)                |
| pRS-sdh5         | pRS415                  | (19)                |
| Figure 1. Organization of the regulatory sites in the sdhC promoter region. The nucleotide sequence between –330 and +470 with respect to the transcription start site of the promoter is shown. Lines above the sequence indicate the three ArcA binding sites and one presumable CRP binding site on the sdhC promoter, and the transcription start point and the translation start codon are marked in boxes. The dashed arrows below the sequence indicate the oligonucleotides SdhF0, SdhF1, SdhF2, SdhF3, SdhF4, SdhP, SdhR, and engineered restriction sites are shown below the arrows. The transcriptional start site and ArcA binding regions were from the previous report (9).
Construction of transcriptional lacZ fusions
To prepare the sdhC-lacZ fusion plasmid pRS-sdh0, the DNA fragment covering from positions –312 to +450 relative to the transcription start site of sdhC was amplified by PCR using SdhF0 and SdhR containing an engineered BamHI site as the upstream and downstream primers, respectively (Figure 1). The PCR product digested with EcoRI and BamHI was ligated into the corresponding cloning sites of pRS415 (19) to generate the sdhC-lacZ operon fusion plasmid pRS-sdh0. Similarly, the sdhC-lacZ fusions pRS-sdh1, pRS-sdh2, pRS-sdh3 and pRS-sdh4 containing the engineered EcoRI sites as the upstream primers, respectively, and SdhR as the downstream primer (Figure 1). To generate mutation in the CRP binding site (CGTGACCT-0) of pRS-sdh0, pRS-sdh1, pRS-sdh2, pRS-sdh3 and pRS-sdh4 were constructed first by the PCR amplification method and subsequently cloned into EcoRI/BamHI-digested pRS415 after digestion with the same enzymes. The DNA fragments covering from positions –183 to +126 (pRS-sdh1), –60 (pRS-sdh3) and +26 (pRS-sdh4) to +450 bp relative to the sdhC transcription start were amplified using oligonucleotides SdhF1, SdhF2, SdhF3 and SdhF4 containing the engineered EcoRI sites as the upstream primers, respectively, and SdhR as the downstream primer (Figure 1). To generate mutation in the CRP binding site (CGTGACCT-0 to CTGGCGTTGACTGCA, changed bases underlined), two sequential PCR steps were carried out. In the first round of PCR, the mutagenic primer SdhCRP1 (5′-GGT TTT ATC CTG AAC TGC AGT CCA GGC AGA 0) was used in combination with SdhR for the amplification of the 5′ region from the CRP binding site, while the mutagenic primer SdhCRP2 (5′-GT TGG TTT ATC TCT GCC TGG ACT GCA GCA GTT CAG GAT AAA ACC 3′) was used in combination with SdhR for the amplification of the 3′ region. The two PCR products were combined and used as template in the second round of PCR with SdhF0 and SdhR as the upstream and downstream primers, respectively. The second round PCR product digested with EcoRI and BamHI was ligated into the corresponding cloning sites of pRS-sdh0 to generate TSDH00, TSDH10, TSDH20, TSDH30, TSDH40 and TSDH50, respectively, as described previously (19). Several independent lysogens were analyzed to obtain monolysogens.

β-Galactosidase assays
Cells were grown to A600 of 1.0, and β-galactosidase activities were measured using permeabilized cells as described previously (14). Enzymatic activities are given in units of μmol ONPG hydrolyzed per min. Average values of at least four independent samples were determined.

Detection of EIIIC<sup>Δ</sup>Glc-interacting proteins
E.coli GI698 harboring pHisEIIIB was used for overexpression of His-EIIIB. Cell culture and induction of protein overexpression was performed as described previously (12). Purification of His-EIIIB was carried out using the BD TALON<sup>™</sup> metal affinity resin (BD Biosciences) following the manufacturer’s instructions. EIIIB was purified as described previously (12). E.coli MG1655 and MG1655 Δmle (21) cells grown in 500 ml of LB media were resuspended in the binding buffer (20 mM HEPES, pH 8.1 containing 200 mM NaCl and 5 mM imidazole) in the presence of 100 μg/ml phenylmethylsulfonyl fluoride. The cell suspensions disrupted by passing through a French press at 10 000 p.s.i. were centrifuged at 12 000 g for 15 min at 4°C, and the supernatant solutions were used as crude extracts. Each crude extract was used for the ligand fishing experiment to search for a protein(s) interacting with His-EIIIB by employing the BD TALON<sup>™</sup> metal affinity resin. Proteins specifically interacting with His-EIIIB were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry as described previously (21). Protein concentration was determined by the bicinchoninic acid protein assay (Pierce).

Gel mobility shift assay
Gel mobility shift assays were performed essentially as described previously (12). DNA fragments covering the promoter regions of sdhC and ptsG (from –183 to +156 and –264 to +180, respectively, relative to their transcription start sites) were amplified by PCR and labeled with [γ-<sup>32</sup>P]ATP by using T4 polynucleotide kinase. The DNA binding reaction mixtures in the binding buffer contained 100 μM of cAMP, 1 nM of <sup>32</sup>P-labeled DNA fragments and indicated amounts of CRP. The binding mixtures were incubated at room temperature for 10 min and analyzed by electrophoresis on 6% polyacrylamide gels in 0.5× TBE at room temperature for 90 min.

In vitro transcription
Reactions were carried out as described previously (11) in a 20 μl total volume containing 20 mM Tris-acetate, pH 8.0, 150 mM potassium glutamate, 1 mM DTT, 3 mM MgSO<sub>4</sub>, 1 nM supercoiled DNA template pTSDHpro, 100 μM CAMP, 40 μg/ml BSA, 1 mM ATP, 100 μM each GTP and UTP, 10 μM CTP, 5 μCi of [γ-<sup>32</sup>P]CTP (3000 Ci/mmol) and 0.2 U of E.coli RNA polymerase. CRP and phosphorylated ArcA were prepared as described previously (21) and added to the reaction as described in the legend to Figure 6. All components except nucleotides were incubated at 37°C for 10 min. Transcription was started by the addition of nucleotides containing 100 μg/ml of heparin and terminated after 30 min by adding 20 μl of formamide loading buffer. mRNA was electrophoresed on an 8 M urea, 5% polyacrylamide gel and visualized by autoradiography.

Western blot analysis
The phosphorylation state of EIIA<sup>Glc</sup> was determined according to the procedure developed by Takahashi et al. (22). Cell culture (0.2 ml at A<sub>600</sub> = 1.0) was quenched by adding 20 μl of 10 M NaOH followed by vortexing for 10 s, and then 180 μl of 3 M sodium acetate (pH 5.2) and 1 ml of ethanol were added. Samples were chilled at –70°C for at least 15 min, thawed and centrifuged at 4°C. The pellet was rinsed with 70% ethanol and resuspended in 100 μl of the SDS sample buffer, and 20 μl of this solution was analyzed by 15% SDS–PAGE. Proteins were then electrotransferred onto immobilin-P (Millipore, MA) following the manufacturer’s protocol and were detected with immunoblotting using antiserum against EIIA<sup>Glc</sup> raised in mice as described previously (5). The protein bands were visualized by using the SuperSignal West Pico kit (Pierce) following the manufacturer’s instructions. The amounts of
phosphorylated EIIC*Glc were quantified by densitometric tracing of the film using Eagle Eye™ II and Eagle sight software version 3.2 (Stratagene). To detect the intracellular levels of CRP, growing cells were taken at A600 of 1.0 and total cellular proteins were analyzed by SDS–PAGE using a 15% polyacrylamide gel. Proteins were then electrotransferred onto immobilon-P and western blot analysis was carried out using polyclonal antibody raised in mice against CRP. The protein bands were visualized by using the SuperSignal West Pico kit (Pierce) following the manufacturer’s instructions.

**Measurement of intracellular cAMP concentrations**

Intracellular cAMP concentrations were measured as described previously (23) with some modifications after cells were grown to an A600 of 1.0. Cells from 1 ml culture were collected by centrifugation and resuspended in 500 μl of the cell lysis buffer provided with the cAMP enzyme immunoassay system (Amersham Biosciences). After boiling cell suspensions in lysis buffer for 15 min and centrifugation, the cAMP concentrations in supernatants were determined by using the kit. The average intracellular cAMP concentration was expressed in femtomoles per 10⁹ cells assuming an A600 of 1.0 corresponds to 8 x 10⁸ cells/ml (24). Average values of four independent cultures were determined.

**RESULTS**

**Deletion of the glucose-specific PTS genes affects the glucose repression of *sdhCDAB* expression**

Although CRP and Cra are well-characterized global transcription factors regulating carbon catabolite repression of more than 100 genes, they have been dismissed from the glucose repression of *sdhCDAB* expression (7). To elucidate the mechanism of *sdhCDAB* repression by glucose, we first tested whether the glucose repression occurs at the transcriptional level or post-transcriptionally. Expression from the *sdhC* promoter was monitored by primer extension assay of the total RNA extracted from *E.coli* MG1655 cells grown in the presence or absence of glucose. The level of the *sdhC* transcript from the cells grown in the absence of glucose was much higher than that of cells grown in the presence of glucose (Figure 2). Since this result indicated that the glucose effect on *sdhCDAB* occurs at the transcriptional level, we constructed a series of transcriptional *sdhC-lacZ* fusion strains. The strain TSDH00 contains a single copy of the *sdhC-lacZ* transcriptional fusion gene in which *sdhC* promoter region extends from −312 to +450 relative to the transcription start site (Figure 1). Growth in the presence of glucose caused ∼3.4-fold decrease in *sdhC-lacZ* expression when compared with growth without glucose (Figure 3) in agreement with the mRNA level determined by the primer extension assays in Figure 2 and previous reports (7,10). As the ArcA anaerobic repressor is known to serve as the major transcriptional regulator of the *sdhCDAB* operon, we monitored the effect of *arcA* deletion on the glucose repression of *sdhC-lacZ* expression. While deletion of the *arcA* gene resulted in increase of *sdhC-lacZ* expression as expected from the previous reports (6,7,9,10), it did not show any remarkable effect on the glucose repression of *sdhC-lacZ* expression. As a previous study had shown that the glucose repression of *sdhC-lacZ* expression is *ptsG*-dependent (10), we tested the effect of the two glucose-specific PTS genes, crr and *ptsG* encoding EIIC*Glc* and EIICBGlc respectively, on *sdhC-lacZ* expression. Deletion mutations of crr and *ptsG* were transduced into the TSDH00 strain to generate strains TSDH02 and TSDH03, respectively, and β-galactosidase activities of these strains were measured. As shown in Figure 3, the glucose repression was negligible in the crr deletion mutant when compared with wild type: growth of the TSDH02 strain in LB with glucose resulted in only a marginal decrease (∼1.5 fold) of *sdhC-lacZ* expression when compared with that without glucose. Deletion of *ptsG* resulted in the complete loss of glucose repression.
repression of sdhC-lacZ expression in agreement with the previous study (10). Loss of glucose repression of sdhC-lacZ expression in TSDH02 and TSDH03 implies that the glucose-specific PTS proteins play crucial roles in the glucose repression of sdhCDAB expression.

In agreement with the previous study by Takeda et al. (10), the results in Figure 3 demonstrate the complete loss of the glucose repression of sdhC-lacZ expression in ptsG mutant. It was previously shown that the induction of ptsG and ptsHcrr expression by glucose was also ptsG-dependent, and various studies showed that Mlc is the repressor responsible for this glucose induction (18,25–28). Further studies revealed that the dephospho-form of EIICB\textsuperscript{Glc} could sequester the global repressor Mlc through the direct protein–protein interaction and induce expression of the Mlc regulon (11–13). Thus, the simplest model that could account for the glucose repression of sdhCDAB expression and its dependence on ptsG was the existence of a transcription regulator interacting with EIICB\textsuperscript{Glc} and repressing the expression of the sdhCDAB operon in the presence of glucose. To search for a protein(s) interacting with EIICB\textsuperscript{Glc} and thus mediating the glucose repression of sdhCDAB expression, we carried out a ligand fishing experiment. When the crude extracts prepared from MG1655 and its isogenic mlc mutant were mixed with EIIB or 6His-tagged form of EIIB (His-EIIB) and subjected to pull-down assays using the BD TALON\textsuperscript{TM} metal affinity resin, we could not find out any proteins other than Mlc that specifically interacted with the glucose-sensing EIIB domain of the ptsG gene product (data not shown). Although it is well established that Mlc is the global transcription repressor regulating expression of many genes in response to the presence of glucose, the possibility that Mlc may participate in the glucose repression of sdhCDAB expression was ruled out as the mlc null mutant, TSDH04, still exhibited the glucose repression of sdhC-lacZ expression (Figure 3), in agreement with a previous report (10).

**Regulation of sdhCDAB expression by the CRP·cAMP complex**

Considering the fact that only Mlc, which is known to exist in a very limiting concentration in *E. coli* (18,25), could be fished out from the crude extract of MG1655 using EIIB as bait (data not shown), we assumed that the glucose repression of the sdhCDAB operon might not involve any transcription regulators interacting with EIICB\textsuperscript{Glc} in *E. coli* and that the effect of ptsG on the glucose repression of sdhCDAB expression might be indirect.

To search for the cis-acting region(s) responsible for regulation of the glucose repression of the sdhCDAB operon, a series of single-copy transcriptional lacZ fusion constructs containing various promoter regions of sdhC were generated and introduced into the *E. coli* strain MC4100. The glucose repression of sdhC expression in TSDH00 and four different 5′ deletion constructs of sdhC-lacZ fusion, TSDH10, TSDH20, TSDH30 and TSDH40, was monitored in cultures grown aerobically in the presence or absence of glucose (Figure 4).

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**Figure 4.** 5′ Deletion analysis of the sdhC promoter region. Each construct was inserted into the MC4100 chromosome and monolysogens were selected and grown in LB media with or without glucose to search for the cis-acting region(s) responsible for regulation of the glucose repression of the sdhCDAB operon. (A) 5′ Deletion constructs of the transcriptional sdhC-lacZ fusions. Four ArcA binding sites, one CRP binding site and the translation start site are schematically shown. Mutated CRP binding site on TDH50 is shown as hatched box. The numbers refer to the nucleotide positions relative to the transcription start site of sdhC. Effect of glucose on sdhC expression is presented on the right side of each construct as the ratio of β-galactosidase activities in cells grown in LB to those in cells grown in LB with glucose. (B) β-Galactosidase activities of 5′ deletion constructs of sdhC-lacZ fusion. Cells harboring each fusion construct were aerobically grown in LB medium or LB medium supplemented with 40 mM glucose, and β-galactosidase activities were measured as described above. Values represent the average of at least four independent determinations ± SD.
Disruption of the upstream ArcA site centered at -205 bp relative to the transcription start site (the TSDH10 strain) modestly reduced the level of aerobic gene expression as previously reported (9), while deletion of the DNA region containing the ArcA site centered at -119 elevated the level of aerobic sdhC-lacZ expression (TSDH20 strain). Regardless of the changes in the aerobic sdhC-lacZ expression levels, both the promoter fusions TSDH10 and TSDH20 still exhibited the glucose repression of sdhC-lacZ expression to significant levels similar to wild-type TSDH00 (Figure 4). On the construct TSDH30, lacZ was fused to the region covering from -60 to +450 bp relative to the sdhC transcription start, and thus the presumed CRP-binding site centered at -79.5 (29) was deleted but it still retains the ArcA binding site centered at -28 (9). This construct resulted in the significant reduction of β-galactosidase activity and growth of the TSDH30 strain in LB with glucose resulted in only a marginal decrease (1.49 fold) of sdhC-lacZ expression when compared with growth without glucose (Figure 4). These results indicate that the factor mediating the glucose repression may bind to the region extending from -126 to -60 relative to the transcription start site of the sdhC promoter, where the presumed CRP-binding site is located (29) (Figures 1 and 4). Since it was reported that the crp deletion mutant cell still showed the glucose repression of sdhC-lacZ expression (7), it had been believed that a regulator other than CRP would be responsible for the glucose repression of sdhCDAB expression (10). The promoter deletion experiments in this study, however, led us to speculate that CRP may be the direct regulator of the glucose repression of sdhCDAB expression. Therefore, we mutated the putative CRP binding site centered at -79.5 to check whether it is directly involved in the glucose repression of sdh expression. TSDH50, which contains a single copy of the sdhC-lacZ transcriptional fusion gene with mutated CRP binding site (CGTGACCTGGATCCTGACTGCATGCT to CTCTGCTGGAGACTGCA), resulted in the significant reduction of β-galactosidase activity and almost complete loss of the glucose repression of lacZ expression similar to the TSDH30 strain (Figure 4). From these results, we concluded that the CRP binding site on the sdhC promoter region is directly involved in the glucose repression of sdhCDAB expression.

The involvement of CRP and cAMP on the glucose repression of sdhC expression was further investigated using two deletion mutants lacking either CRP or cAMP production. Cells of the crp mutant strain, SA2777, were used to generate an isogenic crp deletion mutant of the parental strain TSDH00 by P1 transduction. After crp deletion was confirmed by western blot analysis using anti-CRP polyclonal antibody in this mutant strain, TSDH05 (data not shown), β-galactosidase activities were measured in TSDH05 cells grown in LB media in the presence and absence of glucose. Contrary to the previous report, the glucose repression was completely abolished in this mutant strain (compare data for the crp mutant with wild type in Figure 3A and B). The sdhC-lacZ expression of TSDH05 grown in LB was even lower than that of wild-type cells grown in the presence of glucose, indicating that CRP is directly involved in the regulation of sdhCDAB expression. To determine whether the glucose repression of sdhC-lacZ expression is dependent on CRP, an isogenic cya deletion mutant of TSDH00 was also generated by P1 transduction from the CA8000Δcya strain (30). The sdhC-lacZ expression in this mutant strain TSDH06 showed a similar pattern with that of TSDH05 and was not affected by the presence of glucose (cya mutant in Figure 3). From these results, we concluded that the CRP-cAMP complex is one of the major transcriptional regulators of the sdhCDAB operon and it is directly involved in the glucose repression of sdhCDAB.

To confirm the direct involvement of CRP and cAMP in the glucose repression of the sdhCDAB operon, we tested the effect of episomal expressed CRP and cAMP added in the medium on sdhC-lacZ expression in the two mutant cells. The genomic DNA fragment containing the crp gene including its own promoter was cloned into the low copy number plasmid pBR322, and the product pBRcrp was transformed into the crp deletion mutant to see whether the glucose repression phenotype is recovered. The episomal expression of CRP increased the sdhC-lacZ expression in TSDH05 cells and the TSDH05 cells transformed with pBRcrp showed the glucose-dependent repression of sdhC-lacZ expression (Figure 5A). The sdhC-lacZ expression of TSDH05 cells harboring pBRcrp grown in the absence of glucose showed

![Figure 5. Restoration of sdhC-lacZ expression by the addition of cAMP and episomal expression of CRP in the cya and crp mutants, respectively.](image)

(A) Complementation of the crp mutation phenotype on sdhC-lacZ expression by episomally expressed CRP. The open bars represent the sdhC-lacZ expression in the TSDH05 (Δcrp) strain harboring pBRcrp grown in LB and the shaded bars represent that in LB supplemented with glucose (40 mM). The TSDH05 strain harboring pBR322 was used as a control. (B) Addition of cAMP in growing medium increases the expression of sdhC-lacZ in cya mutant cells. Freshly grown TSDH06 (Δcya) cells were inoculated into LB medium. After incubation for 2.5 h (marked with arrow) under aerobic condition at 37°C, cAMP (1 mM) was added to the medium (triangle), β-galactosidase activities were determined in cells taken at the indicated times and compared with those in cells grown without addition of cAMP (circle).
regardless of the presence of glucose. These results suggest that expression of sdhC promoter by cAMP was also investigated in the cya mutant strain (Figure 5B). The sdhC-lacZ expression level in TSDH06 cells was not significantly changed during the cell growth (filled circles). When 1 mM of cAMP was added to the growth medium, however, the sdhC-lacZ expression of TSDH06 cells was increased to ~3-fold within 1 h. These results support that intracellular cAMP production as well as crp expression plays a crucial role in the regulation of sdhCDAB expression.

The CRP-cAMP complex binds to the sdhC promoter and regulates transcription in vitro

To show the direct binding of the CRP-cAMP complex to the sdhC promoter in vitro, gel shift assays were carried out using purified CRP and the sdhC promoter fragment in the presence of cAMP. The results showed that CRP-cAMP specifically binds to the sdhC promoter (Figure 6A). As the amount of CRP added in the reaction mixture increased, the amount of the CRP–promoter DNA complex also increased. Binding affinity of the CRP-cAMP complex toward the sdhC promoter was comparable with that toward the ptsG promoter.

To investigate the effect of CRP-cAMP binding to the promoter on sdhCDAB transcription, the in vitro transcription assay was performed with a supercoiled DNA template (pTSDHpro) containing base pairs –183 to +209 relative to the transcription start site, covering the sdhC promoter and its CRP and ArcA binding sites. When RNA polymerase alone was present in the reaction, transcription from the sdhC promoter did not occur efficiently (Figure 6B, lane 1). The addition of CRP and cAMP, however, remarkably increased the promoter activity (Figure 6B, lanes 2–5). Most intriguingly, incubation of the reaction mixture with ArcA-P repressed the CRP-activated promoter activity in a dose-dependent manner (Figure 6B, lanes 6–8). The specificity of CRP-cAMP function in sdhCDAB transcription was confirmed by the consistent activity of rep that originates from replication origin of the DNA template regardless of the presence of CRP and ArcA-P. These data confirm that the CRP-cAMP complex affects the sdhCDAB transcription initiation and is directly involved in the glucose repression of sdhCDAB expression.

The level of phosphorylated EIIAGlc correlates with the intracellular cAMP concentration and sdhC-lacZ expression

It was reported that expression of sdhC-lacZ varied depending on the type of carbon source added in the medium (7). From the above results, it could be assumed that the different expression levels of sdhC-lacZ on various sugars might result from the change in the intracellular cAMP concentration depending on the type of carbon source. To verify this assumption, the relationship between the intracellular cAMP concentrations and β-galactosidase activities was determined in TSDH00 cells grown in LB with various carbon sources. The β-Galactosidase activities of TSDH00 revealed the carbon source-dependent expression of sdhC-lacZ (Figure 7A). Growth with mannose, fructose or maltose did not affect the expression level of sdhC-lacZ, while N-acetylglucosamine and galactose showed the similar effect with glucose on sdhC-lacZ expression. To investigate the effect of cAMP on sdhC-lacZ expression, the levels of intracellular cAMP were also measured (Figure 7A). The intracellular cAMP level in TSDH00 cells decreased when glucose was added to the medium, in agreement with the previous reports [reviewed in (1)]. The intracellular cAMP level in cells grown on glucose, N-acetylglucosamine or galactose was lower than that in cells grown on mannose, fructose or maltose. The result showed that carbon source-dependent expression of sdhC-lacZ is in accordance with the intracellular cAMP concentration. Although the mechanism for the regulation of the intracellular cAMP level is not fully understood, a popular model for the regulation of adenylate cyclase activity is that phosphorylated EIIAGlc stimulates adenylate cyclase activity and increases the intracellular cAMP concentration (1). Therefore, we measured the level of EIIAGlc phosphorylation in the cells grown with various carbon sources by western blot analysis according to the procedure developed by Takahashi et al. (22) as described under ‘Materials and Methods’ (Figure 7B). It is well
phosphorylation state in the mutant that affects the level of intracellular cAMP.

**DISCUSSION**

It was reported that activities of *E. coli* TCA cycle enzymes such as succinate dehydrogenase are remarkably reduced during anaerobiosis and in the presence of glucose in the medium almost 40 years ago (31). The recent studies in the transcriptomic and proteomic levels also revealed that the genes involved in the TCA cycle are strongly repressed by glucose and/or anaerobiosis (32–34). While the mechanism underlying the anaerobic repression of *sdhCDAB* was well documented in previous studies (7,9), the mechanism of the glucose repression of *sdhCDAB* expression still remains as a puzzling issue.

Although CRP was excluded from the regulatory circuit of *sdhCDAB* expression (7), several reasons prompted us to re-consider the CRP-cAMP complex as the direct mediator of the glucose repression of *sdhCDAB*: (i) the CRP-cAMP complex has been established as the major regulator of the glucose-mediated carbon catabolite repression of more than 100 genes (1); (ii) a putative CRP-binding site was proposed to be located on the *sdhC* promoter region (29) (Figure 1), although binding of CRP to the promoter has never been demonstrated; (iii) we could not find any transcription regulators other than Mlc that interact with the glucose-sensing EIIB domain of the *ptsG* gene product (data not shown), while it was shown that the *ptsG* gene acts as a crucial mediator of the glucose repression of the *sdhCDAB* operon (10). Furthermore, the mlc mutant still showed the glucose repression of *sdhCDAB* (10) (Figure 3); (iv) in a previous review, it was proposed that catabolite repression of the *sdhCDAB* operon is controlled presumably by the CRP-cAMP complex (35). (v) Finally, recent reports on the transcriptome analyses of the *crp* mutant using microarray techniques indicated that expression of the *sdhCDAB* operon might actually be regulated by the CRP homologue Fnr in vivo (37), based on the facts that Fnr has been shown to regulate *sdhCDAB* expression in response to anaerobiosis (7), the consensus sequence for Fnr is similar to that for CRP, and both proteins can bind to the DNA site for the other protein (38).

From the results in this study, it is evident that CRP is directly involved in the regulation of *sdhCDAB* expression and the glucose repression of *sdhCDAB* occurs in a cAMP-dependent manner. Genetic studies using *cya* and *crp* mutants and the *sdhC-lacZ* fusion strain harboring the mutated *crp* binding site on the *sdhC* promoter region suggest that both cAMP and CRP are required for *sdhCDAB* expression and its glucose repression (Figures 3–5). In *vitro* studies demonstrate binding of CRP to the *sdhC* promoter and activation by the CRP-cAMP complex of *sdhC* transcription (Figure 6). Furthermore, the phosphorylation level of EIIAGlc in cells grown with different carbon sources correlates with the intracellular concentration of cAMP and the *sdhC-lacZ* expression level (Figure 7). It was previously reported that the phosphorylation level of EIIAGlc is dependent on the type of carbon source in the medium (39). Although no biochemical evidence has been
provided, it is generally believed that the phosphorylated form of EIIGlc stimulates adenylate cyclase activity (1). In the presence of glucose, N-acetylglucosamine or galactose in the medium, the level of the phospho-form of EIIGlc decreased (Figure 7B). This decrease seems to be responsible for reduced activity of adenylate cyclase and reduced production of cAMP required to activate sdhCDAB expression after binding to its receptor protein CRP. Thus, we conclude that the CRP-cAMP complex mediates the glucose repression of the sdhCDAB operon. There still remains one question why the ptsG mutant exerts a more profound effect than mutation of crr on the glucose repression of the sdhCDAB promoter (Figure 3), that is in conflict with our conclusion that the crr gene product EIIGlc is the major regulator in orchestrating glucose repression of the sdhCDAB promoter. One possibility for this conflict may be due to the pleiotropic effect of the ptsG and crr mutants on expression of many genes expected from the fact that both EIICBglc and EIIGlc interact with and regulate activities of many regulatory proteins (1,5,11–13). More studies need to be carried out to fully understand this question.

Under fully aerobic conditions, the TCA cycle in E. coli operates as an oxidative pathway that needs the activities of succinate dehydrogenase, encoded by sdhCDAB, and α-ketoglutarate dehydrogenase. In the presence of readily fermentable sugars and/or under anaerobic conditions, however, the TCA cycle hardly operates in an oxidative way because coupling of the pathway to terminal respiration is absolutely required to maintain the activities of the succinate dehydrogenase complex and α-ketoglutarate dehydrogenase complex that produce FADH2 and NADH, respectively. On the other hand, the reactions that make oxaloacetate, succinylcoenzyme A and α-ketoglutarate are necessary because these intermediates are still required for the biosynthesis of amino acids and tetrapyrroles. Under these conditions, the TCA cycle is converted from an oxidative and cyclic into a reductive and branched pathway to solve the problem. In the reductive pathway, succinyl-coenzyme A is made by reversing the reactions between oxaloacetate and succinyl-coenzyme A, using the enzyme fumarate reductase instead of succinate dehydrogenase (40). Thus, the decreased sdhCDAB expression by carbon catabolite repression in the presence of glucose provides one of the mechanisms to maintain the TCA cycle in a reductive pathway, leading to accumulation of succinate and succinyl-coenzyme A (41). The sdhCDAB operon in this study is not the first example of genes encoding the TCA cycle enzymes whose expression are activated by CRP and repressed by ArcA and Fnr. The acnB, encoding one of the two acanotases differentially expressed in E. coli, has been shown to be regulated in the same way as the sdhCDAB operon (42). Intriguingly, expression of both fumA and sdhCDAB was recently shown to be down-regulated by the small RNA, RyhB (8). Expression of the fumA and fumC genes encoding two fumarase isozymes of the TCA cycle in E. coli was also shown to be subject to the glucose repression and require cAMP (43). Thus, decrease in the CRP-cAMP level in the presence of readily fermentable glucose seems to be responsible for the reduced expression of genes encoding enzymes necessary to maintain the TCA cycle in an oxidative pathway and conversion of the cycle into a reductive pathway.

ACKNOWLEDGEMENTS

This work was supported by the Korea Research Foundation Grant (KRF-2004-015-C00480) and the 21C Frontier Microbial Genomics and Applications Center Program (Grant MG05-0202-6-0), Republic of Korea. T.-W.N. and Y.-H.P. were supported by BK21 Research Fellowships from the Korean Ministry of Education and Human Resources Development. The authors thank Dr A. Peterkofsky for his generous gifts of strains and plasmids. Funding to pay the Open Access publication charges for this article was provided by Korea Research Foundation.

Conflict of interest statement. None declared.

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