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Expression of *ptsG* Encoding the Major Glucose Transporter Is Regulated by ArcA in *Escherichia coli**

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Because the phosphoenolpyruvate:sugar phosphotransferase system plays multiple regulatory roles in addition to the phosphorylation-coupled transport of many sugars in bacteria, synthesis of its protein components is regulated in a highly sophisticated way. Thus far, the cAMP receptor protein (CRP) complex and Mlc are known to be the major regulators of *ptsHIcrr* and *ptsG* expression in response to the availability of carbon sources. In this report, we performed ligand fishing experiments by using the promoters of *ptsHIcrr* and *ptsG* as bait to find out new factors involved in the transcriptional regulation of the phosphoenolpyruvate:sugar phosphotransferase system in *Escherichia coli*, and we found that the anaerobic regulator ArcA specifically binds to the promoters. Deletion of the *arcA* gene caused about a 2-fold increase in the *ptsG* expression, and overexpression of ArcA significantly decreased glucose consumption. *In vitro* transcription assays showed that phospho-ArcA (ArcA-P) represses *ptsG* P1 transcription. DNase I footprinting experiments revealed that ArcA-P binds to three sites upstream of the *ptsG* P1 promoter, two of which overlap the CRP-binding sites, and the ArcA-P binding decreases the CRP binding that is essential for the *ptsG* P1 transcription. These results suggest that the response regulator ArcA regulates expression of enzyme IICB^{Glc} mediating the first step of glucose metabolism in response to the redox conditions of growth in *E. coli*.

The bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS)[1] consists of two general cytoplasmic proteins, enzyme I and histidine phosphocarrier protein HPr, and some sugar-specific components collectively known as enzyme II (1, 2). Glucose-specific enzyme II of *Escherichia coli* consists of two subunits, soluble enzyme IIA^{Glc} (EIIA^{Glc}) and membrane-bound enzyme IICB^{Glc}. Thus, glucose transport in *E. coli* involves three soluble PTS components (enzyme I, HPr, and EIIA^{Glc}, encoded by the *ptsHIcrr* operon) and one membrane-bound protein, enzyme IICB^{Glc} (EIICB^{Glc}), encoded by the *ptsG* gene. During translocation of glucose, a phosphoryl group derived from phosphoenolpyruvate is transferred sequentially along a series of proteins to the transported glucose molecule, eventually converting it into glucose 6-phosphate. The sequence of phosphotransfer is from phosphoenolpyruvate to the general PTS proteins enzyme I and HPr and further to the carbohydrate-specific cytoplasmic EIIA^{Glc}, membrane-bound EIICB^{Glc}, and glucose.

The PTS takes an important part in metabolic adaptation to environmental changes to compete effectively with ambient organisms by sensing the availability of nutrients in the environment. In addition to sugar transport, multiple roles are exerted by the PTS and these include chemoreception (3), catabolite repression (4), carbohydrate transport and metabolism (1, 5, 6), carbon storage (7, 8), and the coordination of carbon and nitrogen metabolism (9). More recently, we found that EIIA^{Glc} of the PTS also regulates the flux between respiration and fermentation pathways by sensing the available sugar species via a phosphorylation state-dependent interaction with the fermentation/respiration switch protein FrsA (10). For this reason, synthesis of the PTS components should be regulated in a highly sophisticated way.

It was reported that expression of the *pts* operon and the *ptsG* gene increases a few fold during growth on glucose or other PTS substrates (1), and the functional cAMP·CRP complex is essential for the expression of the *ptsG* gene (1, 11). From further studies, it was revealed that the expression of the *ptsHIcrr* operon and the *ptsG* gene is subject to complex regulation. It was shown that the *ptsH* P1 promoter is regulated by the repressor of the fructose PTS, FruR (12). Moreover, both the *ptsH* P0 promoter and the *ptsG* P1 promoter have been shown to be subject to activation by the cAMP·CRP complex and repression by Mlc (13–18). Subsequently, it was demonstrated that the unphosphorylated form of EIICB^{Glc} can relieve the repression of the Mlc regulon by sequestering Mlc through the direct protein-protein interaction to induce the expression of the Mlc regulon including PTS proteins (19–21). Recently, it was also found that glucose-induced expression of *ptsG* is enhanced significantly under heat shock, and it was thus proposed that this enhancement may counteract the highly increased *mlc* expression upon heat shock, whereby normal glucose metabolism is maintained at high growth temperatures (22). In addition to the regulations at the transcriptional level, expression of *ptsG* is regulated post-transcriptionally via modulation of *ptsG* mRNA stability in response to glycolytic flux in the cells (23). These observations suggest that the expression of the PTS proteins is regulated in a highly complex and dynamic

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[1] The abbreviations used are: PTS, phosphoenolpyruvate:sugar phosphotransferase system; ArcA-P, phospho-ArcA; CRP, cAMP receptor protein; DTT, dithiothreitol; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; ORF, open reading frame.

TABLE I
Bacterial strains and plasmids used

Strains and plasmids	Genotype or phenotype	Source or Ref.
Strains		
DY330	W3110 Δ*lacU169 gal490 λCI857 Δ(cro-bioA)*	24
GI698	F^- λ^- *lacI*^{q} *lacPL8 ampC*::P_{trp} cI	38
MC4100	F^- *araD*139 Δ(*argF-lac*)U169 *flbB*5301 *thiA deoC*1 *relA*1 *rbsR rpsL*150 *ptsF*25	39
MG1655	Wild type *E. coli*	40
SR702	MC4100 *suhX*1 *ptsG*::*cat*	10
YJ2000	DY330 *arcA*::*cat*	This study
YJ2001	MC4100 *arcA*::*cat*	This study
YJ2002	MC4100 λ [Φ(*ptsG′-′lacZ*)]	This study
YJ2003	YJ2002 *arcA*::*cat*	This study
YJ2004	DY330 *mlc*::Tet^R	This study
YJ2005	YJ2002 *mlc*::Tet^R	This study
YJ2006	YJ2003 *mlc*::Tet^R	This study
Plasmids		
pNS200	*ArcA* (*Nde*I/*Bam*HI) in pRE1, Amp^r, overexpression vector for ArcA	This study
pRE1	Expression vector under control of λP_L promoter, Amp^r	25
pRS415	*LacZ lacY*$^+$ *lacA*$^+$, *Amp*^{r}	28
pRSptsG	pRS415[Φ(*ptsG′-′lacZ*)], Amp^r	This study

manner in response to various growth environments as well as the availability of carbon sources.

In addition to the regulators of PTS expression mentioned above, we thought that there might be other factor(s) regulating PTS expression in *E. coli*. This prospective stimulated us to embark on ligand fishing experiments to search for other transcriptional regulator(s) exhibiting high affinity interaction to promoters of the *pts* operon and the *ptsG* gene. Consequently, we found that ArcA specifically binds to these promoters. Here we have investigated the negative regulation of *ptsG* expression by phospho-ArcA.

EXPERIMENTAL PROCEDURES

Materials—cAMP was obtained from Sigma. RNA polymerase saturated with σ^{70}, nucleotide triphosphates, [γ-^{32}P]ATP, and [α-^{32}P]UTP were purchased from Amersham Biosciences. The cycle sequencing kit was from Epicenter Technologies (Madison, WI).

Bacterial Strains and Growth Conditions—The bacterial strains and plasmids used in this study are listed in Table I. The *arcA* mutant was constructed using *E. coli* DY330 as described previously (24). The *arcA* gene (from the start codon to the stop codon) was replaced by a chloramphenicol acetyltransferase gene (*cat*). The *cat* gene was amplified by PCR from SR702 (*ptsG*::*cat*) with the following primers: forward primer, 5′-TAACACGCAACACGTTGAAAAGTATTTTCGAAGCGGAAGGCTATGATGTTATGGAGAAAAAAATCACTGG-3′ and reverse primer, 5′-ATAAAAACGGCGCTAAAAAGCGCCGTTTTTTTTTGACGGTGGTAAAGCCGATTACGCCCCGCCCTGCCACT-3′. The PCR product was electroporated into *E. coli* DY330 to make the strain YJ2000. MC4100 Δ*arcA* (YJ2001) was constructed by P1 transduction of the Cm^r region of DY330 Δ*arcA*. Disruption of the *arcA* gene was confirmed by PCR. Strains were grown in Luria-Bertani (LB) medium at 37 °C unless otherwise specified. The *mlc* deletion strain DY330 *mlc*::Tet^R (YJ2004) was prepared by using the following primers: 5′-CACCATAGCCTACAGATTATTTCGGAGCGCGAAAATATAGGGAGTATGCGTTAAGACCCACTTTCACATT-3′ and 5′-AAAATGTTAACCCTGCAACAGACGAATCAACAAAGAACCGTTATACATCGCTAAGCACTTGTCTCCTGTT-3′. The Tet^R region was moved from YJ2004 to YJ2002 and YJ2003 by P1 transduction to make YJ2005 and YJ2006, respectively (see Table I).

Ligand Fishing Experiments—The DNA fragments used for ligand fishing were amplified by PCR using biotinylated primers (biotin-5′-CCT TCA ATC CAT CCG TTG AA-3′ and 5′-GTA CAG GGC ATC TAA GCG CC-3′ for amplification of the *ptsG* promoter, biotin-5′-GCC AGC TTG TTA AAA ATG CG-3′ and 5′-ACC GCT GAC GTT TCG TAC GT-3′ for the *ptsH* promoter, and biotin-5′-GTT GCC TGG TAC CGT TTG GT-3′ and 5′-TAC CGC CCA CTT TCG CGC GG-3′ for *ptsG* ORF as a control). The PCR mixture contained 250 units of Taq polymerase, 200 μM of each dNTP, and 2 μM of each primer in a total volume of 10 ml. After PCR, the solution containing amplified DNA fragments (about 50 μg/ml) was loaded onto a NeutraAvidin column (Pierce) containing 1 ml of resin. The wild type *E. coli* strain MC4100 was grown to A_{600} = 2 in 1 liter of LB medium buffered with 20 mM potassium phosphate (pH 7.0) in an atmosphere of 80% nitrogen, 10% hydrogen, and 10% carbon dioxide. Cells were harvested and resuspended in the binding buffer (40 mM HEPES-KOH (pH 7.9), 100 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, and 10% glycerol) and then passed three times through a French press cell at 10,000 pounds/square inch. The extract was centrifuged at 20,000 × *g* for 30 min. The soluble fraction was loaded onto the column immobilized with the DNA fragments. After agitation on a rotator at room temperature for 20 min, the affinity column was drained and washed with 5 ml of binding buffer twice, and the bound proteins were eluted with 1 ml of 0.1% SDS. The solution containing eluted proteins was freeze-dried and resuspended in 200 μl of deionized water. Then 10 μl of the protein sample was analyzed by SDS-PAGE.

In-gel Digestion—The polyacrylamide gel was stained with Coomassie Brilliant Blue R, and the bands of interest were excised from the gel. The gel fragments were destained by incubation in 30–50 μl of 20% isopropyl alcohol for 20 min twice, dehydrated in 50% acetonitrile, and washed with deionized water. The gel fragments were equilibrated in 40 μl of 50% acetonitrile containing 100 mM NH_4HCO_3 for 15 min twice and reduced with 40 μl of 10 mM DTT containing 100 mM NH_4HCO_3 for 1 h at 50 °C. Then cysteine residues in proteins were alkylated by incubation in 40 μl of 50 mM iodoacetamide and 100 mM NH_4HCO_3 at room temperature for 40 min in the dark and washed with 20 μl of 50% acetonitrile containing 100 mM NH_4HCO_3 twice. The washed gel fragments were dried in vacuum for 20 min. The polypeptides in the gel fragments were digested by overnight incubation with 200 ng of trypsin in 10 μl of 50 mM NH_4HCO_3. The peptides digested by trypsin were eluted twice with 10 μl of 5% trifluoroacetic acid containing 50% acetonitrile and concentrated to 4–5 μl in vacuum. MALDI-TOF mass spectra for peptide mapping of tryptic digests were acquired using a Voyager-DE STR (Applied Biosystems Inc.) MALDI-TOF mass spectrometer equipped with a nitrogen laser (337 nm).

Purification and Phosphorylation of ArcA—Primers possessing the synthetic restriction enzyme sites NdeI, located 3 bp upstream from the ATG start codon (in boldface type) (5′-GTTGGCAATTTAGGTAGCACATA**TG**CAG-3′) of the *arcA* gene, and BamHI, located 8 bp downstream from the TAA stop codon (in boldface type) (5′-TTTTGACGGTGGTGGATCCGA**TTA**ATCT-3′), were constructed (restriction sites underlined). By using these primers, the *arcA* gene from the MG1655 genomic DNA was amplified by PCR. After digestion, the NdeI-BamHI fragment was inserted into the corresponding sites of pRE1 (25), and the recombinant plasmid (pNS200) was electroporated with a Bio-Rad *E. coli* pulser into *E. coli* strain GI698 (26), which encodes the gene for the λ repressor under control of the *trp* promoter. Expression of ArcA was induced by the addition of tryptophan (100 μg/ml) in 1 liter of M9 salts-based rich medium as described previously (10, 26). The cells containing overexpressed ArcA were harvested and resuspended in 10 mM Tris-Cl (pH 7.5) buffer containing 50 mM NaCl and then passed twice through a French press cell at 10,000 pounds/square inch. Cell debris was removed by centrifugation at 100,000 × *g* for 90 min. The supernatant was chromatographed through an FPLC MonoQ 10/10 column (Amersham Biosciences) equilibrated with Buffer A (10 mM Tris-Cl (pH 7.5), containing 50 mM NaCl and 1 mM DTT) using a gradient of 50–500 mM NaCl. The fractions containing ArcA were concentrated in a Centriprep filter (Millipore) and loaded onto a gel filtration column (Superdex 200, Amersham Biosciences) equilibrated with Buffer A. The purified ArcA was concentrated to suitable concentration before use. ArcA was phos-

phorylated using carbamoyl phosphate as the phospho donor as described previously (27). The carbamoyl phosphate reaction mixture containing the indicated amount of ArcA protein, 50 mM dilithium carbamoyl phosphate, 100 mM Tris-HCl (pH 7.0), 125 mM KCl, and 10 mM $MgCl_2$ in a total volume of 10 μl was incubated at 30 °C for 30 min and immediately used for experiments.

Gel Retardation Assay—DNA fragments encompassing the promoter region of *ptsG* and *ptsH* and the ORF region of *ptsG* were amplified by PCR using the primers used in the ligand fishing experiments. The promoter of *lctPRD* was amplified by new primers (5′-CTG GAT CAA CAA CCT AAG GGC AAT TCT CTG-3′ and 5′-ATC TCC TTG TCA CAC GTT GTG TAA AAG TGG-3′). The DNA fragments were labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. The binding buffer for electrophoretic mobility shift assay contained 100 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 2 mM DTT and 10% glycerol. Also, each reaction mixture included sonicated herring sperm DNA (Promega) as a nonspecific competitor at the concentration of about 300-fold molar excess over the labeled DNA binding substrate. The binding mixture was incubated at 30 °C for 30 min and analyzed by electrophoresis on the 6% polyacrylamide gel containing 1× TBE (Invitrogen) at 4 °C.

In Vitro Transcription Assay—Reactions were done as described by Ryu and Garges (13) in a 20-μl volume containing 20 mM Tris acetate (pH 8.0), 3 mM magnesium acetate, 200 mM potassium glutamate, 1 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 0.2 mM CTP, 0.02 mM UTP, 10 μCi of [α-^{32}P]UTP (800 Ci/mmol), 2 nM supercoiled DNA template, 5–20 nM RNA polymerase, 100 μg/ml bovine serum albumin, and 5% glycerol. Regulator proteins such as CRP and ArcA were added to the reaction as described under "Results."

DNase I Footprinting Analysis—DNA fragment carrying the *ptsG* promoter region was amplified by PCR using either 5′-end-labeled PG1 or PGR2 (5′-ATAACTTCGCCCGTCTGTTTCACATCG-3′, −248 to −222). The PCR product was purified using polyacrylamide gel. Purified protein(s) and DNA fragment were incubated in 40 μl of *in vitro* transcription assay buffer. DNase I solution (10 ng of DNase I in 5 μl per reaction) was added to the binding mixture, which was then placed at room temperature for 1 min. DNase I reaction was terminated by the addition of 200 μl of stop solution containing 0.4 M sodium acetate, 10 mM EDTA, and 100 μg/ml yeast tRNA. After phenol extraction and ethanol precipitation, the pellet was dissolved in sequencing dye and resolved on 7% polyacrylamide gel containing 8 M urea. For the identification of the binding sites of proteins, the sequencing ladder prepared by sequencing the *ptsG* promoter region using PG1 or PGR2 primer was applied to the gel electrophoresis.

Construction of a ptsG-lacZ Fusion and β-Galactosidase Assays—The DNA fragment covering from 363 bp upstream to 77 bp downstream of the *ptsG* start codon was amplified by PCR using primers 5′-CAGGTAACCACCGATGAATTCGCCCGTCTG-3′ (an engineered EcoRI site underlined) and 5′-GCGATAGGCAGTACGGATCCCGGCAGCATC-3′ (an engineered BamHI underlined). The PCR product, digested with EcoRI and BamHI, was inserted into pRS415 (28) to make pRSptsG. The sequence of the *ptsG* insert was verified by sequencing, and the fusion was transferred to λRS45 by *in vivo* recombination as described previously (28). Bacteriophage λ carrying the fusion was used to lysogenize MC4100 at low multiplicity, and several independent lysogens were analyzed to obtain a monolysogen (YJ2002). YJ2003 (YJ2002*arcA*::*cat*) was constructed by P1 transduction of the Cm^r region of YJ2001. Disruption of the *arcA* gene was confirmed by PCR. After cells were grown in LB media adjusted to pH 7.0 using 20 mM potassium phosphate to the mid-logarithmic growth phase, β-galactosidase activity was measured as described previously (29).

RESULTS

Ligand Fishing Experiment Shows That ArcA Specifically Binds to the Promoter Regions of the pts Operon and the ptsG Gene—We performed ligand fishing experiments to search for a protein factor(s) involved in the regulation of PTS expression by using the promoter regions of *ptsG* and the *pts* operon as baits. The DNA fragments encompassing the two promoter regions were amplified to a large quantity (10 ml each) by PCR using biotinylated primers and immobilized onto the NeutraAvidin resin (Pierce). Cell lysate was then mixed with the DNA fragments in the columns, and proteins bound specifically to the two promoters were identified by in-gel digestion and MALDI-TOF analyses (see "Experimental Procedures"). As shown in Fig. 1, we found that ArcA as well as CRP bound to

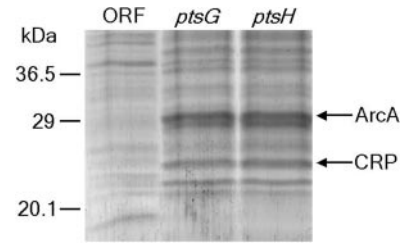

FIG. 1. **Ligand fishing shows that ArcA as well as CRP specifically binds to the promoter regions of the *pts* operon and the *ptsG* gene.** The promoter regions of *ptsG* and the *pts* operon and the *ptsG* ORF region were amplified by PCR using biotinylated primers, and ligand fishing experiments using these amplified DNAs as baits were carried out as described under "Experimental Procedures." The profiles of bound proteins were visualized by SDS-PAGE and staining with Coomassie Blue (*lane 1*, *ptsG* ORF; *lane 2*, *ptsG* promoter; *lane 3*, *ptsH* promoter), and proteins bound specifically to the two promoters were identified by in-gel digestion and MALDI-TOF analyses as indicated. Molecular masses (in kDa) of some standards are presented on the *left side*.

the two promoter regions, but not to the ORF of *ptsG*, to a significant level, implying specific binding of these two proteins to the promoter regions. Although it is not surprising that CRP shows specific binding to the two promoters, since its binding sites to the promoters are well characterized (15, 18), it is intriguing that ArcA binds specifically to the two promoters.

To confirm specificity of binding of ArcA to the two promoter regions, we performed gel retardation assays. We used the promoter region of the *lctPRD* operon, which was shown to interact with ArcA previously (27), as a positive control. Electrophoretic mobility shifts were observed when ArcA was incubated with DNA fragments covering the promoter regions of the *lct* operon, the *ptsG* gene, and the *pts* operon, but not with the *ptsG* ORF (Fig. 2), confirming specific interaction of ArcA with the promoters as shown in Fig. 1. Although both phospho-ArcA (ArcA-P) and dephospho-ArcA showed mobility shifts of the three promoter regions, electrophoretic mobility shifts observed with ArcA-P (Fig. 2*A*) were greater than its dephosphoform (Fig. 2*B*), showing general characteristics of DNA binding of ArcA to target promoters (27) and indicating that the two forms of ArcA protein may show different effects on transcription from each other.

ArcA-P Represses Transcription of ptsG—Although the affinity of ArcA-P to the *ptsG* promoter was comparable with that of the *lctPRD* promoter, binding of ArcA-P to the promoter region of the *pts* operon was much weaker than to the other promoters. Thus we focused on the effect of ArcA on the *ptsG* expression. *E. coli* is a typical facultative anaerobe, which implies that the expression of large numbers of genes should be regulated differentially in response to changing respiratory conditions of growth. ArcA has been well characterized as the response regulator of the two-component sensory system regulating gene expression in response to the redox conditions of growth in *E. coli* (30). When the quinone electron carriers are reduced, its cognate membrane-bound sensor kinase ArcB autophosphorylates and transfers the phosphoryl group to ArcA (31). The ArcA-P then controls the expression of numerous operons involved in respiratory or fermentative metabolism. Because $EIICB^{Glc}$ mediates the first reaction for the glucose metabolism, we decided to examine whether ArcA-P or its dephospho-form affects expression of the *ptsG* gene.

First, to investigate the effect of *arcA* deletion on the expression of the *ptsG* gene encoding the glucose-specific transporter $EIICB^{Glc}$, we constructed a *ptsG-lacZ* fusion strain. As shown in Fig. 3, *arcA* deletion caused an ~2-fold increase in β-galactosidase activity. Because it is well established that Mlc is the major regulator of the *ptsG* expression, it was uncertain

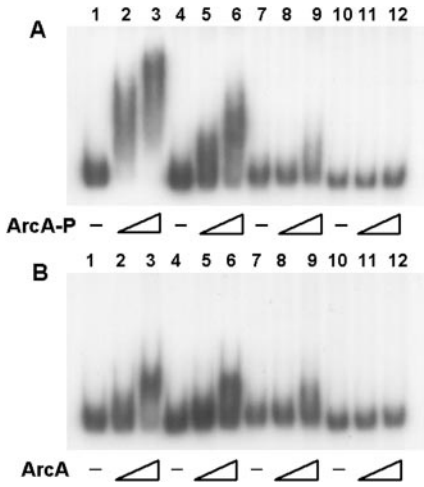

FIG. 2. **The electrophoretic mobility shift assays of the binding of ArcA to the *ptsG* and *ptsH* promoters.** The promoter regions of *lctP* (*lanes 1–3*), *ptsG* (*lanes 4–6*), and *ptsH* (*lanes 7–9*) and the ORF region of *ptsG* (*lanes 10–12*) were amplified by PCR, labeled with [γ-^{32}P]ATP, and used as probes for electrophoretic mobility shift assays as described under "Experimental Procedures." Elecrophoretic mobility shift assays containing the ^{32}P-labeled DNA probes (5 nM for each reaction) were performed by using ArcA with (*A*) and without (*B*) phosphorylation (no ArcA in *lanes 1, 4, 7,* and *10*; 1.25 μM ArcA in *lanes 2, 5, 8,* and *11*; and 2.5 μM ArcA in *lanes 3, 6, 9,* and *12*) on 6% polyacrylamide gels. Sonicated herring sperm DNA (Promega) was contained in each reaction mixture at about 1.5 μM as unlabeled competitor DNA.

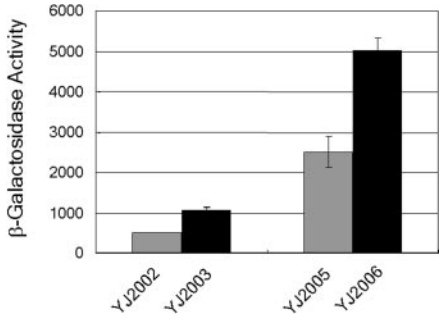

FIG. 3. **β-Galactosidase activity expressed by the *ptsG-lacZ* transcriptional fusion genes.** *E. coli* MC4100 (YJ2002) and its *arcA* deletion derivative (YJ2003), *mlc* deletion derivative (YJ2005), and *arcA mlc* double deletion mutant (YJ2006), each carrying the *ptsG-lacZ* transcriptional fusion gene on the chromosome, were grown in LB (pH 7.0 using 20 mM potassium phosphate buffer) under aerobic conditions. The cells were harvested at the mid-logarithmic growth phase, and then β-galactosidase activities were measured. The values are the means of three independent cultures measured in Miller units (29).

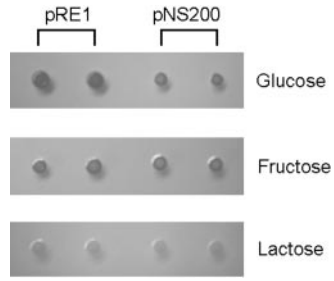

FIG. 4. **Effect of ArcA overexpression on utilization of various sugar substrates.** The *E. coli* GI698 strains harboring the control vector pRE1 or the ArcA overexpression vector pNS200 (two of each) were grown in M9 salts-based rich media (10, 26) until the late exponential growth phase, and 5 μl (~5 $\times$ 10^5 cells) from each culture was spotted on MacConkey agar plates containing the indicated sugars (0.5%) to check rates of growth and color development. Pictures were taken about 10 h after inoculation.

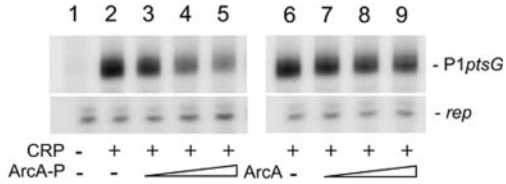

FIG. 5. **Inhibitory effect of ArcA on *ptsG* P1 transcription analyzed by *in vitro* transcription assay.** The supercoiled template was incubated with CRP (40 nM) and ArcA-P (*lanes 3–5*; 0.5, 1.0, and 2.0 μM, respectively) or ArcA (*lanes 7–9*; 0.5, 1.0, and 2.0 μM, respectively) for 10 min at 37 °C. Transcription was started by adding NTP solution, stopped after 10 min by adding loading dye, and analyzed on 6% sequencing gel. The transcripts from the plasmid origin of replication (106/107 nucleotides) are marked as *rep*.

whether regulation of the *ptsG* expression by ArcA was an indirect result of the reduced expression of Mlc in the *arcA* deletion strain. To address this issue, we constructed *mlc*::Tet^R derivatives of MC4100 and its *arcA* mutant, and the expression profile of *ptsG-lacZ* was examined in these mutant cells grown in LB. The results showed that the 2-fold increase in the *ptsG* expression by *arcA* deletion was independent of the regulation by Mlc in that increased *ptsG* expression in the *mlc* strain was still doubled by the introduction of *arcA* mutation. The fact that the presence of a null mutation in *arcA* leads to an increase in *ptsG* expression regardless of Mlc suggests that ArcA should be the direct repressor of *ptsG*.

If ArcA is the direct repressor for *ptsG* expression, it was expected that utilization of glucose might be more significantly inhibited than that of other sugars by overexpression of ArcA. To test this idea, the phenotype of *E. coli* GI698 strains harboring the ArcA overexpression plasmid pNS200 was compared with cells harboring the control vector pRE1. When ~5 $\times$ 10^5 cells were spotted, colonies of the *E. coli* strain GI698 harboring the control vector pRE1 on a glucose-MacConkey agar plate developed a red color about 6 h after incubation at 30 °C. In contrast, growth and color development of colonies of GI698 harboring pNS200 were significantly retarded (Fig. 4). However, growth and color development of the *E. coli* strain GI698 on MacConkey agar plates with fructose (transported through its own PTS independent of *ptsG*) or lactose (taken up through lactose permease encoded by *lacY*) were barely affected by overexpression of ArcA, as expected. These results support that ArcA is the repressor of the *ptsG* gene.

To confirm the inhibitory effect of ArcA on *ptsG* transcription, the *in vitro* transcription assay was performed by using the purified components. When RNA polymerase alone was present in the reaction, transcription at the *ptsG* P1 promoter did not occur (Fig. 5, *lane 1*). The addition of CRP and cAMP, however, increased the P1 promoter activity dramatically (Fig. 5, *lane 2*), suggesting that the transcription initiation at *ptsG* P1 is totally dependent on cAMP·CRP as expected from a previous report (11). Most intriguingly, incubation of the reaction mixture with ArcA-P reduced the CRP-activated P1 promoter activity in a dose-dependent manner (Fig. 5, *lanes 3–5*), whereas addition of unphosphorylated ArcA showed little effect on *ptsG* P1 transcription (Fig. 5, *lanes 6–9*). The specificity of ArcA-P function in *ptsG* P1 transcription was confirmed by the consistent activity of *rep* that originates from replication origin of DNA template regardless of the presence of ArcA-P. These data confirm that ArcA-P inhibits the *ptsG* P1 transcription initiation.

ArcA-P Binds to Three Sites in the ptsG Promoter Region—To identify the binding location(s) of ArcA-P on the *ptsG* promoter region and to understand the mechanism of the inhibitory effect of ArcA-P on *ptsG* P1 transcription, we performed DNase I footprinting experiments. When the end-labeled *ptsG* pro-

moter DNA fragment was incubated with increasing amounts of ArcA-P, the DNA regions between +12 and −12 (site I), −36 and −62 (site II), and −73 and −110 (site III) were protected from DNase I attack (Fig. 6, *lanes 1–5* and *8*). The ArcA-P-binding site I, which centered at +1.5, overlapped the P1 promoter. It has been well known that the cAMP·CRP complex binds to the *ptsG* promoter centered at −40.5 and −95, respectively (15). The binding sites II (centered at −51.5) and III (centered at −97.5) of ArcA-P overlapped these two CRP-binding sites, respectively (Fig. 6). Then we tested the effect of ArcA-P binding on CRP binding to the *ptsG* promoter region. As expected from the previous report (32), CRP in the reaction mixture produced two clear DNase I protection regions (Fig. 6, *lane 7*). The addition of CRP decreased the ArcA-specific protection on binding sites II and III (Fig. 6, compare *lanes 8* and *10*), and the CRP-specific protection was decreased as the ArcA-P level was increased (*lanes 9–11*), implying that ArcA-P and CRP compete with each other for the binding to the promoter. The presence of CRP did not show any effect on ArcA-P binding to the site I.

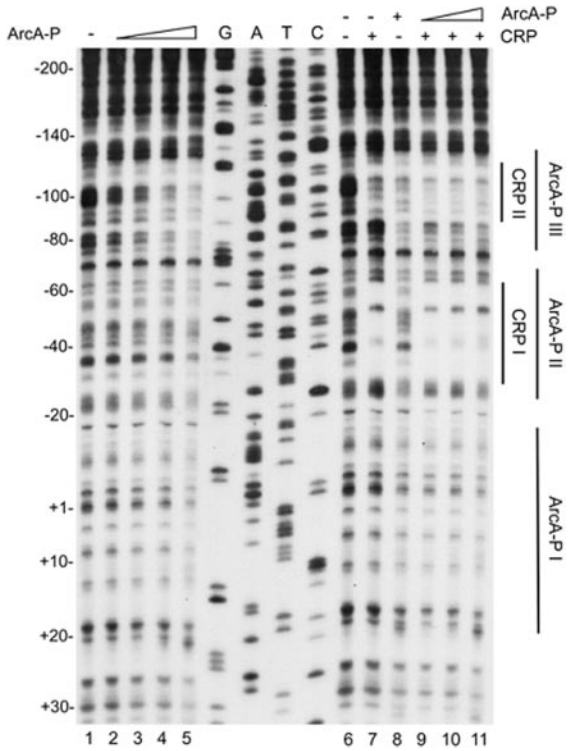

FIG. 6. **DNase I footprinting analysis of ArcA binding to the *ptsG* promoter.** End-labeled *ptsG* DNA fragment was incubated with phosphorylated ArcA and/or cAMP·CRP as indicated at the *top* of the figure. *Lane 1*, 0 μM; *lane 2*, 0.25 μM; *lanes 3* and *9*, 0.5 μM; *lanes 4, 8,* and *10*, 1.0 μM; *lanes 5* and *11*, 2.0 μM ArcA-P. Final concentration of CRP was 20 nM. The products were resolved on 7% polyacrylamide gel containing 8 M urea. The protection regions by the binding of the proteins are indicated with *vertical lines* on the *right side* of the figure.

DISCUSSION

In addition to sugar transport, protein components of the *E. coli* PTS have been shown to regulate many other metabolic activities (1, 2). Considering the importance of PTS as the central system for bacterial metabolism, the expression of PTS should be under highly complex regulation. It has been reported that the level of PTS expression increases about 3-fold during growth on PTS substrates (1). Factors thus far known to regulate PTS expression include CRP (1), Mlc (19–21), FruR (12), mRNA turnover rate (23), σ^{32} (22), and Fis (32).

Although many factors are known to regulate PTS expression, we tried to search for new protein factor(s) involved in the expression of the PTS proteins from *E. coli* extracts employing ligand fishing experiments by using the promoter regions of *ptsG* and the *pts* operon as baits in this study. As a result, we found that ArcA as well as CRP specifically binds to the two promoters (Fig. 1). ArcB and ArcA consist of a two-component signal transduction system that mediates adaptive responses of *E. coli* to changing redox conditions of growth. During respiration, oxidized forms of quinone electron carriers increase and act as direct negative signals that inhibit autophosphorylation of ArcB (30). When quinones are reduced by the depletion of electron acceptors, however, the ArcB sensor kinase autophosphorylates and then transphosphorylates ArcA, a global transcriptional regulator that controls the expression of numerous operons involved in respiratory or fermentative metabolism (30). Included in the ArcA regulon are mainly the factors involved in pathways generating cellular energy from sugar substrates: several dehydrogenases of the flavoprotein class, terminal oxidases, tricarboxylic acid cycle enzymes, enzymes of the glyoxylate shunt, enzymes of the pathway for fatty acid degradation, and enzymes involved in fermentative metabolism (33, 34). Because the PTS plays important roles in carbohydrate metabolism, we thought that ArcA might be involved in the regulation of PTS expression in response to the cellular redox status.

In agreement with the result of the fishing experiment, ArcA was shown to bind *in vitro* to the promoters of *ptsG* and the *pts* operon specifically (Fig. 2). Because the binding of ArcA to the promoter region of the *pts* operon was much weaker than to the

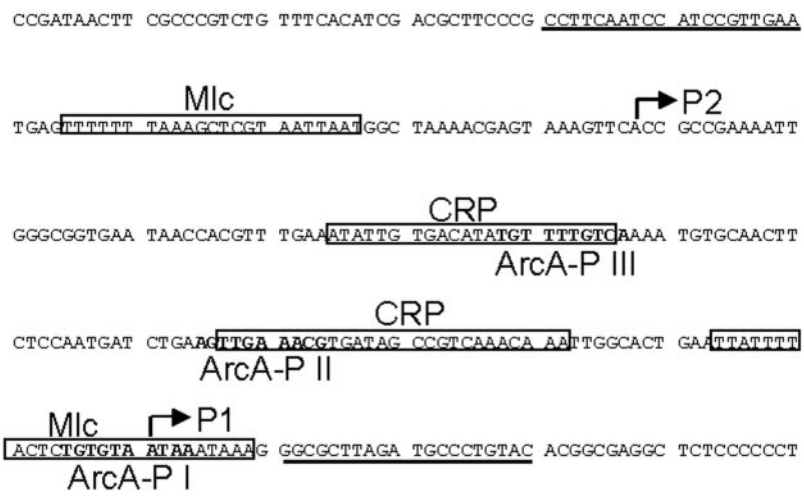

FIG. 7. **Organization of regulatory sites upstream of *ptsG*.** The *ptsG* gene is expressed from two promoters separated by 141 bp. P1 indicates the major promoter whose transcription is strongly dependent on the cAMP·CRP complex. The locations of the two Mlc-binding sites and the two CRP-binding sites are shown in *boxes.* Bases *underlined* correspond to the primers used to amplify the DNA for the ligand fishing and gel retardation assays carried out in this study as indicated under "Experimental Procedures." The sequences showing similarity with the consensus ArcA box (27) within the ArcA-P-mediated DNase I protection region obtained in Fig. 5 are in *boldface.*

ptsG promoter, we focused on the effect of ArcA on the *ptsG* expression in this study. The *lacZ* fusion data revealed that expression of *ptsG* is negatively regulated by ArcA, and this effect is independent of Mlc (Fig. 3). Furthermore, cells overproducing ArcA showed significantly retarded growth and color development on the MacConkey indicator plate containing glucose but not on the indicator plates with fructose or lactose compared with wild type, supporting that ArcA-P is the negative regulator of the *ptsG* expression (Fig. 4). The *in vitro* transcription assay confirmed that ArcA-P is the direct repressor of the *ptsG* gene (Fig. 5). Decreased transcription of *ptsG* and the *pts* operon was also observed in recent research examining the global gene expression profiling during the shift to anaerobiosis in *E. coli* (35), implying ArcA-P might be involved in this regulation.

Involvement of ArcA in the regulation of *ptsG* expression has never been addressed, although more than 30 genes have been identified as the ArcA-P modulon in *E. coli* (36). DNase I footprinting experiment in this study revealed that ArcA-P binds to three sites upstream of *ptsG* P1 promoter (Fig. 6). Binding to multiple sites with rather low affinity at regulated promoters seems to be characteristic of ArcA (36). Through the homology search of ArcA-P-protected promoter regions of more than 20 operons belonging to ArcA modulon, the ArcA-P binding consensus was suggested as 5′-(A/T)GTTAATTA(A/T)-3′ (27). The sequences showing similarity with the consensus ArcA box within the ArcA-P-mediated DNase I protection regions of the *ptsG* promoter were found (Fig. 7). The ArcA-P sites I–III have 6, 6, and 7 bases matching the 10-base ArcA box, respectively. These sites are comparable with the four distinct ArcA-P protected segments found in the *pflA* promoter region, which have 6–8 and 10 bases matching the ArcA box (27, 37). The ArcA-P sites II and III overlap the two CRP-binding sites, and ArcA-P and CRP compete with each other for the binding to the promoter (Figs. 6 and 7). It is assumed that the inhibitory effect of ArcA-P on the *ptsG* P1 transcription results from the binding of ArcA-P to the P1 promoter region (site I) and decreasing the CRP binding that is essential for the transcription.

Under certain conditions where the transport rate of electron acceptors does not meet the metabolic rate of sugars and thus the quinone electron carriers are more reduced, pathways to generate cellular energy from sugars might work less efficiently, and imported sugars might even be harmful to the cells by driving them to make more acidic products. The bacterial PTS plays many important roles for metabolic adaptation in changing environment in addition to the sugar transport (2). Thus, regulation of the appropriate intracellular level of $EIICB^{Glc}$ mediating the first step of the glycolytic pathway, leading to fermentation and/or the tricarboxylic acid cycle, seems to be one of the most important ways to compete with other organisms in the changing environments. Because ArcA-P has been known to control fermentation, the tricarboxylic acid cycle, the glyoxylate shunt, as well as many respiratory enzymes, regulation of the *ptsG* expression by ArcA-P seems to be a natural phenomenon.

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