A Novel Fermentation/Respiration Switch Protein Regulated by Enzyme IIA\textsuperscript{Glc} in Escherichia coli\textsuperscript{*}

Byoung-Mo Koo\textsuperscript{†}, Mi-Jeong Yoon\textsuperscript{†}, Chang-Ro Lee\textsuperscript{†}, Tae-Wook Nam\textsuperscript{†}, Young-Jun Choe\textsuperscript{†}, Howard Jaffe\textsuperscript{§}, Alan Peterkofsky\textsuperscript{∥}, and Yeong-Jae Seok\textsuperscript{‡‡}

From the \textsuperscript{†}Laboratory of Macromolecular Interactions, School of Biological Sciences and Institute of Microbiology, Seoul National University, Seoul 151-742, Korea, the \textsuperscript{§}NINDS, National Institutes of Health, Bethesda, Maryland 20892, and the \textsuperscript{∥}Laboratory of Cell Biology, NHLBI, National Institutes of Health, Bethesda, Maryland 20892

The bacterial phosphoenolpyruvate:sugar phosphotransferase system regulates a variety of physiological processes as well as effecting sugar transport. The crr gene product (enzyme IIA\textsuperscript{Glc} (IIA\textsuperscript{Glc})) mediates some of these regulatory phenomena. In this report, we characterize a novel IIA\textsuperscript{Glc}-binding protein from Escherichia coli extracts, discovered using ligand-fishing with surface plasmon resonance spectroscopy. This protein, which we named FrsA (fermentation/respiration switch protein), is the 47-kDa product of the yafA gene, previously denoted as “function unknown.” FrsA forms a 1:1 complex specifically with the unphosphorylated form of IIA\textsuperscript{Glc}, with the highest affinity of any protein thus far shown to interact with IIA\textsuperscript{Glc}. Orthologs of FrsA have been found to exist only in facultative anaerobes belonging to the \gamma-proteobacterial group. Disruption of frsA increased cellular respiration on several sugars including glucose, while increased FrsA expression resulted in an increased fermentation rate on these sugars with the concomitant accumulation of mixed-acid fermentation products. These results suggest that IIA\textsuperscript{Glc} regulates the flux between respiration and fermentation pathways by sensing the available sugar species via a phosphorylation state-dependent interaction with FrsA.

The bacterial phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) plays an important role in the transport of a variety of sugar substrates. This system catalyzes phosphorylation coupled to translocation of numerous simple sugars across the cytoplasmic membrane. The PTS is composed of two general cytoplasmic proteins, enzyme I (EI), and histidine phosphocarrier protein (HPr), which are used for all sugars, and, in addition, some sugar-specific components collectively known as enzymes II (1). The glucose-specific enzyme II of Escherichia coli consists of two components: soluble enzyme IIA\textsuperscript{Glc} (IIA\textsuperscript{Glc}) and membrane-bound enzyme IICB\textsuperscript{Glc} (IICB\textsuperscript{Glc}). Thus, glucose transport in E. coli involves three soluble PTS components (EI, HPr, and IIA\textsuperscript{Glc}, encoded by the ptsHcrr operon) and one membrane-bound protein, enzyme IICB\textsuperscript{Glc} (encoded by the ptsG gene). Glucose uptake entails sequential phosphoryl transfer via the PTS, as follows: phosphoenolpyruvate (PEP) $\rightarrow$ EI $\rightarrow$ HPr $\rightarrow$ IIA\textsuperscript{Glc} $\rightarrow$ IICB\textsuperscript{Glc} $\rightarrow$ glucose.

Components of the PTS also participate in several regulatory mechanisms (1). Catabolite repression allows for the preferential utilization of sugars transported by the PTS. Consequently, when E. coli are cultured in a medium containing both glucose and a non-PTS sugar, the glucose is consumed first. The currently accepted mechanism for this effect is that, when PTS sugars are transported, the steady-state condition of IIA\textsuperscript{Glc} is mainly in the dephospho-form. The unphosphorylated form of IIA\textsuperscript{Glc} inhibits transport of non-PTS sugars such as lactose, maltose, melibiose, and raffinose by interacting with transporters for these sugars (a process termed inducer exclusion) (1, 2–4). Other allosteric regulatory functions of IIA\textsuperscript{Glc} include inhibition of the phosphorylation of glyceraldehyde3-phosphate dehydrogenase (5) and either inhibition or activation of adenyl cyclase (6).

E. coli IIA\textsuperscript{Glc} is a protein with a $M_\text{r}$, predicted from its amino acid sequence, of 18,250 and which is phosphorylated by P-HPr at the N-3 position of His-90. In E. coli, the level of phosphorylated IIA\textsuperscript{Glc} reflects not only the availability of extracellular glucose but also the intracellular ratio of [PEP] to [pyruvate] (7). In addition to the regulatory roles of IIA\textsuperscript{Glc} listed above, we thought that there might be regulation of other physiological activities by IIA\textsuperscript{Glc} in E. coli. This perspective stimulated us to embark on ligand-fishing experiments aimed at detecting other soluble protein(s) exhibiting high affinity binding to IIA\textsuperscript{Glc}. In our previous study, employing ligand-fishing in E. coli extracts with surface plasmon resonance spectroscopy and immobilized HPr as the bait, we discovered a high affinity binding of glycogen phosphorylase to HPr; thus, HPr mediates cross-talk between sugar uptake through the PTS and glycogen breakdown (8–10). In this study, ligand-fishing, using surface plasmon resonance spectroscopy, was also employed to search for a high affinity binding of IIA\textsuperscript{Glc} to a protein in E. coli extracts. Consequently, we discovered a new IIA\textsuperscript{Glc}-binding protein in E. coli extracts. The protein was purified and established to be the previously uncharacterized yafA gene product, which we now refer to as a fermentation/respiration switch protein (FrsA). This report also describes experiments aimed at deducing the locus of action of FrsA.

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\textsuperscript{†}To whom correspondence should be addressed. Tel.: 82-2-880-8827; Fax: 82-2-888-4911; E-mail: yjeok@plaza.snu.ac.kr.

\textsuperscript{§}The abbreviations used are: PEP, phosphoenolpyruvate; PTS, phosphoenolpyruvate:sugar phosphotransferase system; EI, enzyme I of the PTS; IIA\textsuperscript{Glc}, the glucose-specific enzyme II of the PTS; HPr, histidine phosphocarrier protein; FrsA, a novel fermentation/respiration switch protein interacting with IIA\textsuperscript{Glc} described in this work; SPR, surface plasmon resonance; LC, liquid chromatography; MS, mass spectrometry; BSA, bovine serum albumin; PM, phenotype microarray.

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EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Growth—The bacterial strains and plasmids used in this study are listed in Table I. Luria-Bertani (LB) medium was used for routine bacterial growth except with strain G1698 and its derivatives. M9 salts-based rich medium (supplemented with 2% casamino acids and 1% glycerol) was used for routine culture of G1698 and its derivatives. DY330, G1698, and their derivatives were grown at 30 °C, while other strains listed in Table I were grown at 37 °C. For the overproduction of proteins using G1698 and the pRE1-based vector system, cells were grown in M9 salts-based induction medium (0.2% casamino acids, 1% glycerol, and M9 salts) at 30 °C. Tryptophan (final concentration: 100 μg/ml) was added to the culture medium when the culture reached an A600 of 0.4, and cells were harvested 18–20 h after induction. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 20 μg/ml; chloramphenicol, 30 μg/ml. To construct pKY103, the DNA sequence from nucleotide 3227 to 4541 of the E. coli frsA gene (GI:2367098) was amplified by PCR, using mutagenic primers to create an Ndel site (underlined) at the ATG start codon. The PCR product, digested with Ndel and BamHI, was cloned into the vector pRE1 (11), resulting in the recombinant plasmid pKY103 for FrsA overproduction.

To construct pKY13, in which FrsA expression is under the control of its own promoter, the sequence covering its own promoter and coding regions was amplified by PCR using mutagenic primers to create an Eagl site (underlined) at the ATG start codon (5′-TCTGGAGGCTGCACATATGCACAGGCAAA-3′) and a BamHI site (underlined) 39 nucleotides downstream from the TAA stop codon (5′-TATCTCCTGTTGGATCCAGCTACACTTC-3′). The internal Ndel site located at codons 186 and 187 was removed by mutagenesis (CATATG to CACATG) so that this did not result in any amino acid changes. The PCR product, digested with Ndel and BamHI, was cloned into the vector pRE1 (11), resulting in the recombinant plasmid pKY103 for FrsA overproduction.

Measurement of Protein-Protein Interaction—The interaction of IIA\textsuperscript{Glc} and its binding factor was monitored by surface plasmon resonance (SPR) detection using a BIAcore 3000 (BIAcore AB) as described previously (13). IIAGlc and IIBGlc were separately immobilized onto the sensor chip at 1 ng/mm\textsuperscript{2}, the proteins IIAGlc and IIBGlc were a surface concentration of 1 ng/mm\textsuperscript{2}, the proteins IIAGlc and IIBGlc were allowed to flow over a sensor chip at 10 μl/min to allow the proteins to the matrix by a N-hydroxysuccinimide-N-ethyl-N-(3-diehydralylaminopropyl)-carbodiimide reaction (80 μl of mix). Unreacted N-hydroxysuccinimide was inactivated by injecting 80 μl of 1 μM ethanolamine HCl, pH 8.0. Assuming that 1000 resonance units correspond to a surface concentration of 1 ng/mm\textsuperscript{2}, the proteins IIAGlc and IIBGlc were immobilized to a surface concentration of 1.5 and 1.8 ng/mm\textsuperscript{2}, respectively. The standard running buffer was 10 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM KCl, 1 mM MgCl\textsubscript{2}, and 1 mM dithiothreitol, and all reagents were added at a flow rate of 10 μl/min. The sensor surface was regenerated between assays by injecting 10 μl of 2 M NaCl to remove bound analyte.

To examine the \textit{in vivo} interaction between IIA\textsuperscript{Glc} and FrsA, G1698 cells harboring pKY103 expressing FrsA or pKY103H expressing His-

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**Table I**

**Bacterial strains and plasmids used**

| Strains | Relevant genotype or phenotype | Source or Ref. |
|---------|--------------------------------|---------------|
| MG1655  | Wild type E. coli              | 32            |
| DY330   | MC1050 ΔlacZ169 galD490 Δcya875 (Δcro-bioA) | 17            |
| G1698   | F′ lacI5 lacZ857 proAB plasmid CPCR-1008 | 33            |
| G1698pts | ΔptsH, ptsI, crr, K\textsuperscript{+} | 34            |
| SR702ΔptsG | araD139 SgrF-lacU1696psP150 thiA1 relA1 fbb3801 deco1 ctsE25 rbsR suhX1 | Gift from S. R. Ryu |

FrsA were grown in 200 ml of M9 salts-based rich medium to an A\textsubscript{600} of 1.5, harvested, and washed with 30 ml of 20 mM HEPES buffer, pH 8.0, containing 100 mM NaCl. The cell pellets were resuspended in 3 ml of the buffer in the presence of 500 μM phenylmethylsulfonyl fluoride. The cell suspension was disrupted in a French pressure cell and centrifuged at 100,000 g for 30 min at 4 °C, and the supernatant was used as the crude extract. The crude extract was incubated with 200 μl of BD TALON\textsuperscript{TM} resin. After the mixture was loaded onto a Poly-Prep chromatography column (8 × 40 mm) (Bio-Rad), the column was washed twice with the wash buffer (20 mM HEPES, pH 7.0, with 300 mM NaCl) containing 10 mM imidazole. The proteins bound to the column were eluted with the wash buffer containing 200 mM imidazole and analyzed by SDS-PAGE followed by staining with Coomassie Blue and Western blotting using anti-IIA\textsuperscript{Glc} serum raised in mice.

**Identification of Coomassie Blue-stained Bands**—Coomassie Blue-stained gel bands were excised and subjected to in-gel proteolytic digestion with trypsin according to the method of Moritz et al. (13). Digests were subjected to LC-MS analysis on a previously described (14) Michrom Biore sources Magic 2002 model microhore high performance liquid chromatography coupled to a model LCQ ion trap mass spectrometer (Finnigan) equipped with an electrospray interface utilizing a 0.3 × 150 mm Magic MS C18 column (Michrom Biore sources) eluted at 8 μl/min and 40 °C with a linear gradient of 2–65% Solvent B over 30 min. Solvent A was 10/10/980/1/0.005 CH\textsubscript{3}CN/1-PrOH/H\textsubscript{2}O/acetic acid/ (v/v/v/v). The mass spectrometer was operated in the “Top Five” mode in which the instrument was set up to automatically acquire (a) a full scan between 300 and 600 mass units to allow for acylamidation of cysteine and enzyme specificity was set to “no enzyme.” Fragment ions were labeled using the Bioworks software.
Interaction of Enzyme IIA<sup>Glc</sup> with FrsA

RESULTS

Immobilized IIA<sup>Glc</sup> Complexes with a Factor in E. coli Extracts—The technique of ligand-fishing by SPR (8) was used to search for a protein that might interact with and be regulated by IIA<sup>Glc</sup>. SPR detects a change in refractive index resulting from the interaction of a soluble protein with another protein covalently linked to a surface. IIA<sup>Glc</sup>, immobilized on a CM-5 sensor chip (see “Experimental Procedures”), was used as the bait. To minimize nonspecific interactions between the crude extract and IIA<sup>Glc</sup>, a cytoplasmic crude extract from a stationary phase culture of an E. coli strain (TP2811) carrying a deletion of the pts operon (see “Experimental Procedures”) (19) was chromatographed through a Mono Q 10/10 column (Amersham Biosciences) using a linear gradient of 50–500 mM NaCl (80 ml total volume). Each fraction was allowed to flow over the immobilized IIA<sup>Glc</sup> for 10 min; we found that fractions eluting near 100 mM NaCl showed a detectable increase in SPR response (Fig. 1A).

Since IIA<sup>Glc</sup> interacts with glycerol kinase, it seemed possible that the detected interaction might be due to formation of the glycerol kinase-IIA<sup>Glc</sup> complex. However, when purified glycerol kinase (120 μl of 10 μg/ml protein) was allowed to flow over the immobilized IIA<sup>Glc</sup> in the absence of Zn<sup>2+</sup> (20 for 10 min, no interaction was detectable (data not shown). Furthermore, because the ptsH<sup>lcr</sup> operon is deleted in strain TP2811 (19), the possibility of interaction of HPr and IIA<sup>Glc</sup> was also excluded. This result prompted us to purify this new IIA<sup>Glc</sup>-binding protein.

Purification of the Factor Interacting with IIA<sup>Glc</sup>—The approach to purification of the factor that interacts with IIA<sup>Glc</sup> was based on SPR analysis; fractions from each purification step were examined for binding to immobilized IIA<sup>Glc</sup> using the Biacore (Fig. 1). E. coli strain TP2811 was grown to stationary phase in 6 liters of Luria broth at 37 °C. The harvested cell pellet was washed and resuspended in buffer A (10 mM Tris-HCl, pH 7.5, containing 50 mM NaCl); this suspension was passed three times through a French pressure cell at 10,000 p.s.i. The lysate was centrifuged at 100,000 × g for 90 min to obtain the soluble cytoplasmic fraction. This fraction was chromatographed through a DEAE-Sephadex (Sigma) column (8 × 30 cm) using a linear gradient of 50–500 mM NaCl (2 liters total volume). Fractions eluting around 100 mM NaCl demonstrated interaction with immobilized IIA<sup>Glc</sup>; these fractions were pooled and concentrated in a 3 K Macrosep centrifugal concentrator (Pall Gelman Laboratory). The concentrated pool was further purified on a hydroxyapatite (Bio-Rad) column (1.5 × 10 cm) using a linear gradient of 0–500 mM potassium phosphate buffer, pH 7.5 (80 ml total volume). Fractions eluting around 100 mM potassium phosphate demonstrated interaction with immobilized IIA<sup>Glc</sup>, and these fractions were pooled and concentrated as described above. The concentrated pool was chromatographed on a Superdex 75 column (Amersham Biosciences) equilibrated with buffer A. Once again, the eluted fractions were analyzed using SPR, and fractions showing affinity toward immobilized IIA<sup>Glc</sup> were pooled and concentrated as described for previous steps. As a final purification step, a Mono Q 5/5 column (Amersham Biosciences) was used with a 15-volume NaCl gradient (50–300 mM) in buffer A. The fractions eluting near 100 mM NaCl, showing interaction with IIA<sup>Glc</sup>, were pooled, and the protein was precipitated by adding acetone to a final concentration of 80% after BSA (1 mg/ml) was added to assist in recovery.

The IIA<sup>Glc</sup>-binding Protein Is the yapA Gene Product—The precipitated proteins were solubilized in SDS-loading buffer and subjected to SDS-PAGE. Staining of the gel with Coomassie Blue revealed a protein band migrating with an apparent molecular...
mass of 47 kDa in addition to a band corresponding to BSA. The bands in the vicinity of the 47-kDa protein were cut out of the gel and subjected to in-gel proteolytic digestion. Digests were analyzed by LC/MS/MS (see “Experimental Procedures”) to identify proteins. Protein in the slower migrating (presumptive BSA) band was identified as BSA on the basis of the following tryptic peptides: (K)SEIAHR, (K)DLGEEHFK, (R)FKDLGEEHFK, (K)HLVDEPQNLK, (R)KVPQVSTPTLVEVSR, and (K)TESGELHGLTTEDK. Protein in the faster migrating band was identified as a mixture of BSA on the basis of the following tryptic peptides: (R)LGMHDASDEALR, (K)GDDLAEQAQALSNNR, (R)VAAFGFR, (R)TDDDLYDVTGYR, and (M)TQANLSETLFKPR (see Fig. 1B).

On the basis of the sequence analyses, we concluded that the BSA sequence was a carryover from the added protein (see above) and that the YAFA_ECOLI sequence was likely to correspond to the IIA^{Glc}-binding factor. The correspondence of the apparent molecular mass by SDS-PAGE of the IIA^{Glc}-binding protein with the calculated molecular mass of YAFA_ECOLI (47,008 Da) was consistent with this prediction.

**Overexpression and Purification of the frsA Gene Product**—To further explore the possibility that the IIA^{Glc}-binding protein corresponded to the product (YAFA_ECOLI) of the yafA gene (we refer to yafA as frsA, since we report here that its gene product (FrsA) acts as a fermentation/respiration switch), the gene was cloned into an expression vector so that sizeable amounts of the pure protein could be produced. For overexpression of FrsA, we constructed the pRE1-based recombinant plasmid, pKY103. In this plasmid, genes are under the control of the strong λP1 promotor-cII ribosome binding site combination (11, 15). *E. coli* GI698, transformed with the pKY103 recombinant plasmid, was used for overexpression of the gene product. This strain was grown as described under “Experimental Procedures,” and the overexpressed protein with molecular mass

Fig. 1. Purification of the factor interacting with IIA^{Glc}. A, SPR analysis of fractions showing interaction with immobilized IIA^{Glc}. E. coli TP2811 cell extract was fractionated by Mono Q 10/10 chromatography. An aliquot of each fraction was allowed to flow over the IIA^{Glc} surface to test for binding as described under “Experimental Procedures.” Solid bars indicate the signal from the IIA^{Glc} immobilized surface. The solid line shows the salt gradient. Fraction 13 corresponds to the portion of the salt gradient containing about 100 mM NaCl. B, the scheme of purification and identification of IIA^{Glc}-binding protein. Full-scale MS/MS spectrum of the doubly charged ion was obtained at *m/z* 753.3. The spectrum was analyzed by the SEQUEST program to give the N-terminal tryptic peptide TQANLSETLFKPR. Observed y and b ions, including a continuous y2–y11 series and b2, b4–b7, b10, and b11 ions, consistent with those predicted for the peptide, are labeled.
FrsA, the cellular concentration of the IIA Glc-binding protein

Generally, the regulatory functions of the PTS a Heterodimer electrophoresis (Fig. 2). To phosphorylate immobilized IIAGlc, affinity for its binding protein was checked by SPR and native gel eluted as a symmetrical peak at 59 ml, corresponding to the monomeric form (47 kDa), while isolated IIAGlc eluted at 61 ml (Fig. 3A). When IIAGlc and FrsA (4 mg each) were mixed in a buffer (20 mm Tris-HCl buffer, pH 7.5, with 50 mm NaCl) and loaded onto the column, a unique peak eluted at 55 ml (Fig. 3A). When fractions (18–20) corresponding to this peak were run on a SDS-PAGE gel, they resolved into two bands migrating at the same elution volume, suggesting that the complex was stable even after its dilution resulting from the two successive gel filtration chromatography runs (data not shown). Based on the elution profile of the complex through the gel filtration column and the band intensity on SDS-PAGE of the corresponding fractions, it appears that IIAGlc interacts with FrsA in a one-to-one ratio to form a heterodimer.

Fig. 2. Specific interaction between FrsA and unphosphorylated IIAGlc. A, interaction of purified FrsA with immobilized unphosphorylated or phosphorylated IIAGlc. SPR analysis was carried out as described under “Experimental Procedures.” Phosphorylated IIAGlc was formed by passing a solution containing PEP, EI, and HPβ over a surface of immobilized IIAGlc. Significant interaction signal was obtained when FrsA was allowed to flow over the dephospho-form of IIAGlc (sensogram a), but not with the phosphorylated form of IIAGlc (sensogram b). The arrows indicate the starting points of injections. B, electrophoretic mobility shift of IIAGlc complexed with FrsA. The specificity of interaction between FrsA and the unphosphorylated form of IIAGlc was tested on a nondenaturing PAGE gel. Lane 1, FrsA; lane 2, IIA Glc; lane 3, FrsA; lane 4, FrsA + IIA Glc; lane 5, HPβ + FrsA; lane 6, IIβGlc + FrsA; lane 7, IIAGlc + FrsA; lane 8, EI + HPβ + IIAGlc + FrsA + PEP (phosphorylated form of IIA Glc); lane 9, IIAGlc; lane 10, HPβ.

about 47 kDa corresponded to about 30% of the total cell protein after induction. After sequential purification steps, as described under “Experimental Procedures,” ~98% pure protein was obtained as judged by SDS-PAGE (data not shown). The purified protein bound to immobilized IIAGlc with high affinity, indicating that the product of the frsA gene is the IIA Glc-binding protein. The dissociation constant (Kd) for the interaction between IIA Glc and its binding protein, using BIAevaluation 2.1 software, was determined to be ~1.8 × 10^{-7} M, assuming interaction of the monomeric forms of both IIA Glc and its binding protein (data not shown). From the comparison of SPR response units of the IIAGlc-binding protein toward immobilized IIAGlc in Fig. 1 with that of the purified recombinant FrsA, the cellular concentration of the IIAGlc-binding protein was calculated to be about 0.27 μM in wild type cells.

FrsA Binds Specifically to Unphosphorylated IIA Glc to Form a Heterodimer—Generally, the regulatory functions of the PTS depend on the phosphorylation state of the involved protein (1). Accordingly, the effect of phosphorylation of IIA Glc on the affinity for its binding protein was checked by SPR and native gel electrophoresis (Fig. 2). To phosphorylate immobilized IIA Glc, PEP, EI, and HPβ were mixed and incubated for 20 min, and then this mixture was allowed to flow over the immobilized IIA Glc surface. When IIAGlc was phosphorylated, binding of the IIAGlc-binding factor to the immobilized IIAGlc surface was almost completely abolished (Fig. 2A, sensogram b) while there was significant binding to the unphosphorylated form of IIA Glc (Fig. 2A, sensogram a). The specificity of the binding was supported by the observation that FrsA did not bind to immobilized cloned IIβGlc (12). When EI, HPβ, or the soluble IIβGlc domain (4 μg/ml, respectively) was allowed to flow over a surface immobilized with FrsA, no signal was detected (data not shown).

The biomolecular interaction studies by SPR indicated a high affinity interaction of IIAGlc and its binding protein. It was therefore anticipated that such a stable complex might survive under electrophoretic conditions. Consequently, gel mobility shift experiments using non-denaturing polyacrylamide gel electrophoresis were carried out (Fig. 2B). The band corresponding to FrsA smeared just below the loading wells (lanes 3–6 in Fig. 2B) unless IIAGlc was co-loaded (lane 7). While FrsA significantly retarded the migration of IIAGlc in the gel (lane 7 in Fig. 2B), it did not lead to a mobility shift of EI, HPβ, or IIβGlc, consistent with the SPR data. When FrsA was run with IIAGlc under phosphorylating conditions (incubating the reaction mixture in the presence of EI, HPβ, and PEP before loading), the band corresponding to the complex between IIAGlc and FrsA was hardly visible (compare lanes 7 and 8 in Fig. 2B). These data confirm the previous observation that FrsA interacts specifically with IIAGlc and that this interaction depends on the phosphorylation state of IIA Glc.

The tight interaction between IIA Glc and FrsA was also confirmed by gel filtration analysis. The elution profile from a Superose 12 gel filtration column (25 × 500 mm) of the complex was compared with those of individual proteins. FrsA alone eluted as a symmetrical peak at 59 ml, corresponding to the monomeric form (47 kDa), while isolated IIAGlc eluted at 61 ml (Fig. 3A). When IIA Glc and FrsA (4 mg each) were mixed in a buffer (20 mm Tris-HCl buffer, pH 7.5, with 50 mm NaCl) and loaded onto the column, a unique peak eluted at 55 ml (Fig. 3A). When fractions (18–20) corresponding to this peak were run on a SDS-PAGE gel, they resolved into two bands migrating at the positions expected for IIA Glc and its binding protein (Fig. 3B). Fraction 21 corresponds to unbound IIA Glc. When the fractions (19 and 20) corresponding to the complex peak were reloaded onto the same column, they eluted again as a single peak at the same elution volume, suggesting that the complex was stable even after its dilution resulting from the two successive gel filtration chromatography runs (data not shown). Based on the elution profile of the complex through the gel filtration column and the band intensity on SDS-PAGE of the corresponding fractions, it appears that IIA Glc interacts with FrsA in a one-to-one ratio to form a heterodimer.

Evidence for the in Vivo Interaction between IIA Glc and FrsA—The in vitro experiments described above demonstrated a phosphorylation state-dependent high affinity interaction of IIA Glc with FrsA. To examine whether formation of the direct complex occurs in vivo, FrsA with N-terminal 6 histidine residues (His-FrsA) was expressed in cells lacking the chromosomal frsA gene. The crude extract prepared from these cells was subjected to a pull-down assay using BD TALON™ resin (Clontech). As shown in Fig. 4, IIA Glc, but not its phospho-form, was co-eluted with His-FrsA bound to the beads, although about 50% of total IIA Glc existed in its phosphorylated form as judged from Western blot analysis, while no IIA Glc was recovered from the bound fraction when the crude extract was prepared from cells expressing unmodified FrsA. These results provide confirmation that the phosphorylation state-dependent interaction between FrsA and IIA Glc also occurs in vivo.

Phenotype Microarray Analysis Indicates That Deletion of frsA Increases Cell Respiration Rate on Several Sugars—To assay the effects of frsA disruption on cell respiration, phenotype analysis was carried out using phenotype microarrays

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(PMs) (Biolog, Inc.). This technique involves the deduction of cellular phenotypes using cell respiration as the reporter system. The assay chemistry uses a tetrazolium dye to colorimetrically detect the respiration of cells. Reduction of this dye due to cell respiration during growth results in formation of a purple color, and it accumulates in the well over the incubation period; the data (see Fig. 5) are presented as a plot of the respiration over time (21, 22). Therefore, the more respiratory activity growing cells have, the more purple color should accumulate. Total loss of function will result in no growth and no purple color. Therefore, the colorimetric assay of respiration can provide a virtually universal reporter system for phenomic testing.

PM tests were performed on BM101, compared with the control strain MG1655, in a set of 20 96-well microplates containing different nutrients or inhibitors; this allowed measurement of cell respiration to examine nearly 2000 cellular phenotypes in a sensitive, highly controlled, reproducible format (further extensive information concerning PM technology can be obtained at www.biolog.com). The results indicated that BM101 showed faster purple color development using carbon sources such as fructose, arabinose, glucose, and lactose than its parental strain (MG1655), while there was no significant change in

Fig. 3. Gel filtration chromatography of IIA_Glc, FrsA, and the IIA_Glc-FrsA complex. Samples were preincubated and injected on a Superose 12 column as described under “Experimental Procedures.” A, the three chromatograms were obtained separately and two were superimposed to compare the elution profiles. The dashed line corresponds to injection of FrsA alone (4 mg), and the solid line corresponds to the mixture of IIA_Glc and FrsA (4 mg each). The chromatogram for IIA_Glc was not included because it showed little absorbance at 280 nm. Arrows indicate elution volumes of molecular weight markers used to calibrate the column: arrow 1, RNase (14.7 kDa); arrow 2, chymotrypsinogen (20.2 kDa); arrow 3, ovalbumin (47.2 kDa); and arrow 4, albumin (61.6 kDa). B, Coomassie Brilliant Blue-stained SDS-PAGE gel loaded with gel filtration chromatography fractions (3 ml for each fraction) of the IIA_Glc-FrsA mixtures. Lane M indicates Mark 12™ protein molecular mass standards (Invitrogen), and numbered lanes indicate fractions from gel filtration chromatography of the mixture. Molecular masses (in kDa) of some standards are presented on the left side.

Fig. 4. In vivo interaction between dephospho-IIA_Glc and FrsA. GI6983/frsA cells harboring pKY103 expressing FrsA or pKY103H expressing His-FrsA were grown as described under “Experimental Procedures.” The cell suspension disrupted in a French pressure cell was centrifuged, and the supernatant was mixed with BD TALON™ resin. The mixture was loaded on a column (Bio-Rad) and the proteins bound to the resin were eluted with 200 mM imidazole and analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue (A) and Western blotting using anti-IIA_Glc serum raised in a mouse (B). Lane 1, crude extract of GI6983/frsA/pKY103; lane 2, crude extract of GI6983/frsA/pKY103H; lane 3, eluate of crude extract of GI6983/frsA/pKY103 from the resin; and lane 4, eluate of crude extract of GI6983/frsA/pKY103H from the resin. Molecular masses (in kDa) of some standards are presented on the left side.

Fig. 5. Phenotype microarray comparison of MG1655 and BM101. Significant changes (more than 60% difference compared with MG1655) are enclosed in boxes and indicated by arrows. Yellow, red, and green colors indicate similar growth of the wild type and mutant, faster growth of the wild type, and faster growth of the mutant, respectively. Growth was analyzed by Biolog, Inc. Detailed information of PM experiments is available at www.biolog.com.
other phenotypes. From these studies, it was apparent that, out of about 2000 tests, an increase in respiration rate on several sugars is the only phenotype detected due to the deletion of frsA (Fig. 5).

To check whether episomal frsA expression can complement the deletion phenotype, an expression plasmid (pKY513) in which frsA expression is under the control of its own promoter was constructed and transformed into BM101. To construct pKY513, the sequence from 432 bp upstream of the frsA start codon to 39 bp downstream of the stop codon was amplified by PCR and cloned into pBR322. The expression level of FrsA from this plasmid was determined to be about 5-fold greater than wild type but still much less than that of IIAGlc by Western blot analysis (data not shown). To confirm the phenotype microarray data, we carried out phenotype tests of MG1655, BM101, and BM101/pKY513 using GN2 microplates (Biolog, Inc.) containing 96 different carbon sources. Although carbon catabolic activities of the three tested strains showed no significant differences, the rate of color development was different from strain to strain. BM101 showed faster purple color development using the carbon sources fructose, arabinose, glucose, lactose, and mannitol as expected from the PM test described above. It is worth noting that introduction of pKY513 into the BM101 strain resulted in the same phenotype as the wild type (MG1655), indicating that the increased respiration rate of BM101 on several sugars was correlated with deletion of the frsA gene from MG1655 (data not shown).

**FrsA Increases Accumulation of Fermentation Products**—From repeated measurements of growth and glucose consumption, we found that the frsA strain (BM101) harboring pKY513 consumed glucose slightly faster than the wild type (MG1655) or BM101 harboring pBR322, while MG1655 showed a slightly faster growth rate than the other strains in M9 medium containing glucose as the sole carbon source under aerobic culture conditions. Fig. 6 shows representative data of several independent reproducible experiments. Since either disruption or higher expression of FrsA does not affect the expression level of IIAGlc or IICBGlc (data not shown), these results suggest that FrsA might affect metabolic flux through the glycolytic pathway. It should also be noted that color changes in PM analyses do not always correlate with growth rate, because cell respiration is not necessarily coupled to cell growth (21).

To elucidate the reason for the discrepancy between growth and glucose utilization rates in these strains, we carried out $^1$H NMR spectroscopy of the growth medium after 12 h (Figs. 7, A and B). More acetate, ethanol, lactate, pyruvate, and succinate were accumulated in the glucose medium for BM101 harboring pKY513 than for MG1655 (Fig. 7A). BM101, in which the frsA gene is deleted, produced lower amounts of these fermentation products than did the wild type. In the growth medium containing fructose as the sole carbon source, production of acetate, ethanol, formate, and lactate increased in strain BM101 harboring pKY513 (Fig. 7B).

Enzymatic analyses of the concentration of acetate in 20 h growth medium also confirmed these data (Fig. 7C). The concentration of acetate from BM101 harboring pKY513 was significantly higher than MG1655 or BM101 harboring pBR322 in synthetic medium containing all the indicated carbon sources except for glycerol. These results suggest that FrsA stimulates mixed-acid fermentation in *E. coli* by acting as a fermentation/respiration switch that down-regulates respiration and up-regulates fermentation rates.

**DISCUSSION**

Each protein component of the *E. coli* PTS involved in glucose uptake has been shown to regulate the activity of a partner protein by a direct protein-protein interaction. These regulatory functions of the PTS depend on the phosphorylation state of the involved component; the phosphorylation state of PTS proteins has been shown to increase in the absence and decrease in the presence of a PTS sugar substrate. The ratio of phosphorylated to dephosphorylated proteins in turn serves as signal input for the control of these physiological processes. Unphosphorylated EI interacts with and regulates the auto-phosphorylation activity of CheA to trigger chemotaxis toward PTS carbohydrates (23). Unphosphorylated IIAGlc inhibits glycogen phosphorylase and several sugar permeases by a mechanism termed inducer exclusion (1, 5). Phosphorylated IIAGlc stimulates CAMP synthesis (6). Unphosphorylated HPr interacts with and stimulates the activity of glycogen phosphorylase *in vitro* (8, 10) and in *vivo* (9). More recently, it was also discovered that the membrane-bound glucose transporter enzyme IICBgc interacts with Mlc to modulate the cellular localization and activity of the global repressor protein Mlc in a phosphorylation state-dependent manner (12, 24, 25).

We have been interested in searching for other physiological activities exerted by the PTS in *E. coli*. Consequently, we performed ligand-fishing experiments aimed at detecting soluble protein(s) exhibiting binding to IIAGlc with a higher affinity than to glyceral kinase (26). The same methodology, employing SPR, as used before to demonstrate the high affinity interaction of HPr with glycogen phosphorylase (8), was followed. Our preliminary data showed that the interaction between glyceral kinase and IIAGlc was detectable by SPR only in the presence of Zn$^{2+}$ (data not shown). This result is supported by the previous report that Zn$^{2+}$ promoted the association between glyceral kinase and IIAGlc (20). Furthermore, it is worth noting that no interaction was detectable when purified EI was exposed to

**FIG. 6.** Growth rate-dependent glucose consumption of MG1655 and its derivatives. Growth rates (A) and the amount of glucose remaining in the medium (B) are recorded as a function of incubation time. Cells were grown aerobically with shaking at 200 rpm in M9 minimal medium supplemented with 0.2% casamino acids and 0.5% glucose at 37°C. This experiment was repeated several times and the growth profiles were reproducible. Representative data are shown here. *Squares*, MG1655/pBR322; *circles*, BM101/pBR322; *triangles*, BM101/pKY513.
immobilized HPr in the BIACore in our previous study (8) or when purified HPr or IIBGlc were exposed to the IIA\textsuperscript{Glc} surface in this study (data not shown), although the association constants were reported to be \(-1.5 \times 10^5 \text{ M}^{-1}\) between EI and HPr (27) and \(-10^8 \text{ M}^{-1}\) between HPr and IIA\textsuperscript{Glc} (28) and between IIA\textsuperscript{Glc} and IIBGlc (29). We concluded that it was unlikely that an interaction with a \(K_a\) in this range would be detected by SPR under our experimental conditions.

The search for IIA\textsuperscript{Glc}-interacting protein(s) employing ligand-fishing by SPR in the absence of Zn\textsuperscript{2+} ion turned up a protein with the expected tight binding; after purification to near homogeneity, sequencing experiments identified the novel IIA\textsuperscript{Glc}-interacting protein as the product specified by the \(yfA\) gene, which we now refer to as \(frsA\) (Fig. 1B). This gene was previously only characterized as encoding a hypothetical protein of unknown function. Sequence comparison of the protein with other proteins (HPr, IIBGlc, glycerol kinase, lactose permease, and melibiose permease) interacting with IIA\textsuperscript{Glc} revealed no homology.

We cloned the \(frsA\) gene under the control of the \(\lambda\) promoter to produce the expression vector, pKY103. Consequently, FrsA could be overexpressed to about 30% of the total cell protein. Purification of the overexpressed protein, using a combination of several chromatographic procedures, resulted in more than 98% pure protein (data not shown). The interaction between FRSa and IIA\textsuperscript{Glc} was dependent on the phosphorylation state of IIA\textsuperscript{Glc}, and only the dephospho-form of IIA\textsuperscript{Glc} showed an interaction with FRSa, both in vitro and in vivo (Figs. 2–4).

A BLAST search revealed that orthologs of FRSa exist only in some Gram-negative bacteria such as \textit{E. coli}, \textit{Salmonella typhimurium}, \textit{Shigella flexneri}, \textit{Yersinia pestis}, \textit{Vibrio cholerae}, \textit{Vibrio vulnificus}, \textit{Vibrio parahaemolyticus}, and \textit{Photobacterium luminescens} (amino acid sequences at www.ncbi.nlm.nih.gov). It is worthy of note that all of these species are facultative anaerobes belonging to the \(\gamma\)-proteobacterial group, and most of them are highly pathogenic. FRSa has been referred to as a hypothetical protein and studies related to this protein have not previously been reported. Thus, elucidation of the function of FRSa might help us understand the physiology of facultative anaerobiosis and pathogenesis in these strains. A BLAST conserved domain search suggested that FRSa might be an enzyme of the \(\alpha/\beta\) hydrolase family, which includes dienlactone hydrolase, dipetidyl aminopeptidase, and acetyl esterase. With purified FRSa, however, we could not show any of these enzymatic activities nor find the consensus sequence patterns for these enzymes from the FRSa amino acid sequence.

It is noteworthy that the cellular concentration of FRSa is about 0.27 \(\mu\)M while that of IIA\textsuperscript{Glc} is about 40 \(\mu\)M (30). Since there is a tight interaction of the two proteins, it is likely that all of the cellular FRSa protein exists in a complex with IIA\textsuperscript{Glc} when PTS sugars are being transported (the condition under which the PTS proteins are mainly in their dephospho-forms). Thus we assumed that it probably interfaces with the status of phosphorylation of the PTS.

For facultative anaerobes like \textit{E. coli}, fine tuning between respiratory and fermentation pathways may be important for more efficient utilization of sugars available in the environment. To test the physiological importance of the interaction between FRSa and IIA\textsuperscript{Glc}, we prepared \textit{E. coli} strains with different genotypes. Since the precise function of FRSa has not been elucidated and its orthologs are found only in facultative anaerobes, we carried out PM experiments. This technology can be used to find new functions of genes by testing mutants for a large number of phenotypes simultaneously using cell respiration as the reporter system (22). Based on phenotype microarray experiments (Fig. 5), it was found that disruption of \(frsA\) resulted in an increase of cellular respiration in medium containing glucose or some other sugars as the sole carbon
source compared with wild type; introduction of pKY513 into
the BM101 strain resulted in the same phenotype as the wild
type MG1655, indicating that increased respiration rate of
BM101 on several sugars was correlated with deletion of the
frsA gene from MG1655 (data not shown). NMR spectroscopic
and enzymatic analyses showed that more fermentation prod-
ucts are accumulated in the growth medium of the pKY513-
harborin strain than the BM101 and MG1655 strains despite
thorouigh aeration with atmospheric air (Fig. 7). Since all PTS
and several non-PTS sugars can also lower the phosphorylated
forms of the PTS proteins (7), the specific interaction between
dephosphorylated IIA^Glc and FrsA may regulate FrsA activity
to balance utilization of sugars through fermentative or respir-
atory pathways in Gram-negative bacteria.

Data in this study suggest that FrsA promotes fermentation
and that IIA^Glc complexes with it to negate the effect. A previous
study reported that the modulation in the synthesis of specific proteins during aerobic glucose starvation is similar to
the response of cells shifted to anaerobiosis in that the glucose-
starved cell increases the flow of metabolites through glycolysis
to fermentative pathways although the trigger mechanisms
could not be elucidated (31). The present studies may provide
the molecular mechanism for this phenomenon. When cells are
starved of glucose or other energy-rich carbon sources, IIA^Glc
will exist in its phospho-form, and the FrsA protein would be
depleted of IIA^Glc and thus be fully active. FrsA as a down-
regulator of respiration or up-regulator of fermentation should
make cells increase the flow of metabolites into fermentative
pathways. Increased excretion of acetate, lactate, pyruvate,
succinate, ethanol, and formate in cells harboring pKY513 supports
this model. Conservation of amino acid sequences of IIA^Glc
and FrsA in facultative anaerobes suggests that regulation
of the metabolic switch from respiration to fermentative
pathways during aerobic glucose starvation by IIA^Glc might be
a general mechanism in facultative anaerobes. Further experi-
ments will be necessary to define the precise metabolic reaction(s)
influenced by FrsA, subject to regulation by IIA^Glc.

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Interaction of Enzyme IIA^Glc with FrsA
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A Novel Fermentation/Respiration Switch Protein Regulated by Enzyme IIA<sub>Glc</sub> in <i>Escherichia coli</i>
Byoung-Mo Koo, Mi-Jeong Yoon, Chang-Ro Lee, Tae-Wook Nam, Young-Jun Choe, Howard Jaffe, Alan Peterkofsky and Yeong-Jae Seok

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