Adaptation in bacterial flagellar and motility systems: from regulon members to ‘foraging’-like behavior in E. coli

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ABSTRACT

Bacterial flagellar motility and chemotaxis help cells to reach the most favorable environments and to successfully compete with other micro-organisms in response to external stimuli. Escherichia coli is a motile gram-negative bacterium, and the flagellar regulon in E. coli is controlled by a master regulator FlhDC as well as a second regulator, flagellum-specific sigma factor, σF. To define the physiological role of these two regulators, we carried out transcription profiling experiments to identify, on a genome-wide basis, genes under the control of these two regulators. In addition, the synchronized pattern of increasing CRP activity causing increasing FlhDC expression with decreasing carbon source quality, together with the apparent coupling of motility activity with the activation of motility and chemotaxis genes in poor quality carbon sources, highlights the importance of CRP activation in allowing E. coli to devote progressively more of its limited reserves to search out better conditions. In adaptation to a variety of carbon sources, the motile bacteria carry out tactical responses by increasing flagellar operation but restricting costly flagellar synthesis, indicating its capability of strategically using the precious energy in nutrient-poor environments for maximizing survival.

INTRODUCTION

The DNA-dependent RNA polymerase of bacteria is the sole enzyme capable of producing messenger, transfer and ribosomal RNA by catalyzing the 5' to 3' synthesis of phosphodiester bonds between adjacent ribonucleoside triphosphates. In E. coli, core DNA-dependent RNA polymerase consists of four different subunits and has the composition α₂ββ'α₀. It was discovered that an additional and separable factor, the sigma factor (σF), was required for transcription of certain types of DNA (1). Numerous sigma factors have been described in E. coli and other prokaryotic organisms (2–5) since then. The seven known E. coli sigma factors are σ⁷₀, σ⁵₄, σ₃₂, σ₂₈, σ₁₅, σ₁₀ and σ⁹-/. The holoenzyme complex (Eσ) (1,6) constituted by core RNA polymerase (E) together with a sigma factor (σ) is required to initiate transcription at specific DNA sequences termed promoters.

Through the specificity of its σ subunit, holoenzyme is directed to two conserved DNA hexamers centered ~10 and 35bp upstream of the transcription start to initiate transcription. Each sigma factor recognizes and directs RNA polymerase to a different set of promoters. While sigma factors provide the primary interaction with those consensus two-block promoter DNA sequences and play a central role in the regulation of global gene expression, additional transcriptional activators such as FIS (7,8) and CRP (9,10) can be utilized to strengthen the promoter–holoenzyme interaction.

Flagellar, chemotaxis and motility genes are organized into large complex units. The genes that constitute the flagellar regulon are expressed in a cascade that closely parallels the assembly hierarchy of the flagellar structure (11,12). The flagellar operons are divided into three gene classes with respect to this transcriptional hierarchy (classes I, II and III or early, middle and late classes, respectively). At the top of the hierarchy is the FlhDC master operon that encodes FlhDC complex as a positive transcriptional activator of σF-dependent transcription from class II promoters which also include the fliA promoter. σF is made, binds to core RNA polymerase, and then transcribes from class III promoters. The synthesis of the flagellar system can be tightly regulated by a master regulator, the transcriptional activator FlhDC complex, as well as by the secondary regulator, the alternative sigma factor, σ₁₀.
In the previous characterizations of the flagellar regulon, genetic mutagenesis analysis was used as a main approach to study those non-flagellate mutants which included spontaneous, Mu phage-induced, and some transposon-induced flagellar mutants to define genes involved in the formation of a functional flagellar apparatus (13–17). Although several new flagellar genes have been recently identified in *Salmonella enterica* (18), no additional experiment has been published so far to systematically study the FlhDC and σ^F^ regulons in *E. coli*. Due to the high transcriptional and translational level of these two regulators in log-phase, our basic strategy was to delete these two regulators as well as to minimally perturb steady-state cell growth by moderate induction of these genes in defined MOPS minimal medium. We then monitor global RNA transcript abundance change using Affymetrix GeneChip *E. coli* Antisense Genome Arrays. While transcription by σ^F^ is known to be modulated by the anti-sigma FlgM (19), we will not consider effects of FlgM here. We believe that the genes which are dependent on σ^F^ can be identified by the σ^F^ deletion or induction experiments presented.

In addition, to characterize how the activity of motility is regulated by different carbon sources, we measured the transcriptional level of FlhDC and FliA as well as the level of several well-known genes under the direct control of these two regulators in a range of carbon source conditions by a quantitative RT-PCR analysis. The correlation between the activity of CRP and the motility of cells indicates that activation of CRP plays an important role in triggering *E. coli* ‘foraging’-like behavior (20–22) to actively search for better conditions as the quality of the available substrate(s) decreases. On the basis of these first systematic studies of the FlhDC complex and σ^F^ regulons in *E. coli*, including the effects of carbon source conditions on motility of cells, we gain insight into the complex networks regulated by these two regulators and how they contribute physiological adaptation to the changes in the external environment.

**MATERIALS AND METHODS**

**Reagents, strains and plasmids**

All reagents were purchased from Sigma Chemical Company (St Louis, MO) unless otherwise indicated. 10X MOPS minimal media was prepared as described in Neidhardt *et al.* (23). The media was filter sterilized through a 0.2 μm filter and stored at 4°C. The defined media for log-phase cell growth contained 1 X MOPS minimal media, 0.1% glucose, 0.66 mM K_2HPO_4_.

Because the *E. coli* Genechip probe set is based on the sequenced *E. coli* K-12 strain MG1655 (λ^- F^- ilvG^- rfb^- 50 rpl-1, prototroph) (24), we chose this bacterial strain for use in our study. In order to disrupt the expression of FlhDC/σ^F^ in *E. coli*, we used a simple and highly efficient method (25,26) to prepared in-frame deletion strains for FliA (σ^F^) and for the master regulator FlhDC. In this procedure (as shown in supplemental material Figure S1), we generated PCR products by using primers with 60- to 70-nt extensions that are homologous to regions adjacent to *flhDC* or *fliA* gene(s) and a template plasmid carrying kanamycin-antibiotic resistance genes. Using linear DNA to do homologous recombination in *E. coli* requires the phage lambda Red recombinase (λRed system), which is synthesized under the control of an inducible promoter on a low copy number plasmid. Deletion mutants of the *fliA* gene or *flhDC* operon were isolated as kanamycin antibiotic-resistant colonies after the introduction the respective PCR products into bacteria carrying a λRed expression plasmid. The replication of the temperature-sensitive plasmid pKD46 was inhibited and the loss of this plasmid in mutant strains occurred when clones were grown at 43°C. For controllable induction of individual regulators in *vivo*, we used the P_Ltet promoter which is controlled by the repressor TetR to construct these overexpression vectors as described previously (27). A downstream gene can be induced in the presence of inducer, αTc. All strains used in this study were derivatives of *E. coli* K12 MG1655.

**Microarray experiments**

*Escherichia coli* strains were grown overnight in MOPS minimal media at 37°C in an air shaker with vigorous aeration (225 rpm). Two milliliters of the overnight culture was used to inoculate 100 ml of fresh MOPS minimal medium. For preparing the total RNA for microarray experiments, 15 ml samples of culture (corresponding to 7.5 × 10^8 _cells_ were taken for wild-type and deletion mutant strains when the culture density OD_600_ value reached 0.2 and the same amount of culture was taken before and 5 min after induction in FlhDC or σ^F^ overexpression strains (based on our previous results from σ^F^ time-course experiments (27), we choose 5 min after induction in this assay because it is a reasonable time point that provides sufficient time to induce sigma-dependent genes and also reduce potential post-transcription or other indirect effects]. RNA was stabilized immediately by mixing with a double volume of RNAProtect Bacterial Reagent (Qiagen) and incubated at room temperature for 10 min. Cells were centrifuged at 5800 g for 20 min and cell pellets were stored at −80°C prior to RNA extraction. Preparation of labeled probes and microarray procedures were performed exactly as described previously (27) and in Supplemental Material.

**Real-time quantitative RT-PCR**

Quantitative reverse transcription (RT)-PCR primers were designed using Primer Express software (Applied Biosystems) and were synthesized by the UW Biotechnology Center. Two steps of real-time quantitative RT-PCR are performed. Five micrograms of the DNase-treated total RNA was reverse transcribed for first strand cDNA by using Superscript II system (Invitrogen). Reactions were then performed using 1 ng cDNA and 100 nM of each primer in a 50 μl volume with 1X SYBR Green I mixture. Controls lacking AmpliTaq Gold DNA Polymerase or template were used. Reactions were run on an ABI 7700 instrument (Applied Biosystems) using the following cycling parameters: 95°C for 10 min, 40 cycles of
denaturation at 94°C for 15 s and extension at 60°C for 1 min. Relative gene expression data analysis was carried out with the standard curve method (28). Assays were performed in triplicate.

Multiple-round in vitro transcription following by filter-binding assay for radioactive incorporation

The incorporation of radioactivity in the newly synthesized RNA can be measured by the DE81 filter-binding assay. The DNA fragments used for in vitro transcription assays were amplified by PCR with/without the upstream sequence (~350 bp) of those candidate σF-dependent genes with high confidence identified in our microarray data. The DNA fragment (~50 nM) was incubated with ~15 nM purified σF-associated holoenzyme in a buffer containing 50 mM Tris–HCl (pH 7.9), 150 mM K Glu, 150 μM 3 NTP's (CTP, ATP, GTP) and 20 μM UTP plus 1 μl [α-32P]UTP (~10 μCi), 100 μg/ml BSA, 1 mM EDTA, 10 mM MgCl2, 1 mM DTT in a total volume of 20 μl. The mixtures were incubated at 37°C and transcription was stopped by addition of 0.5 M EDTA (final 100 mM) 30 min after reactions were initiated. The samples were loaded directly onto DE81 filter discs of 22.5-mm diameter (Whatman). The 32P-labeled transcripts were bound to DE81 filters by absorbing the total reaction mixture on the filters. Unincorporated nucleoside triphosphates were removed by washing the filters three times with 500 mM Na2HPO4 buffer, pH 7.6 and twice with 95% ethanol. The filter discs were dried, and nucleotide incorporation was quantified by Cerenkov counting. Incorporation was corrected for apparent incorporation in the absence of holoenzyme.

Electrophoretic mobility-shift assays (EMSA)

The DNA fragments (~350 bp) used for gel mobility shift assays were amplified by PCR from the upstream sequence of flhDC operon. The DNA fragment was 32P-labeled at the 5’ end using T4 polynucleotide kinase. Samples of <40 ng of the labeled DNA fragments were included in 20-μl reaction mixtures containing DNA-binding buffer (10 mM Tris–HCl, pH 7.5, 50 mM KCl, 0.5 mM EDTA, 5% glycerol, 1 mM dithiothreitol), 500 μg/ml bovine serum albumin (BSA) and 25 μg/ml herring sperm DNA. The CRP was added at the following concentrations: 0, 5, 10 and 25 nM, respectively. cAMP was included in all reaction mixtures at a final concentration of 2 mM. Reaction mixtures were incubated for 15 min in 37°C and then were stopped by the addition of 1 μl of loading buffer (0.1% xylene cyanol and 50% glycerol in H2O). The samples were loaded on a 4–10% native Tris–glycine Novex Gel (Invitrogen) and dried on a Slab Dryer (BioRad) as described previously (27). Biomax MS film (Kodak) was used for autoradiography. The gels were scanned using a PhosphorImager (Molecular Dynamics), and the intensities of the bands were determined using ImageQuant version 5.2 software.

Additional experimental procedures are provided in Supplemental Material.

RESULTS

The inactivation and overexpression of flhDC and fliA in E. coli MG1655 strains

The sequenced E. coli K-12 strain MG1655 (λ- F- ilvG- rfb- 50 rph-1, prototroph) (24), on which E. coli Affymetrix Genechip probe design is based, was chosen for our studies. E. coli FlhDC or σF in-frame deletion strains as well as FlhDC or σF overexpression strains were constructed as described in Zhao et al. (27) and in Experimental Procedures. RT-PCR was used to examine the expression of flhD, flhC and fliA in the respective deletion mutants before microarray analysis. As expected, the FlhDC mutant did not express flhD and flhC, and the σF mutant failed to express fliA (Figure 1A), confirming inactivation of these genes.

Soft tryptone swarm agar plates (29) were used to evaluate motility or swimming ability of the strains. In the tryptone swarm agar (Figure 1B), the flhDC and fliA deletion mutants were totally non-motile and did not form any swarm rings compared with the wild type. When the flhDC or fliA mutant was complemented with a cloned flhDC or fliA gene on a low-copy plasmid, the motility was restored as shown in Figure 1B. The empty vector pACYC184 served as a control and had no influence on the motility of the flhDC or fliA mutant (data not shown).

RT-PCR (semi-quantitative) was also performed to detect the mRNA level of flhDC or fliA genes before and after induction of a plasmid-borne flhDC or fliA genes in E. coli. Instead of 35 PCR cycles as performed earlier to test gene disruption, the semi-quantitative PCR was performed for 24 cycles. This reduces the chance of saturation of final PCR product. The synthesized DNA was loaded onto an agarose gel and stained with ethidium bromide. A significant increase of RNA level from target genes was observed after a 5-min induction as shown in Figure 1C, confirming induction of those genes.

FlhDC regulon

To characterize the effects of the decreasing or increasing FlhDC protein level in vivo on gene expression, global RNA transcript abundance was monitored in the deletion mutant strain and the overexpression strain 5 min after FlhDC induction with cells grown in log-phase (OD600 = 0.2) in MOPS minimal medium at 37°C. Expression profiles were obtained as described in Materials and Methods’ section. Expression profiling of transcripts corresponding to the complete set of ORFs in the E. coli genome revealed that the response to deletion of FlhDC in vivo was quite broad. There are 117 genes (2.7% of the genome) downregulated 2-fold or more in the flhDC deletion mutant strain. The wide distribution of FlhDC-dependent genes in E. coli genome (as shown in Figure 2) indicates that FlhDC might play a larger role in the global gene transcription regulation than just to serve as a master regulator for the flagellar regulon. There are 53 genes in E. coli known to be directly involved in flagellar structure and motor function (11,12). Compared with the transcriptional level of genes in the wild-type strain, DNA microarray results showed...
through genetic mutagenesis analysis and can be divided into two functional groups: (1) chemotaxis and mobility; (2) surface structures.

Comparing the DNA microarray results before and 5 min after FlhDC induction in the overexpression strain, there are no significant changes (no RNA level changed more than 2-fold compared to the uninduced control) of RNA level of genes in the flagellar regulon (data not shown). We are not too surprised with this result. Compared with previous experiments (27) where initial $\sigma^{32}$ protein level is low and then is induced almost 8-fold after a 5-min induction, the fold induction of FlhDC under the same inducible $P_{let}$ promoter control must be limited due to the high initial abundance of this protein. Therefore, we expect the reason for no significant increased transcription of FlhDC-dependent genes is due to the high initial protein level of FlhDC before induction. The low fold increase of FlhDC in this short time period (5 min) is not enough to further increase transcription of FlhDC-dependent genes using $\sigma^{70}$-associated holoenzyme.

In Table S2 (Supplemental Material), we show a group of 12 new candidate genes whose transcriptional level decreased more than 3-fold as well as a non-flagellar gene, $yecR$, known to be regulated by FlhDC complex in E. coli (30). Computer prediction of FlhDC-related binding element (27) shows that the consensus is represented as tNAacGc(N)2AAATAgcg (Figure 3C), where lowercase letters indicate less highly conserved sites. This consensus agrees well with the previously reported FlhDC binding consensus that was aligned from several published FlhDC-dependent genes (31). Note, the general height of these consensus element displayed on SEQUENCE LOGO is not high. This indicates that FlhDC consensus binding sequence might not be as strict as sigma factor-binding sites.

### $\sigma^F$ regulon

There are 21 known $\sigma^F$-dependent genes in E. coli involved in flagellar synthesis and function (11,12). In Table 1, we can see that the transcriptional level of these genes is significantly downregulated in the $flhA^+$ strain and is slightly increased in $\sigma^F$ overexpression strain. These results are consistent with our previous hypothesis that a change of the intracellular level of a given sigma factor will cause a change of the transcriptional level of genes dependent on this sigma factor. Jishage (32) reported that the intracellular level of $\sigma^F$ is maintained at 50% the level of $\sigma^{70}$ during log and stationary phase growth and $\sigma^F$ is thought to be the second most abundant sigma factor among seven sigma factors in E. coli. Loss of $\sigma^F$ in cells will greatly decrease the transcription of $\sigma^F$-dependent genes; especially those genes that can only be transcribed by $\sigma^F$-associated holoenzyme (such as $flhC$ in Table 1). Compared with our previous study where the induction of $\sigma^{32}$ caused high induction of $\sigma^{32}$-dependent genes, induction of $\sigma^F$ in this study did not show a large increase in the transcriptional level of $\sigma^F$-dependent genes. Using a specific monoclonal antibody for $\sigma^F$, we determined that the protein level of $\sigma^F$ increased about

![Image](https://via.placeholder.com/150)

**Figure 1.** Confirmation of FlhDC and $\sigma^F$ deletion and overexpression in respective strains. (A) RT-PCR analysis of $flhD$, $flhC$ and $flhA$ expression in deletion strains. RNA isolated from each strain was converted to cDNA and PCR-amplified with primers specific for $flhD$, $flhC$ and $flhA$. DnaK served as positive control. Expression of DnaK can be detected by RT-PCR in the wild-type strain as well as in $flhDC$ and $flhA$ mutant strains. Expression of $flhD$, $flhC$ or $flhA$ can only be detected in wild-type strain, but the expression of these genes is absent in the respective mutants. (B) Motility in E. coli wild-type strain and $flhDC$ and $flhA$ mutant strains. The $flhDC$ and $flhA$ deletion strains lose their motility on soft tryptone swarm agar. The motility can be recovered from these mutant strains by *in vivo* expression of FlhDC or $\sigma^F$ from a plasmid-borne $flhDC$ or $flhA$ gene, respectively. (C) RT-PCR analysis of $flhD$, $flhC$ and $flhA$ expression in overexpression strains. Compared with the transcript level of control ($dnaK$), a significant increase of transcripts of $flhD$, $flhC$ and $flhA$ expression can be seen after a 5-min induction.
2.3-fold after a 5-min induction (Figure 3A), lower than the 8-fold increase in the previous induction experiments.

Comparative analysis of the microarray data from the set of genes whose transcription is downregulated in the fliA deletion strain (decrease of $\sigma^F$) and the set of genes with increased transcription at 5 min after $\sigma^F$ induction (increase of $\sigma^F$) allow us to assign many additional genes to the $\sigma^F$ regulon. In Table 2, there are 13 new candidate genes in $\sigma^F$ regulon. The transcriptional level changes of three reported $\sigma^F$-dependent genes (33,34) which were not assigned into traditional flagellar regulon before ($flxA$, $b1194$ and $ybhH$) are also listed in Table 2. These three genes are non-flagellar genes or have unknown function in the flagellar system.

We chose the top 10 genes in Table 2 for in vitro transcription assays because no experiment has been performed so far to test if these genes can be directly transcribed by $\sigma^F$-associated holoenzyme. The upstream sequence of $grpE$ gene, encoding a heat shock protein, was chosen as a negative control for the in vitro transcription assay because transcription of this gene is dependent on $\sigma^F$ and is not a $\sigma^F$-dependent gene (35). In vitro transcription assay results (Figure 4A) show that most of these 10 genes can be directly transcribed by $\sigma^F$ holoenzyme. Note both $modA$ and $modB$ genes are in one operon. The $modB$ gene can be co-transcribed by $\sigma^F$-dependent promoter located in the upstream region of the $modA$ gene. Results from promoter region consensus analysis using the algorithms BioProspector (36) and HMMER (37) revealed $\sigma^F$ holoenzyme-binding sites in the upstream regulatory sequences of these genes (Figure 4B). Note that several of these genes have two putative promoters. Based solely on the negative results
from our \textit{in vitro} transcription assay, we are not sure if the \textit{hemL} gene belongs to the \(\sigma^F\) regulon. While these possible two-block DNA consensus sequences might provide the primary interaction with holoenzyme, additional transcriptional activators such as FIS and CRP might be utilized to strengthen the promoter–holoenzyme interaction \textit{in vivo} which are not available in our \textit{in vitro} transcription assay. Due to the virulence role of pathogenic bacteria flagella system in adhesion, biofilm formation and colonization of host organisms and in secretion of virulence determinants to host (38–40), the homologous counterparts of the newly identified genes presented here might be potentially associated with these functions, especially with that of the pathogenicity island SPI-1 TTSS (type three secretion system) which appears to be most commonly found in pathogenic bacteria.

**Motility control by carbon source: CRP binds to and scalably activates FlhDC operon in alternative carbon sources**

Recently, Liu and coworkers (20) proposed a new model for carbon source foraging strategy by \textit{E. coli}. By growing \textit{E. coli} in several different carbon sources, they discovered, as carbon substrate quality declines (defined by growth rate), cells systematically increase the number of genes expressed in a hierarchical manner. Concomitantly, cells also increase their motility. They proposed a RNA polymerase (RNAP) reapportoning model to explain the expansion of genes expression. But the mechanism of increasing the motility in a low-quality carbon source remains unknown.

The gradual increase of motility activity with decreased carbon quality was unanticipated because motility by means of flagella is very expensive for cellular economy in terms of the number of genes and the energy required for flagellar biosynthesis and functioning (11). Using energy-intensive flagella in poor nutrient environments would trigger a high risk of more rapidly exhausting the sole energy supply. In Liu’s paper (20), a strategy known as risk-prone foraging (21,22) has been proposed for this behavior; that bacteria take a risk and use the flagellar system to actively search out better conditions. It was observed many years ago that carbon catabolite repression affects flagellar biosynthesis (41,42). This led us to think that the relief from the carbon catabolite repression might be a key factor involved in the effects on motility activity inversely correlated with different carbon source quality.

Relief from the carbon catabolite repression is a complex regulatory circuit that triggers reprogramming of global gene expression patterns to adapt the changes in external environment. This mechanism will activate the cyclic AMP receptor protein (CRP) (43), a global transcriptional factor that positively regulates most carbon catabolic pathways. While it is known that carbon catabolite repression affects the flagellar synthesis and the CRP activation might be involved in alleviating this repression (41,42,44,45), much less is known regarding the role of CRP in motility regulation under a range of carbon source conditions. To determine the effect of different carbon sources on \textit{E. coli} CRP activity as well as the functional relevance between the active CRP level and the expression of FlhDC operon in a range of conditions with the sequenced \textit{E. coli} strain MG1655, we performed the following assays. CRP protein was purified using the pET expression system (Supplemental Material, Figure S2) for \textit{in vitro} assays. In electrophoretic mobility-shift assays as shown in Figure 5A, the upstream DNA fragment of \textit{flhDC} operon can be shifted by purified CRP protein. CRP is a dimer of identical subunits. The consensus tandem DNA-binding site for CRP dimer has been identified by \textit{in silico} analysis as shown in red color which is approximately palindromic and provides two
Table 1. All 21 known $\sigma^F$-dependent genes in flagellar system are downregulated (or induced) after FliA deletion (or overexpression)

| b no. | Gene | Product | Function | Deletion | Overexpression |
|-------|------|---------|----------|----------|---------------|
| b1923 | fliC | Flagellar biosynthesis; flagellin, filament structural protein | Surface structures | $-78.8^c$ | $3.9^d$ |
| b1924 | fliD | Flagellar biosynthesis; filament capping protein, enables filament assembly | Surface structures | $-9.9$ | $3.2$ |
| b1921 | flgM | Anti-FliA (anti-sigma) factor; also known as RflB protein | Surface structures | $-6.9$ | $2.0$ |
| b1921 | flgK | Flagellar biosynthesis; hook-filament junction protein | Surface structures | $-5.5$ | $2.2$ |
| b1921 | flgL | Flagellar biosynthesis; hook-filament junction protein | Surface structures | $-4.9$ | $1.8$ |
| b1921 | fliS | Flagellar biosynthesis; repressor of class 3a and 3b operons (RflA activity) | Surface structures | $-4.6$ | $2.2$ |
| b1921 | flgN | Flagellar biosynthesis; believed to be export chaperone for FlgK and FlgL | Surface structures | $-4.6$ | $1.9$ |
| b1921 | flgT | Flagellar biosynthesis; putative export chaperone for FliD | Surface structures | $-2.2$ | $3.4$ |
| b3072 | aer | Aerotaxis sensor receptor, senses cellular redox state or proton motive force | Degradation of small molecules: | $-2.0$ | $2.8$ |

ab no. indicates Blattner number.
It is possible that one gene has several different gene names.
Numbers indicate fold decrease to wild-type strain.
Numbers indicate fold increase to pre-$\sigma^F$ induction.

Table 2. Additional genes for the $\sigma^F$ regulon

| b no. | Gene | Product | Function | Deletion | Overexpression |
|-------|------|---------|----------|----------|---------------|
| b1566 | fha | Qin prophage | Unknown | $-28.4^e$ | $8.2^d$ |
| b1194 | hha | Putative regulator | Unknown | $-17.5$ | $3.3$ |
| b4109 | yjdA | Conserved hypothetical protein | Not classified | $-12.3$ | $5.3$ |
| b3525 | yhjH | Conserved protein | Unknown | $-9.6$ | $1.9$ |
| b1760 | ynjH | Conserved hypothetical protein | Unknown | $-6.9$ | $7.9$ |
| b1742 | ydR | Conserved hypothetical protein | Unknown | $-4.7$ | $4.5$ |
| b0250 | ykJ | CP4-6 prophage | Unknown | $-3.7$ | $4.0$ |
| b0764 | modB | Molybdate transport protein | Transport of small molecules | $-3.2$ | $2.0$ |
| b0763 | modA | Molybdate transport protein | Transport of small molecules | $-3.0$ | $1.3$ |
| b0154 | hemL | Glutamate-1-semialdehyde aminotransferase (aminomutase), PLP-dependent | Biosynthesis of cofactors, carriers: | $-3.0$ | $2.3$ |
| b0315 | yahA | Putative transcriptional repressor | Unknown | $-2.5$ | $1.1$ |
| b0049 | apaH | Diadenosine tetraphosphatase | Salvage of nucleosides and nucleotides | $-2.5$ | $1.1$ |
| b4110 | ygrZ | Unknown | Unknown | $-2.3$ | $4.2$ |
| b1126 | porA | Spermidine/putrescine transport protein | Transport of small molecules: | $-2.1$ | $1.4$ |
| b4315 | fimI | Fimbrial protein, internal segment | Surface structures | $-2.0$ | $2.6$ |
| b0775 | galK | Galactokinase | Degradation of small molecules | $-2.0$ | $2.6$ |

ab no. indicates Blattner number.
It is possible that one gene has several different gene names.
Numbers indicate fold decrease relative to wild-type strain.
Numbers indicate fold increase relative to pre-$\sigma^F$ induction.
to catalyze the formation of cyclic AMP (46). The pattern of increasing the transcriptional levels of CRP-dependent genes in low-quality carbon sources, as was observed in the wild-type strain, disappeared in the cyaA deficient strain (as shown in Supplemental Material, Figure S3). This indicates that CRP-cAMP plays an important role in promoter activation in our assays. No significant change of the transcriptional level of CRP was observed (Figure 5C), suggesting that the induction of CRP-dependent genes might be mainly due to the activation of CRP rather than the increase in CRP expression (47–49).

Genes for motility and chemotaxis are systematically induced

In an intact flagellar system, the mechanism by which cells control flagellar operation involves genes that are under the direct control of alternative sigma factor, σF. In *E. coli* motility control, the cell has a family of transmembrane proteins with receptor functions (50, 51) termed methyl-accepting chemotaxis proteins, or MCPs (52). MCPs mediate responses to external environmental stimuli. These receptors bind stimulatory ligands and undergo conformational changes that regulate the activities of a network of signal transduction proteins within the cytoplasm. There are six cytoplasmic signal transduction proteins, the products of the Che genes: cheA, cheB, cheR, cheW, cheY and cheZ in *E. coli*. The signal transduction pathways then deliver information to flagellar apparatus to mediate cell filament (product of *fliC*) as a propeller to do rotation and switching. Genomic organization of these genes in respective operons is presented in Figure 5D.

The apparent coupling of motility activity and *fliDC* operon activation in these experiments prompted us to further measure the σF synthesis rate as well as σF-dependent genes synthesis rates in each culture, since these parameter are known to be correlated with motility control (11). We choose the first gene in each operon for the RT-PCR experiments to test the activity of these genes as a function of various carbon sources. Compared with the transcriptional levels of these motility and chemotaxis genes in the fastest growing culture (glucose), a significant increase of the transcriptional level of these genes in slow growing cultures with alternative carbon sources (succinate, alanine, acetate, proline) can be seen in our assays as shown in Figure 5D. The upregulation of motility genes correlated with increasing motility of cells in poor quality carbon sources, together with the synchronized pattern of increasing CRP activity causing increasing FlhDC transcription, further suggests CRP is a factor (or at least one of multiple factors) to play a positive role in triggering *E. coli* ‘foraging’-like behavior to actively search for better conditions as the quality of the available substrate(s) decreases. Interestingly, the transcriptional level of the costly cell filament encoding gene *fliC* did not have any significant changes across different carbon sources. Recently, the Kelly Hughes group (53, 54) reported studies on the transcriptional and translational control of the *fliC* gene in promoter and 5′ untranslated regions in *Salmonella*.

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**Figure 4.** *In vitro* transcription assays to test the transcription by σF holoenzyme of the DNA fragments carrying putative promoter element(s). (A) SDS–PAGE gel shows purified core RNA polymerase and σF as well as MultiMark Standard. *In vitro* transcription assays are performed using purified σF-associated holoenzyme with DNA fragments from the upstream sequence of candidate σF-dependent genes. The upstream sequence of gppE served as a negative control. Error bars represent standard deviation in three different experiments. (B) Potential σF consensus-binding sites of each gene are predicted and aligned by computer program. Previously known σF two-block promoter element consensus is shown below.
Our observations here suggest, in a poor nutrient environment, that bacteria may strategically use the precious energy on the basis of fine tuning of flagellar-gene expression in response to environmental challenges, which it possibly does by sequestering a transcriptional repressor or other factor(s) to inhibit expensive $fliC$ gene expression. The biological implications of this finding are discussed later.

**DISCUSSION**

In this study, we used two different genetic approaches (1) moderately expressing FlhDC or $\sigma^F$ from anhydrotetracycline (aTc) inducible and Tet repressor-controlled $P_{Ltet}$ promoter in a plasmid-borne $flhDC$ or $fliA$ gene; (2) disrupting the expression of FlhDC or $\sigma^F$ in $flhDC$ or $fliA$ deletion mutant strains, to efficiently and reliably study the regulon members of the two flagellar biosynthesis regulators, FlhDC and $\sigma^F$. Our results demonstrate that there are many more genes than previous known under the control of these two regulators. In our previous studies (27), we have demonstrated that our approaches have a significant advantage over those approaches for stimulons and regulons studies in various stress conditions. In this paper, we further discovered that the strength of these two different regulon study approaches depends mainly on the initial concentrations of sigmas or other regulators in vivo. Low initial concentration of a regulator can be induced to a high level after a short induction, which in turn increases transcription of its dependent genes to a significant level. Loss by deletion of the high initial concentration regulator might totally shut off the transcription of its dependent genes in vivo. Note that, for some important regulators, loss of their

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Figure 5. Carbon source effects on cell motility through activation of CRP. (A) SDS-PAGE gel shows purified CRP protein as well as MultiMark Standard and native gel shift assays show the binding of CRP to upsteam DNA fragment of $flhDC$ operon. The palindromic consensus DNA-binding site for CRP dimer is shown in red. (B) The transcript abundance of two CRP-dependent genes, $cstA$ and $cpdB$, in cells grown on different carbon sources. (C) The transcript abundance of $flhD$, $fliA$ and $crp$ in cells grown on different carbon sources. (D) The transcript abundance of $\sigma^F$-dependent genes, $motA$, $tar$ and $fliC$, in cells grown on different carbon sources. Note, these carbon sources are of differing quality as defined by the resulting log-phase growth rates which are 0.97 generation $h^{-1}$ in glucose, 0.50 generation $h^{-1}$ in succinate, 0.34 generation $h^{-1}$ in alanine, 0.21 generation $h^{-1}$ in acetate and 0.13 generation $h^{-1}$ in proline.
functions might cause severe growth problems. Currently, using our lab collection of the monoclonal antibodies for each sigma factor in *E. coli*, we are using the ChIP-chips assay (55,56) as a complementary approach to pull down the DNA fragment that is bound and crosslinked by a given sigma factor. Combination of two or several different regulon study approaches will give us more confidence in positive results.

In addition to extending the repertoire of FlhDC and \( \sigma^F \) regulon with new candidates, this work demonstrates that the carbon source utility and motility activity are interdependent. This study was motivated by the previous observation that *E. coli* increases its motility in poor nutrition environments. Much less is known about how the activation of CRP is affected as well as how this activation will affect cell motility by different carbon source supplies. Our results shed light on cell motility control in a range of different carbon sources and suggest intriguing hypotheses about its establishment and function during utilizing different energy compounds. The observations reported here have many notable features: in the defined MOPS minimum medium with different carbon sources, CRP is activated in poor quality (defined by growth rate) carbon sources relative to rich quality ones; this activated CRP associates with the *flhDC* operon encoding the master regulator with roles in flagellar regulation and development; the activation of CRP is correlated with the increase of expression of \( \sigma^F \) as well as the increase of cell motility.

Flagellar genes are organized into a transcriptional hierarchy that underlies temporal and spatial control of biogenesis program. A large amount of energy is required to synthesize flagella and a large part of this energy is used for filament synthesis. The filament consists of an assembly of around 20,000 subunits of a single protein, flagellin (*flfC*), and the amount of flagellin alone composes about 8% of the total cell protein when the flagellar operons are expressed optimally (57). Although the motility of cells gradually increase in low-quality carbon sources supplies, we found the transcription of the *flfC* gene, which encodes costly flagellin, does not increase in those minimum growth conditions. This may be a means of conserving energy. The cost to the cell of flagellar synthesis and flagellar operation is about 2% and about 0.1% of total energy expenditure under normal growth conditions, respectively (11). As the growth potential of an environment decreases, the ability to reach a potential food source ahead of siblings is a group competition behavior (58). Also the ability to efficiently utilize cellular resources to conserve energy for individual self-protection, such as hibernation, would provide a significant survival advantage. We show that motile bacteria carry out tactical responses to a variety of carbon sources by increasing flagellar operation but restricting costly additional flagellar synthesis. This may provide a paradigm for cost and benefit behaviors in prokaryotes, which result in maximum benefit for survival. The delicate balance between conserving more energy and using the energy-intensive flagella to search out better conditions is modulated by environmental conditions. The inverse correlation between the increasing motility of cell with carbon quality might be in a certain minimum nutrition range. As the growth condition becomes more and more harsh as shown in lowest-quality carbon source (proline), conserving precious energy outweighs the cost of expending energy for potential benefit of locating and utilizing good sources. In this situation, the cell will gradually turn off both of the costly flagellar synthesis (flagellin) and flagellar operation (motility and chemotaxis) and save more energy for a long time survival to passively wait for less adverse (natural) conditions. The downshift of the expression of flagellar genes as well as motility activity in proline might represent these behaviors.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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