Repression of Flagellar Genes in Exponential Phase by CsgD and CpxR, Two Crucial Modulators of *Escherichia coli* Biofilm Formation

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Escherichia coli adapts its lifestyle to the variations of environmental growth conditions, swapping between swimming motility or biofilm formation. The stationary-phase sigma factor RpoS is an important regulator of this switch, since it stimulates adhesion and represses flagellar biosynthesis. By measuring the dynamics of gene expression, we show that RpoS inhibits the transcription of the flagellar sigma factor, FliA, in exponential growth phase. RpoS also partially controls the expression of CsgD and CpxR, two transcription factors important for bacterial adhesion. We demonstrate that these two regulators repress the transcription of *fliA*, *flgM*, and *tar* and that this regulation is dependent on the growth medium. CsgD binds to the *flgM* and *fliA* promoters around their −10 promoter element, strongly suggesting direct repression. We show that CsgD and CpxR also affect the expression of other known modulators of cell motility. We propose an updated structure of the regulatory network controlling the choice between adhesion and motility.

Microorganisms adapt to changes in their environment in many different ways. Among others, they adjust gene expression, modify their metabolism, and change their surface properties by displaying particular surface proteins. The latter phenomenon is directly linked to the choice of a particular "lifestyle," adhesion (biofilm formation) or motility (planktonic growth). In *Escherichia coli*, expression of flagella at the cell surface predestines the cell for motility. More than 50 genes are involved in the synthesis of flagella. These genes are classified into three groups according to their temporal expression sequence. The master regulator of flagellar synthesis, FlhDC, is expressed first, and the corresponding genes constitute the class 1 flagellar operon. FlhDC activates the expression of class 2 genes, encoding the inner part of the flagellum as well as the flagellar sigma factor FliA (σ^{28} or σ^{F}) and the FlgM protein (anti-σ^{28}). The class 3 genes are transcribed by σ^{28}-RNA polymerase (RNAP) and encode the outer components of the flagellum as well as chemotaxis proteins (for a review, see reference [1]).

Flagellar synthesis is tightly controlled by several environmental conditions, including osmolarity [2] and temperature [3]. Many of these environmental influences affect the transcription of flagellar genes and control in particular the expression of the master regulator, FlhDC. The regulators of the *flhDC* operon include cyclic AMP (cAMP)-cAMP receptor protein (CRP), H-NS [4], OmpR [2], LrhA [5], integration host factor (IHF) [2, 6], RpoN [7], and Fur [8]. Other regulators affect cell motility by controlling *fliA* expression. These factors include NsrR [9], signaling molecules such as cyclic diguanylic acid (c-di-GMP) (reviewed in reference [10]), the alarmone polyphosphate guanosine [(p)ppGpp] [11], and quorum-sensing molecules such as autoinducer-2 (AI-2) [12–14].

Transcriptomic data show that the stationary sigma factor RpoS (σ^{38}) represses the transcription of flagellar genes in *E. coli* [7, 15]. An *rpoS* mutant has an accordingly higher motility on soft-agar LB plates but also in liquid culture [7, 16]. Quantitative reverse transcription-PCR (qRT-PCR) experiments show that

RpoS represses *fliA* transcription without affecting the transcription of *flhDC* in exponential phase in *E. coli* strain MG1655 [15]. However, another recent report demonstrates that *rpoS* (indirectly) represses *flhC* transcription in *E. coli* strain BW25113 [16]. We confirmed the negative regulation of *fliA* transcription by RpoS by using a green fluorescent protein (GFP) transcriptional fusion both in LB and M9 media [17]. Consistently, an *rpoS* mutant also shows higher accumulation of the FliA (RpoF) protein during growth in LB medium [18]. As a consequence, an *rpoS* mutant strain displays more flagella than a wild-type (WT) strain in both exponential and stationary growth phases [18]. RpoS accumulates in different stress conditions and at the onset of stationary phase. This stress sigma factor directs the expression of about 500 genes [15, 19–22]. RpoS also represses transcription, a counterintuitive function for a sigma factor. One hypothesis to explain the repression of motility genes by RpoS involves the competition between three sigma factors (σ^{38}, σ^{70}, and σ^{28}) for the RNAP-core binding at the onset of stationary phase [23, 24]. However, the relatively low affinity of RpoS for the core RNAP and the rapid degradation of RpoS are not favorable for such a competition which can be further influenced by the small protein Crl [25]. Interestingly, the repression of flagellar genes occurs mostly in exponential phase in minimal medium, where RpoS accumulates to a lower concentration than in stationary phase. Thus, repression by RpoS takes place in growth conditions where the concentration of RpoS is relatively low. Another straightforward mecha-

nistic explanation of repression by RpoS is that RpoS activates the transcription of a repressor. For instance, the global regulator FNR (fumarate and nitrate reductase), whose expression is partially induced by RpoS, represses genes of the tricarboxylic acid cycle (21). Furthermore, adhesion and biofilm formation are regulated by RpoS partially through the activation of the curli regulatory protein CsgD and CpxR (26, 27) or the transcription of diguanylate cyclases (28, 29).

In this article, we studied the expression dynamics of regulators of flagellar genes in order to investigate how RpoS represses cell motility. We confirm that RpoS represses flagellar regulators in exponential phase in different growth media. We show that *fliA* expression is cAMP dependent in all growth phases because CRP-cAMP activates FlhDC (4). We found that two additional RpoS-dependent transcription factors are involved in the repression of the flagellar gene cascade, CsgD and CpxR. These two regulators have antagonistic effects on adhesion and biofilm formation. Our data suggest that they regulate distinct targets of the network controlling flagellar biosynthesis and that CsgD directly represses the *fliA* and *flgM* promoters.

MATERIALS AND METHODS

Strains and plasmids. The GFP transcriptional fusions were selected from a collection containing nearly 2,000 constructs on the low-copy-number reporter plasmid pUA66 harboring *gfpmut2* (30). Each fusion was verified for accurate cloning and expression level well above the background fluorescence to allow reliable quantification. We reconstructed the *rpoS*::*gfp* reporter vector by DNA amplification of the *rpoS* promoter region (including parts of *nlpD*; for primer sequences, see Table S1 in the supplemental material) and cloning into the low-copy-number plasmid pZE (31), upstream of the *gfpmut3* reporter gene (32). The fusions were transformed into *E. coli* K-12 strain BW25113 (*rrnB3 lacI$^+$* DE*lacZ4787 hsdR514* DE(*araBAD*)*567* DE(*rhaBAD*)*568 rph-1* [33]) and its isogenic mutants, the *rpoS*, *csgD*, *cpxR*, and *cya* mutants. The other mutant strains, lacking transcriptional regulators whose expression is positively regulated by RpoS (see Table S2), were used only for motility and Congo red (CR) binding assays. Transformations were performed by using a high-throughput method that utilizes a transformation and storage solution (TSS) solution containing polyethylene glycol (PEG), dimethyl sulfoxide (DMSO), and Mg^{2+} at pH 6.5 (34). The kanamycin (Kn) resistance cassette was removed from the mutant strains using FLP recombinase (35) prior to transformation of pUA66 derivatives. Mutant strains and reporter plasmids were verified by PCR. RpoS was overexpressed by cloning its coding sequence into the TA cloning pTOPO2 plasmid (Invitrogen). The functionality of this overexpression, which relies on an increased copy number of the *rpoS* gene, was validated by complementation of the Congo red binding phenotype of an *rpoS* mutant strain (see below). Table S1 in the supplemental material lists the sequences of the primers used in the present study.

Growth conditions. Gene expression was measured for cultures growing either in LB (Difco) or M9 minimal medium (12.8 g/liter Na$_2$HPO$_4$, 3 g/liter KH$_2$PO$_4$, 0.5 g/liter NaCl, 1 g/liter NH$_4$Cl, 2 mM MgSO$_4$, 0.1 mM CaCl$_2$) supplemented with 0.2% glucose and 40 μg/ml kanamycin. To test curli production, bacteria were inoculated on LB agar plates containing 0.004% (wt/vol) of Congo red (Sigma). To test bacterial motility, a fixed amount of bacteria (2 μl of a fresh cell culture in stationary phase diluted at an optical density [OD] of ~0.05) was inoculated on soft LB plates containing 0.35 g/liter agar (Difco).

Dynamic measurements of reporter gene concentration. Measurements of gene expression were carried out at 37°C in 96-well polystyrene microplates (white-frame clear-well Isoplate-96 [product no. 5PA028; PerkinElmer]), inoculated from a fresh overnight culture in glass tubes. After dilution of the precultures, 30-fold (M9) or 50-fold (LB) into 150 μl of the same medium, the bacteria were grown in a thermostated micro-

plate reader (Fusion; PerkinElmer) at 37°C for 15 h. To ensure microaerobic conditions and to prevent cell sedimentation, one glass bead (1 mm in diameter) was added to each well, and the microplates were shaken for 10 s every 3 min, alternatively by circular and linear movements. Absorbance (600 nm) and fluorescence (excitation at 485 nm and emission at 520 nm) were measured about 100 times during each experimental growth in the plate reader. For our microplate reader, the optical density at 600 nm (OD$_{600}$) of the culture is about 2.5 times the measured absorbance, absorbance at 600 nm. The raw data were analyzed using WellReader (36) in order to determine the reporter gene concentration and calculate the promoter activity (32). Promoter activity is the time derivative of the reporter concentration plus the sum of the degradation rate of the reporter protein and the growth rate of the culture multiplied by the reporter gene concentration. For detailed equations, please refer to the supplemental material for reference 37. Absorbance and fluorescence measurements were background corrected as described previously (32, 36). Since the plasmid copy number was found to vary less than 2-fold between exponential versus stationary phases (37), no correction was implemented. Each experiment was repeated between three to five times independently and in triplicate on each microplate. The promoter activity curves of independent experiments were slightly time shifted (less than 30 min) according to their growth curves (identical time of entry into stationary phase) and averaged. Biologically impossible, negative signal values (less than 10% of the data range) were corrected by shifting the curve to positive values. The shaded regions in the figures represent the standard errors of the means of the averaged promoter activities in the same experimental condition.

Mobility shift assay of the CsgD-DNA complexes. Promoter probes carrying the *csgD*, *fliA*, and *flgM* promoter regions were generated by PCR amplification (Ex *Taq* DNA polymerase; TaKaRa) using one 5′-fluorescein isothiocyanate (FITC)-labeled primer and derivatives of the pRS reporter plasmid as the template. Primer sequences are given in Table S1 in the supplemental material. The FITC-labeled PCR products were purified from a polyacrylamide electrophoretic gel prior to their utilization for gel shift assays. The CsgD protein was produced from the pET21a expression vector (38), and the assays were performed under standard conditions as described in reference 39.

RESULTS

RpoS represses the transcription of flagellar regulators. Microarray data show that a large majority of the flagellar genes are downregulated by RpoS in minimal medium (15). An *rpoS* mutant strain is therefore more motile than a wild-type (WT) strain, and the overexpression of RpoS reduces cell motility (15). We confirmed this observation for *E. coli* strain BW25113 (Fig. 1A) where RpoS can be detected in exponential phase (see Fig. S3C in the supplemental material). In order to elucidate the mechanism underlying the regulation of *fliA* expression by RpoS, we monitored the dynamics of the *fliA*::*gfp* transcriptional fusion during growth in a batch culture. The transcriptional fusion is barely active in the WT strain, both in minimal and rich medium (Fig. 1; see Fig. 4). This is consistent with low FliA protein levels detected in the WT strain, in exponential phase in M9 medium containing 0.2% glucose at 37°C (see Fig. S4 in the supplemental material). In the ΔrpoS strain, however, *fliA* is strongly expressed in exponential phase, and promoter activity declines before entry into stationary phase (Fig. 1C). The *fliA* promoter remains barely active in stationary phase both in the WT and the *rpoS* mutant strain. The induction of *fliA* expression in exponential phase in the *rpoS* strain is unchanged at lower glucose concentration and enhanced by addition of Casamino Acids (Fig. S1). Our measurements of promoter activity (Fig. 1C) show that RpoS very strongly represses the expression of *fliA* during exponential growth: the repression factor is on the order of 100-fold.
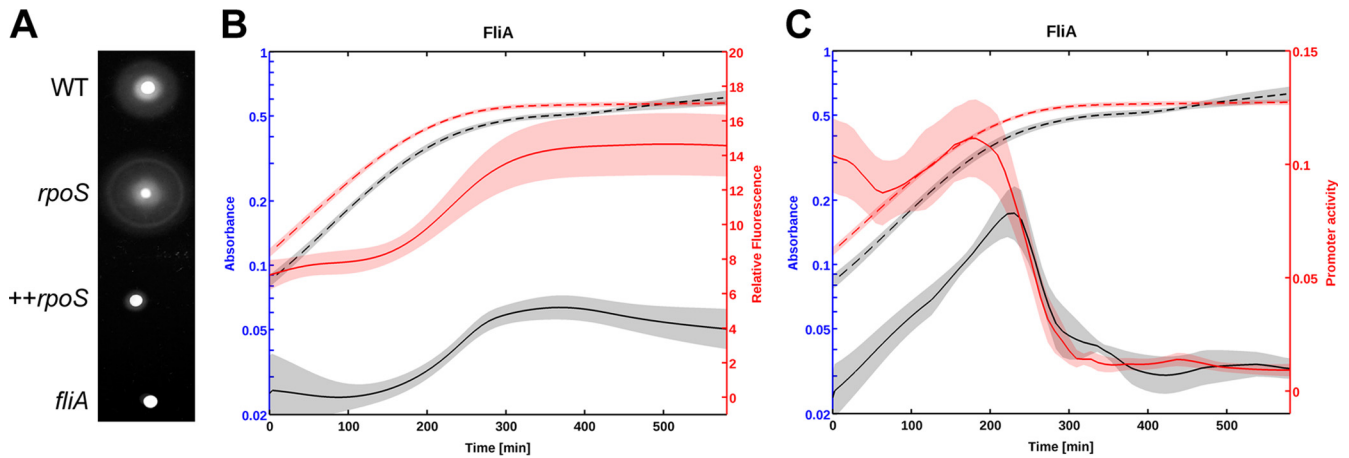
FIG 1 RpoS inhibits motility by repressing *fliA* expression in exponential phase. (A) Motility phenotypes of the wild-type (WT) *E. coli* strain BW25113 and the isogenic *rpoS* and *fliA* mutants. In the strain marked ++*rpoS*, RpoS is overexpressed by providing additional copies of the *rpoS* locus on a multicopy plasmid. Stationary-phase cultures were inoculated on soft LB agar (0.35%) plates and incubated overnight at 30°C. The size of the halo is an indicator of cell dispersion into soft-agar LB plates. (B and C) Relative GFP concentration (B) and computed promoter activity (C) of a *fliA::gfp* transcriptional fusion in a wild-type strain (black) and the *rpoS* mutant (red). M9 stationary-phase cultures were diluted 30-fold into 96-well plates at time zero. Absorbance at 600 nm (broken-line curves) and relative GFP fluorescence (solid-line curves) were monitored for 12 h at 37°C. The standard error of the mean is indicated by the shaded regions of the curves. A minimum of five experimental data sets were used to generate the average of each expression profile. Promoter activity is expressed in relative fluorescence units (RFU)/min (relative fluorescence per time), derived from the kinetics of the absorbance and GFP reporter data.

FliA is a sigma factor directing the transcription of flagellar class 3 genes. If RpoS affects the transcription of *fliA*, we should also be able to observe the (indirect) effect of RpoS on genes downstream of FliA. We tested this prediction for two σ^{28}-dependent genes with measurable expression using GFP fusions: *flgM* and *tar*. As expected, RpoS affects the transcription of *flgM* and *tar* in a comparable way to the effect seen on *fliA::gfp* (Fig. 2). However, the *rpoS* deletion has a stronger effect on the expression of the *flgM* gene, probably because this gene is not only transcribed by FliA. The *flgM* gene is a member of both class 2 and 3 flagellar genes (40, 41) and is transcribed from multiple promoters (42). The expression dynamics of the *flgM* and *tar* genes are entirely explained by the accumulation of FliA predicted from Fig. 1B. These results demonstrate that RpoS represses flagellar genes in exponential phase, despite its low intracellular concentration (15,

23). These observations raise the question of how RpoS represses the transcription of *fliA*?

Identification of intermediate repressors activated by RpoS. RpoS represses transcription of *fliA*, an unusual effect of a sigma factor. The most likely mechanistic explanation would be the activation of an "intermediate" repressor of *fliA* transcription by RpoS. In order to search for such an intermediate regulator, we looked for genes encoding transcriptional factors and whose transcription is affected by the *rpoS* mutation in microarray data performed in four growth conditions (15, 21, 22). Out of 288 transcription factor genes in *E. coli* (reviewed in reference 43), we found 53 genes differentially expressed in an *rpoS* mutant strain: 32 genes activated and 21 genes downregulated by RpoS in at least one medium (LB versus M63) or growth condition (exponential versus stationary). To identify a potential repressor(s) of flagellar
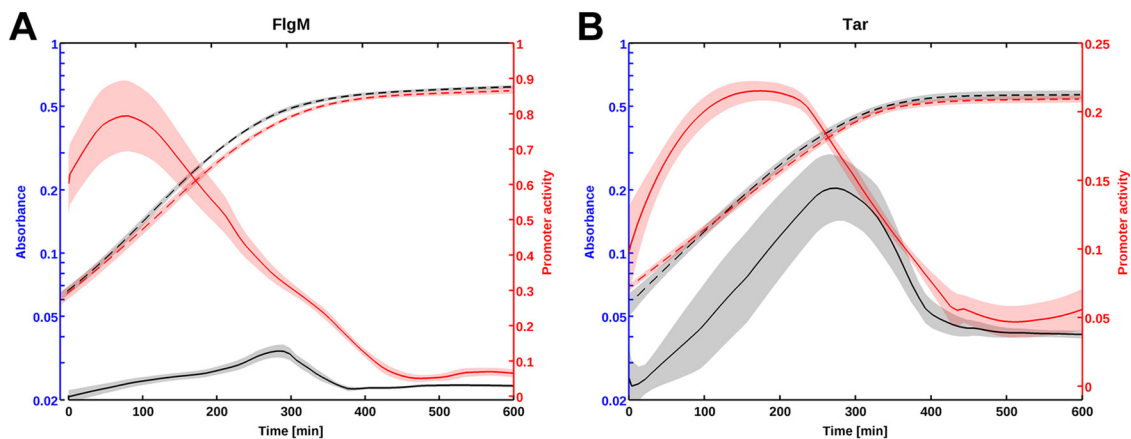

FIG 2 The flagellar gene cascade is repressed by RpoS at several levels. (A and B) Typical gene expression profiles for *flgM::gfp* (A) and *tar::gfp* (B). The average promoter activity computed in the wild-type strain is represented in black, and the average activity in the *rpoS* mutant strain is shown in red. The bacteria were grown in M9 minimal medium at 37°C (see Materials and Methods). Promoter activity is expressed in RFU/min (relative fluorescence per time), derived from the kinetics of the absorbance and GFP reporter data.
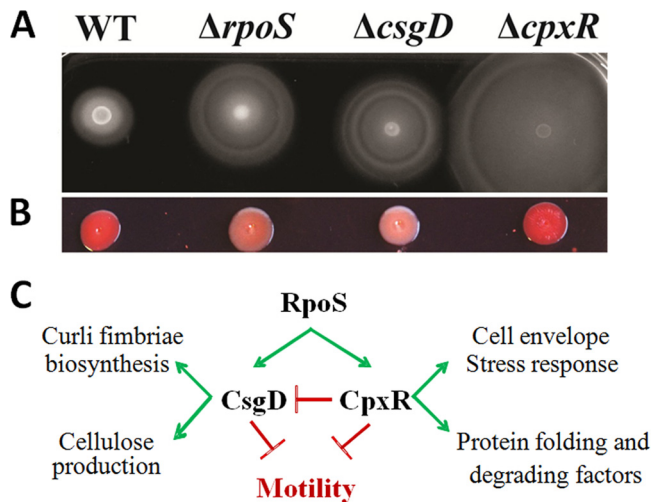
FIG 3 CsgD and CpxR as intermediate repressors of bacterial motility. (A and B) Motility (B) and Congo red (B) phenotypes of the wild-type *E. coli* BW25113 strain and *rpoS*, *csgD*, and *cpxR* mutant strains derived from the WT strain. Stationary-phase cultures were inoculated on the top of the LB soft-agar (0.35%) plates for motility tests and onto LB agar (1.2%) plus Congo red (0.004%) for curli fimbria staining and incubated overnight or 2 days, respectively, at 30°C. (C) Known functions and interactions between RpoS, CsgD, and CpxR.

genes, we selected, from the single mutant library of *E. coli* K-12 strain BW25113 (33), strains that carry insertion in these 32 transcription factors positively regulated by RpoS. From this selection, we eliminated mutants for master regulatory genes such as ArcA and RpoS itself, and ended up with 21 mutant strains (listed in Table S2 in the supplemental material) that were tested for swimming motility.

In order to select regulators affecting cell motility, we inoculated the corresponding mutants on soft-agar (0.35%) LB plates. Since the *rpoS* mutation leads to increased cell motility, we expected that some of these mutations should affect cell motility. Only four mutant strains presented a motility phenotype different from the wild-type strain. Two of them were known to affect RpoS protein levels, the *gadX* and *gadW* mutant strains (44, 45). The two remaining mutants are known regulators of bacterial adhesion and biofilm formation, the *csgD* and *cpxR* mutant strains. CsgD is the central regulator of curli fimbria synthesis and cellulose production (26, 27, 46), and CpxR is involved in the response to cell envelope stress and regulation of protein folding or cell adhesion (26, 47). Because CpxR is known to repress *csgD* transcription (26, 48, 49), the two regulators are important for both curli production and the regulation of cell motility.

Activation of *csgD* transcription by RpoS had been previously shown (27, 50, 51). As expected, the increase of cell motility is of comparable magnitude for the *csgD* and *rpoS* mutants, whereas the *cpxR* mutation has a much more drastic effect (Fig. 3A). The Congo red (CR) dye binds to curli fibers and can be used to assay curli production. RpoS and CsgD stimulate curli production (46), and their deletion reduces CR staining (Fig. 3B). In contrast, the colony formed by a *cpxR* mutant strain appears slightly redder or comparable to a wild-type colony (Fig. 3B). Thus, while the *cpxR* and *csgD* mutations have antagonistic effects on curli production, they both stimulate cell motility (Fig. 3C). CsgD is known to activate curli production at low temperature, and its accumulation

and binding to DNA targets were studied at temperatures below 30°C (38, 52, 53). However, the stimulatory effect of the *csgD* deletion on swimming motility is independent of the growth temperature (see Fig. S2 in the supplemental material). As expected, overexpression of CsgD and CpxR in a wild-type strain results in reduced swimming motility (data not shown). These two regulators are therefore important intermediates, connecting RpoS to the complex regulatory network responsible for the decision between motility and adhesion.

CsgD and CpxR do not affect *rpoS* transcription or activity. To confirm that CsgD and CpxR affect bacterial cell motility without affecting RpoS levels or activity, we measured *rpoS* transcription, RpoS protein levels, and the expression of characterized targets of RpoS in the WT and in the *csgD* and *cpxR* mutant strains. Figure S3 in the supplemental material shows that *rpoS* transcription is not affected by the *csgD* or *cpxR* mutations; RpoS accumulation and the expression of six known targets of RpoS induced in stationary growth phase are also not affected by these mutations. The *cpxR* deletion reduces the expression of only one gene, *gadB*, suggesting a specific regulation of this gene by CpxR. These results confirm that CsgD and CpxR repress cell motility without affecting RpoS activity.

CsgD and CpxR repress flagellar biosynthesis. In order to elucidate the mechanism by which CsgD and CpxR repress cell motility, we measured the expression kinetics of *fliA*, *flgM*, and *tar* in the corresponding mutant strains. These three fusions were selected among the reporter fusions for motility genes available in our GFP collection (30), because they presented sufficient activity above the promoterless GFP vector (17). The expression of *fliA* is strongly increased in both mutant strains during exponential phase in minimal medium (Fig. 4A) and more drastically in rich growth medium (Fig. 4D). This regulation affects the intracellular level of FliA protein in exponential phase: as shown in Fig. S4 in the supplemental material, FliA accumulates to a greater concentration in a *csgD* or *cpxR* mutant strain compared to the WT strain.

An immediate prediction of this observation is that target genes of FliA should be transcribed more strongly in these mutants. Figure 4B and C show the kinetic measurements of *flgM* and *tar* expression in these mutants. As expected, the expression profiles of *flgM* and *tar* are entirely explained by the effects of the *csgD* and *cpxR* deletions on FliA expression. The *cpxR* mutation induces a massive expression peak in exponential phase and affects the expression of *flgM* and *tar* more than the *csgD* or *rpoS* deletion does. These strong effects were confirmed using quantitative RT-PCR from total RNA extracted in mid-exponential phase (see Fig. S5 in the supplemental material). In both experimental approaches, the induction factors are very strong and range from 4- to >2,000-fold. In order to confirm that CsgD and CpxR repress FliA-dependent genes, we measured expression of a selection of nine target genes of FliA using qRT-PCR. As shown in Fig. S5, both regulators exert a strong negative regulation on other class 3 motility genes (namely, *aer*, *fliC*, *fliL*, *trg*, *tsr*, *ves*, and *ycgR*). Interestingly, in M9 minimal medium, the expression profile of *tar* is much more sensitive to the *cpxR* deletion than to the deletion of the adhesion regulators, CsgD and RpoS (Fig. 4C). This observation is true for the other class 3 genes transcribed by the RNA polymerase associated with FliA (Fig. S5). These regulations are qualitatively independent of the growth medium, although the regulatory effects have higher amplitudes in rich medium (Fig. 4D, E, and F).
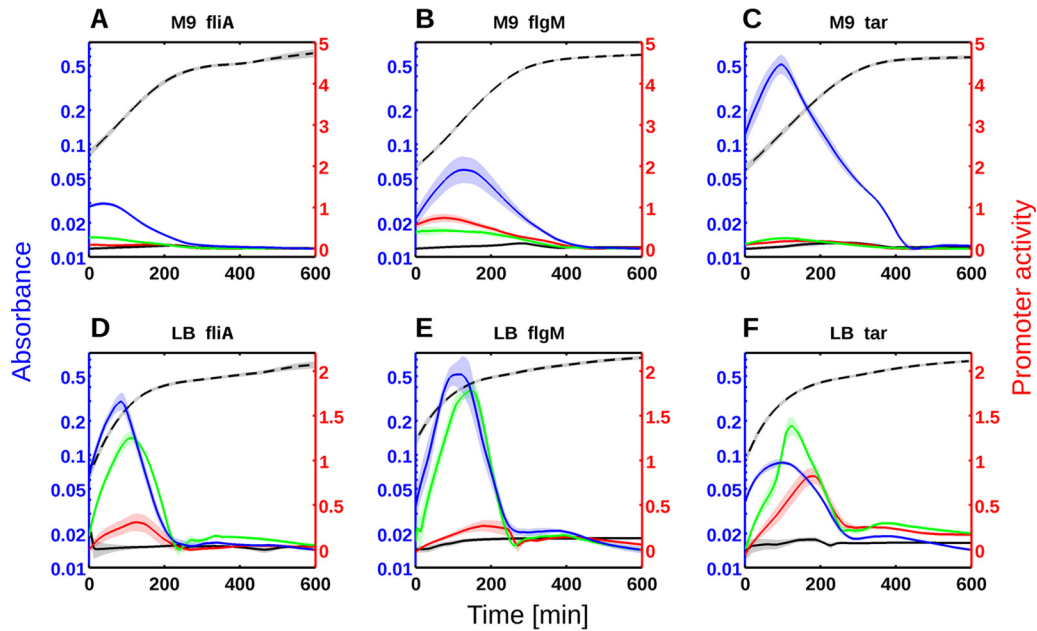
FIG 4 Repression of flagellar class 2 and 3 genes by RpoS, CsgD, and CpxR in M9 minimal medium (A to C) or LB medium (D to F). Gene expression profiles were monitored for *fliA::gfp* (A and D), *flgM::gfp* (B and E), and *tar::gfp* (C and F) in the wild-type (black), *rpoS* (red), *csgD* (green), and *cpxR* (blue) mutant strains. Absorbance at 600 nm is shown on the blue axes, promoter activity (relative units) is shown on the red axes, and time of growth (in minutes) is shown on the black axes. The black broken-line curve represents a typical growth curve (absorbance at 600 nm) of the WT strain at 37°C. A minimum of five experimental data sets were used to generate each expression profile (average activities and standard errors of the means). The standard errors of the means are indicated by the shaded regions of the curves. Promoter activity is expressed in RFU/min (relative fluorescence per time), derived from the kinetics of the absorbance and GFP reporter data.

CsgD directly binds the −10 promoter element of *fliA* and *flgM*. To test whether CsgD directly binds to the promoter regions of the genes encoding the flagellar sigma factor and its targets, we performed gel shift assays. As shown in Fig. 5A, the CsgD protein binds the promoter regions of *fliA* and *flgM*. The bound DNA is clearly shifted by CsgD, forming aggregates in the wells at the top of the gel. These interactions are consistent with known properties of CsgD and the idea of a direct repression of these regulatory genes by CsgD. To demonstrate specific binding of CsgD to these promoters, we added a competitor DNA, the *lacZ* promoter that does not contain a sequence similar to that of the CsgD binding site. As expected, the intensity of unbound DNA decreases proportionally to the increase of CsgD concentration in the assay for each promoter but not for the *lacZ* promoter DNA. This clearly demonstrates that CsgD specifically binds to the promoters of the flagellar regulators (class 2 genes). For an additional control, we tested CsgD binding to its own promoter harboring multiple CsgD binding sites (Fig. 5C). As expected, CsgD binds its promoter with a higher affinity than the one observed at the *fliA* and *flgM* promoters. Figure 5B presents the consensus sequence for CsgD binding (AAAAGNGNNAAAWW [38]) and the potential binding site overlapping the −10 promoter elements of the flagellar regulatory genes. As shown in Fig. 5C, a single putative CsgD binding site is present upstream of the −10 hexamer of *fliA*P2 (σ^{28} dependent), also overlapping the −10 box of the distal promoter *fliA*P1 (σ^{70} dependent). In addition, two putative CsgD binding sites overlap the −10 promoter element of *flgM*, but none were found upstream of the *flhDC* operon, the master regulator of class 2 genes. The two CsgD binding motifs present on the *flgM* promoter have more mismatches to the proposed consensus sequence

for CsgD binding than the unique, high-affinity site found at the *fliA* promoter (Fig. 5B and C). In addition, these two sites contain cytosines instead of adenines at positions 11 and 12, as also observed at other CsgD DNA targets (38).

CsgD and CpxR regulate motility through several known regulators. The control of flagellar gene expression is influenced by numerous genetic factors, summarized in Fig. 6A (for a review, see reference 54). In order to assess the roles of RpoS, CsgD, and CpxR in this network, we have tested the effects of their deletion on expression of these key modulators of cell motility. As shown in Fig. 6B, mutation of *csgD* and *cpxR* directly or indirectly affects the expression of four out of seven regulators of the cascade of flagellar genes. For example, CsgD activates *ihfB*, known to repress FlhD (2, 6). Therefore, CsgD can potentially exert a repressive effect on the two master regulators at two levels of the regulatory cascade: directly on *fliA* and indirectly on *flhD*. In contrast, CpxR directly or indirectly activates *nsrR* and *ompR*, both of which repress cell motility. CsgD represses the transcription of *dksA*, itself known to repress the transcription of *flhD* and *fliA* following starvation (11). Most modulators of *E. coli* cell motility are regulated by RpoS, CsgD, or CpxR both in exponential phase (data not shown) and in stationary phase (Fig. 6B). However, the magnitude of the regulatory effects is higher in stationary growth phase. Taken together, these results show that CpxR and CsgD are major actors of a very complex regulatory network (summarized in Fig. 6C) and these two regulators fine-tune the expression of cellular appendices responsible for cell motility and adhesion.

***fliA* expression is cAMP dependent.** In the proposed network (Fig. 6C), RpoS slightly represses the transcription of CRP itself known to activate the *flhDC* operon (4). FlhDC is the major acti-
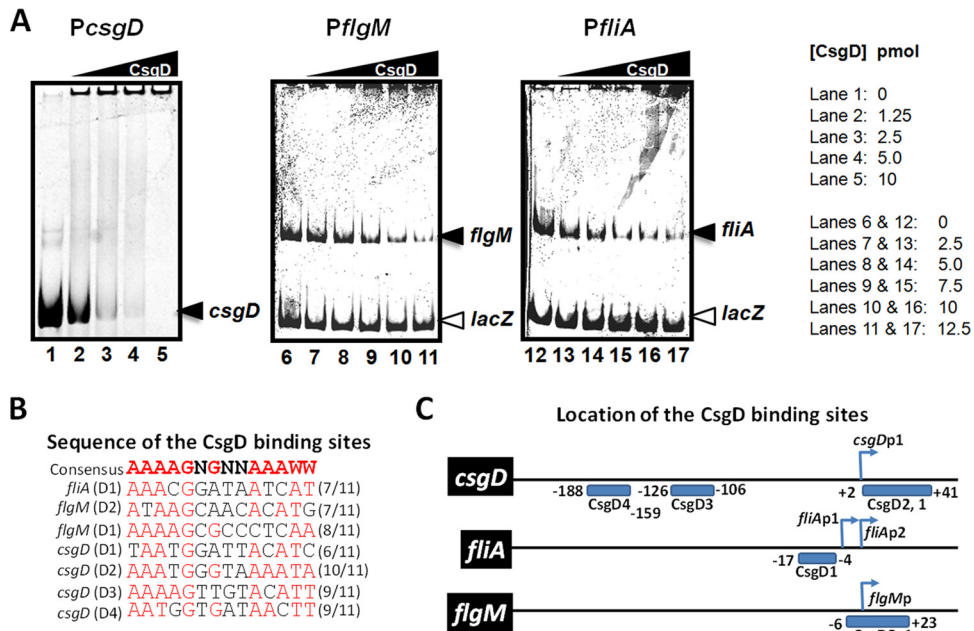
FIG 5 CsgD binds the promoter region of the flagellar class 2 genes. (A) Gel shift assay of CsgD binding to its own promoter and the promoters of *fliA* and *flgM*. The protein was present at 1.25 to 12.5 pmol for 0.5 pmol of labeled DNA in a final volume of 20 μl. The concentration of CsgD is indicated by the height of the triangle above the gel, and the concentration of CsgD (in picomoles) in each lane is given to the right of the gels. (B) Consensus sequence for CsgD binding and sequences of the binding sites found around the −10 promoter element of the *csgD*, *fliA*, and *flgM* promoters. Individual DNA binding sites recognized by CsgD are labelled D1 to D4, which refers to specific positions within the target promoters. (C) Locations of the CsgD binding sites according to the transcription start site.

vator binding the *fliA* promoter (1), and we therefore predicted that *fliA* transcription should be dependent on cAMP-CRP. Indeed, *fliA* is not transcribed in adenylate cyclase mutants (Δ*cyaA*; see Fig. S6A in the supplemental material) or CRP mutants (data not shown). Supplementing a minimal medium with increasing concentrations of cAMP restores transcription of *fliA* in a *cyaA* mutant strain (Fig. S6A). The complementation by cAMP is immediate and functions at all growth phases, although it is less
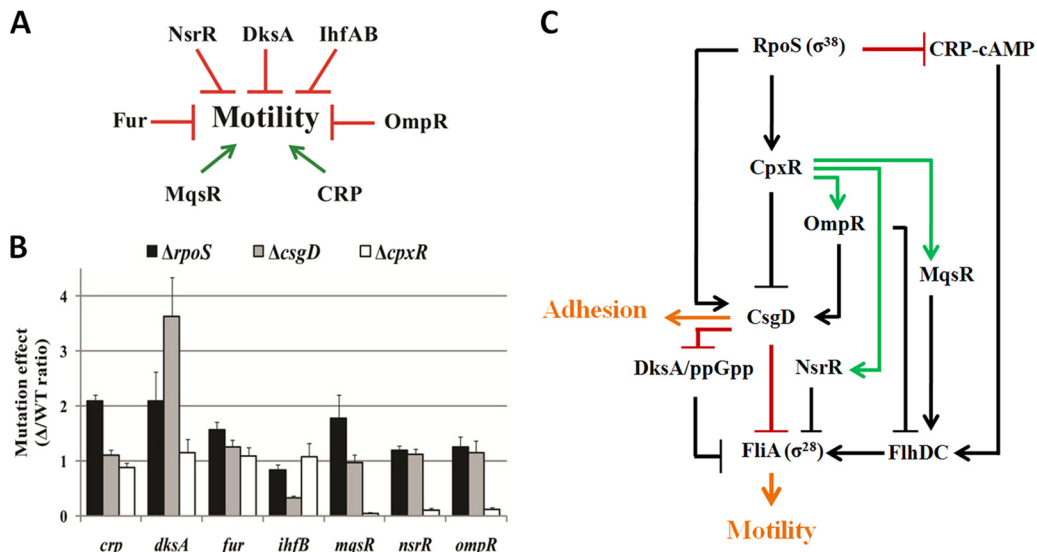

FIG 6 CsgD and CpxR regulate different components in the network controlling FliA expression. (A) Seven major regulators of *E. coli* are known to modulate flagellar biosynthesis, mostly by controlling FlhDC (see introduction). (B) Measurements of GFP transcriptional fusions in the wild-type strain and its *rpoS*, *csgD*, and *cpxR* mutants in M9 medium at 37°C. Gene expression values were calculated, in stationary phase (absorbance at 600 nm of 0.6), as the ratio of the relative fluorescence of the mutant versus the WT strains in at least 3 independent experiments. Very similar gene expression ratios were found in exponential phase (absorbance of 0.2). (C) Proposed model controlling cell adhesion and motility. Black arrows indicate known regulation, while green and red arrows show new stimulatory or repressive interactions, respectively, between key regulators.

efficient in stationary phase (Fig. S6B). These results confirm that cAMP-CRP stimulates *fliA* transcription, most probably through *flhDC* activation. The activation of FlhDC expression by CRP is reduced by repression of *crp* by RpoS, a pathway independent of the *csgD* and *cpxR* genes (Fig. 6).

DISCUSSION

CsgD and CpxR regulate motility and adhesion. Swimming motility in *Escherichia coli* is a highly regulated process. Here, we found that RpoS, the stationary sigma factor, indirectly represses the expression of flagellar genes during growth in both minimal and rich media. This regulation takes place in the exponential phase of growth and confirms previous observations in *E. coli* strains MG1655 and BW25113 (7, 16). These results also agree with previous transcriptomic studies (7, 15, 22) showing that RpoS alters a subset of genes in exponential phase, especially in minimal medium where RpoS accumulates to higher concentrations. Such specific regulatory events in fast-growing cells might rely on improved promoter recognition of a subset of genes (with better affinity for RpoS) and may be enhanced by the Crl protein (17, 27). Since direct repression by a sigma factor is a rare mechanism, we screened for potential transcriptional repressors activated by RpoS and found two regulators, CsgD and CpxR, both of which repress cell motility. They particularly repress the transcription of *fliA* in exponential phase and therefore indirectly downregulate the expression of genes transcribed by FliA (so-called class 3 genes of chemotaxis or encoding the outer part of the flagellum; see Fig. S5 in the supplemental material). Of these genes, FlgM, the modulator of FliA activity, is subject to a similar regulation (Fig. 4) even though the two flagellar regulators were found abundant both in exponential and stationary growth phases (55). Thus, FliA and FlgM are both negatively regulated in exponential phase by major determinants of cell adhesion and biofilm formation of members of the family *Enterobacteriaceae*.

Role of CsgD. CsgD controls the secretion of curli fimbriae and cellulose production, two determinants of bacterial adhesion and biofilm formation (56). CsgD binds directly to flagellar class 2 operons (*fliE* and *fliFGHIJK*) and represses their transcription (38). Pulldown experiments suggest that CsgD interacts with FliA (σ^{28}), probably to modulate its activity or stability (57). Here, we demonstrate that CsgD represses part of the flagellar regulatory cascade through specific inhibition of *fliA* transcription. This repression is logically transmitted to at least nine flagellar class 3 genes (Fig. 4 or Fig. S5 in the supplemental material). In addition, CsgD makes other connections with global regulators, thus diminishing the expression of FliA by alternative routes. For example, CsgD represses the transcription of DksA which, together with ppGpp, plays an important role in reorganizing gene expression in stationary phase, in particular by inducing the RpoS regulon (58). We also found that CsgD activates the transcription of *ihfB* (Fig. 6B) itself known to activate *csgD* transcription in exponential phase (49). Such reciprocal interactions complicate an intuitive understanding of the regulatory network controlling the decision between motility and adhesion (Fig. 6C).

Involvement of nucleoproteins in the regulatory network. The switch in lifestyle is probably also influenced by other nucleoproteins whose concentration varies during growth in batch culture (59). For example, H-NS and IHF control *csgD* (and *ompR*) transcription (49), but only the concentration of IHF varies during the transition from exponential to stationary phase. The time-varying concentration of IHF could thus modulate CsgD accumulation. Furthermore, Fis and Dps are, respectively, the most abundant nucleoid-associated proteins (NAPS) in exponential and stationary phases of growth. It is therefore possible that the high level of Fis in exponential phase affects, indirectly and independently of CsgD, the switch between flagellar and curli expression by reducing (60) the stimulatory effect of CRP on the master activator FlhDC.

Role of CpxR. CpxR and the inner membrane histidine kinase CpxA form a two-component system activated by an increase in osmolarity and/or following attachment to surfaces. CpxR is known to activate protein folding and degrading factors such as DegP, PpiA, and DsbA (61) and to repress curli expression (26), chemotaxis genes, and the *motA* gene, an essential component of the flagellar motor (62). Since CpxR represses *csgD* transcription (26), adhesion to hydrophobic surfaces is impaired when the Cpx pathway is altered (47). Here, we found that CpxR represses *fliA* more strongly than RpoS or CsgD (Fig. 4A and D). However, this strong repression of *fliA* is indirect, passing through several routes that involve different transcription factors. For example, we demonstrate that CpxR is essential for the transcription of the *mqsR*, *nsrR*, and *ompR* regulatory genes (Fig. 6B). These functional dependencies connect CpxR to genes involved in the repression of both FliA and FlhDC. In the proposed model (Fig. 6C), the regulatory path CpxR-OmpR-CsgD-FliA could explain the observed repressive effects of CpxR on *fliA* transcription and cell motility; another route, independent of CsgD (CpxR-OmpR-FlhDC-FliA), also explains the negative regulation of *fliA* by CpxR. In contrast, the straight regulatory path CpxR-CsgD-FliA may not correspond to a predominant route in exponential growth phase, probably because the inhibitory effect of CpxR on *csgD* is impaired by the presence of OmpR that shares the same binding region as CpxR on the *csgD* promoter (26, 49). Thus, CpxR may inhibit the expression of flagellar genes only during exponential growth, and CpxR may inhibit curli expression only in stationary phase. In contrast, CsgD may alternatively repress the biosynthesis of flagella in exponential phase and stimulate curli expression in stationary phase. These growth phase-dependent regulations may be further modulated by fluctuating concentrations of NAPS.

DNA binding sites. A key functional interaction in the model proposed in Fig. 6C is the repression of *fliA* by CsgD. Our gel mobility shift assay shows a direct physical interaction between the CsgD protein and the promoter of this gene (Fig. 5A). This *in vitro* experiment also suggests a direct repression of the *flgM* promoter by CsgD. At least two key interactions of CsgD in the regulatory network therefore correspond to direct physical interactions. Our experiments also add three new binding sites to the list of 18 known binding sites of CsgD (38). Examination of these sequences reveals conserved A or C nucleotides at positions 11 and 12 (Fig. 5B), leading to an updated version of the consensus sequence for CsgD binding, AAAAGNGNNA<u>MM</u>WW. (The underlined nucleotides correspond to two modifications of the consensus sequence for CsgD binding which was previously proposed as AAAAGNGNNAAAWW in reference 38 and as shown in Fig. 5B.)

Negative regulation of cell motility by RpoS is likely mediated by direct binding of CsgD to the promoter of flagellar class 2 genes (*fliE*, *fliF*, *fliA*, and *flgM*). On the other hand, we did not find a putative CsgD box on the *flhD* promoter, which is consistent with the idea that RpoS represses flagellar genes only by acting on *fliA*

transcription as demonstrated in strain MG1655 (7). However, another recent study of strain BW25113 shows that *rpoS* can also repress *flhC* in exponential phase (16). For CpxR, we identify several intermediate regulators (Fig. 6B), but sequence analysis did not reveal any putative binding site for CpxR (GTAAANNNNGT AAA) (63) within the promoters of *flhD*, *fliA*, or *flgM*.

Decision motility versus adhesion. The interactions we have identified in this work further increase the complexity of the network underlying the decision between motility and adhesion. Multiple redundant connections within this network complicate a quantitative interpretation of the proposed interactions. The stress response sigma factor RpoS is at the top of this hierarchical network (Fig. 6C). This master regulator orchestrates the decision between "alternative lifestyles" by promoting the transcription of *csgD* and *cpxR*, two key regulators highly connected to the different modulators of FliA expression. Our work presents the first evidence that this differentiation process shares common transcription factors in addition to the c-di-GMP signaling molecule (28, 29, 64).

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