

## Role of Integration Host Factor in the Transcriptional Activation of Flagellar Gene Expression in *Caulobacter crescentus*

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**In the *Caulobacter crescentus* predivisional cell, class III and IV flagellar genes, encoding the extracytoplasmic components of the flagellum, are transcribed in the nascent swarmer compartment. This asymmetric expression pattern is attributable to the compartmentalized activity of the  $\sigma^{54}$ -dependent transcriptional activator FlbD. Additionally, these temporally transcribed flagellar promoters possess a consensus sequence for the DNA-binding protein integration host factor (IHF), located between the upstream FlbD binding site and the promoter sequences. Here, we deleted the *C. crescentus* gene encoding the  $\beta$ -subunit of the IHF, *ihfB* (*himD*), and examined the effect on flagellar gene expression. The  $\Delta$ *ihfB* strain exhibited a mild defect in cell morphology and impaired motility. Using flagellar promoter reporter fusions, we observed that expression levels of a subset of class III flagellar promoters were decreased by the loss of IHF. However, one of these promoters, *fliK-lacZ*, exhibited a wild-type cell cycle-regulated pattern of expression in the absence of IHF. Thus, IHF is required for maximal transcription of several late flagellar genes. The  $\Delta$ *ihfB* strain was found to express significantly reduced amounts of the class IV flagellin, FljL, as a consequence of reduced transcriptional activity. Our results indicate that the motility defect exhibited by the  $\Delta$ *ihfB* strain is most likely attributable to its failure to accumulate the class IV-encoded 27-kDa flagellin subunit, FljL.**

Cell division in *Caulobacter crescentus* results in the generation of two dissimilar daughter cells, a sessile stalked cell and a motile swarmer cell. The synthesis of the single polarly localized flagellum occurs in a temporally and spatially regulated fashion over the course of each cell cycle (14, 17, 27). Over 50 *C. crescentus* genes are required for construction of the flagellum, with the majority organized within a *trans*-acting regulatory hierarchy of four classes (I to IV). The class I-encoded transcription factor CtrA activates the cell cycle-regulated expression of class II genes, encoding the cytoplasmic membrane-embedded MS ring, the flagellar switch, and the secretory apparatus of the flagellum. The expression and successful assembly of a class II-encoded structure is required for the transcriptional activation of class III genes, which encode the rod transversing the cytoplasmic membrane and the periplasm, the outer membrane rings, and the extracellular hook structure (37, 48, 65). The assembly of the hook structure is, in turn, required for translation of the flagellin subunits (class IV) that comprise the filament.

The temporal regulation of class III and IV flagellar gene expression is influenced by both cell cycle events and the progression of flagellar assembly. These late flagellar genes share a conserved promoter sequence that includes a consensus binding site for RNA polymerase containing the  $\sigma^{54}$  subunit (8, 21, 28, 38, 39, 45, 47) and one or more *ptr* enhancer elements recognized by the class II-encoded transcription factor FlbD (5, 6, 18, 29, 46, 62–64). The activation of FlbD occurs in

response to the successful assembly of early class II-encoded flagellar components. FlbD contains an amino-terminal receiver domain, conserved among two-component regulatory systems, that is phosphorylated in the predivisional stage of the cell cycle (63). The activity of FlbD peaks in the predivisional cell type and becomes restricted to the swarmer compartment of the predivisional cell once a cell division constriction has formed (20, 63). FlbD-mediated transcriptional activation has been shown to be regulated by flagellar assembly through the activity of the *trans*-acting factor FliX (42–44). FliX-regulated coordination of early flagellar assembly with cell division allows FlbD-dependent, swarmer compartment-specific expression of class III and IV flagellar genes in the late predivisional cell.

The *C. crescentus* class III and IV flagellar promoters also contain a conserved binding site for integration host factor (IHF) located between the promoter sequence and the upstream FlbD binding site (18, 19, 38). IHF is a small (approximately 20 kDa in *Escherichia coli*), heterodimeric, sequence-specific DNA binding protein that was first identified in *E. coli* as having a critical role in integration and excision of the prophage form of bacteriophage  $\lambda$  (33). During bacteriophage  $\lambda$  integration, IHF binding induces a bend in the DNA (22, 30, 52, 53, 60) of greater than 160° (53). This DNA bending activity was shown to be required for Int-catalyzed recombination between the *attB* and *attP* sites on the bacterial and lambda chromosomes in vitro (22, 30, 53). Subsequently, IHF was found to play an important role in diverse cellular processes, such as the initiation of DNA replication, certain recombination events, and the regulation of gene expression (1, 11, 12, 23–25, 34, 59).

When present in  $\sigma^{54}$  promoters, the IHF-induced bend is thought to bring RNA polymerase bound to the promoter and

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transcriptional activators bound to the relatively distant enhancer sequences into close proximity with each other, thus increasing the probability of interaction and transcriptional activation (10, 18, 19, 26, 54). Experiments with the *Pseudomonas putida* Pu promoter have shown that IHF can also function to recruit RNA polymerase holoenzyme to promoter sequences (7, 35). IHF has been demonstrated to be required for the transcription of the  $\sigma^{54}$  *nifH* promoter (26) and *C. crescentus* class III and IV flagellar promoters in in vitro experiments (5). Furthermore, mutations in the IHF binding site of either of the class III flagellar gene promoters *fliK* (formerly *flgG*) or *flgK* (formerly *flaN*) indicate that IHF is required for maximal levels of transcription in vivo (18, 19). Interestingly, a mutagenized *fliK* promoter that was shown in vitro to no longer be bound by IHF was still capable of driving FlbD-dependent reporter gene expression, though to a much lesser extent than the wild-type promoter (18, 19). Thus, the higher-order DNA structure created by IHF binding is not strictly required for *fliK* transcription.

Although these previous experiments suggest a role for IHF in fine-tuning the temporal and spatial regulation of class III and IV flagellar gene expression, it is not known what influence IHF has on motility and swarmer cell differentiation in *C. crescentus*. Here we investigate the role of IHF activity on the regulation of transcription of late flagellar genes in *C. crescentus* by determining the effects of deleting *ihfB* (*himD*) on growth, cell morphology, motility, and the transcription of flagellar promoters. Strains containing a deletion of *ihfB* exhibited a motility defect and a reduction in the level of transcriptional activation of several class III/IV flagellar genes. Interestingly, cells lacking IHF displayed a wild-type temporal pattern of class III, *fliK-lacZ* reporter gene expression, although the promoter activity was diminished. Absence of IHF was found to most significantly affect expression and accumulation of the class IV flagellin, FljL.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** Bacterial strains and plasmids used in this work are listed below. *C. crescentus* strains were LS107 (wild-type strain), *syn-1000 bla-6* (58), JG1182, *syn-1000 bla-6 flbD198::Tn5* (44), and JG1216, *syn-1000 bla-6 ihfB1* (this work). *E. coli* strains were DH5 $\alpha$ , *endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* and S17-1, Rp4-2 Tc::Mu Km::Tn7 (57). Plasmids used were the following: pREM85, 747-bp PCR-generated SalI (natural site)-PstI (introduced site) fragment encompassing *ihfB* and endogenous *ihfB* promoter in pBBR1MCS-1 (31); pREM86, 547-bp SalI-PstI *ihfB* fragment lacking an internal 200-bp AvaII fragment originating from pREM85 in pNPTS139; and transcriptional reporters *pflif/lacZ*/290 (62), *pflil/lacZ*/290 (41), *pflIX/lacZ*/290 (44), *pflgF/lacZ*/290 (38), *pflgI/lacZ*/290 (42), *pflgK/lacZ*/290 (18), *pflIK/lacZ*/290 (18), and *pfljL/lacZ*/290 (36). In order to overexpress His-tagged IHF-B, the entire *ihfB* open reading frame was subcloned in frame downstream of the His<sub>6</sub>-encoding sequence in pET21b (Novagen). The overexpressed His-tagged IHF-B was purified by nickel affinity chromatography under denaturing conditions (4). DNA manipulations were performed essentially as described elsewhere (4). Plasmids were introduced into *C. crescentus* by bacterial conjugation. *C. crescentus* strains were grown at 31°C in peptone-yeast extract medium (PYE) (50) or M2 minimal medium (28) either alone or supplemented with one or more of the following: chloramphenicol (2.5  $\mu$ g ml<sup>-1</sup>), kanamycin (25  $\mu$ g ml<sup>-1</sup>), or tetracycline (2.0  $\mu$ g ml<sup>-1</sup>). PYE motility plates contained 0.3% agar. The *E. coli* strains were grown at 37°C in Luria-Bertani medium (4) either alone or supplemented with one or more of the following: chloramphenicol (30  $\mu$ g ml<sup>-1</sup>), kanamycin (50  $\mu$ g ml<sup>-1</sup>), or tetracycline (12.5  $\mu$ g ml<sup>-1</sup>).

**Construction of the  $\Delta$ *ihfB* strain JG1216.** The *ihfB1* deletion strain was generated utilizing the *sacB* selection method (55). Plasmid pREM86 was conjugated into *C. crescentus* LS107, and transformants were selected on PYE agar

containing kanamycin (25  $\mu$ g ml<sup>-1</sup>) and naladixic acid (20  $\mu$ g ml<sup>-1</sup>). Transformants were grown overnight in PYE without selection and plated onto PYE agar containing 2.5% sucrose. A total of 192 sucrose-resistant colonies were isolated and tested for kanamycin sensitivity and assayed for motility in PYE-0.3% agar. Of the 192 colonies chosen, only one was both kanamycin sensitive and displayed a motility defect, seen as a motile swarm much smaller in diameter than any produced from the other 191 colonies assayed. Deletion of the 200-bp AvaII fragment encompasses the ribosome binding site and start of translation through the first 52 encoded residues of the 96 codons which comprise the *ihfB* open reading frame. The presence of this deletion in the chromosome of JG1216 was confirmed by PCR. Note that 30 different kanamycin-sensitive, sucrose-resistant isolates that exhibited wild-type motility did not contain a deletion in *ihfB* as determined by PCR (data not shown). Complementation tests were performed by transducing either the wild-type allele into the  $\Delta$ *ihfB* strain or transducing the deleted *ihfB* allele into wild-type cells. In order to accomplish this, the gene directly adjacent to *ihfB*, *mscL* (CC3585), was amplified by PCR and subcloned into plasmid pNPST139, which cannot replicate in *C. crescentus*. The *mscL*-containing plasmid was then mated into either LS107 (wild-type) or JG1216 ( $\Delta$ *ihfB*) cells, and integrants were selected by kanamycin resistance. Transducing lysates of bacteriophage  $\phi$ CR30 were then prepared from each strain (13). Transduction recipients were selected for resistance to kanamycin and scored for motility defects. The effect of the *ihfB* deletion on the accumulation of IHF  $\alpha$ - and  $\beta$ -subunits was demonstrated by immunoblot analysis, using anti-IHF rabbit antiserum provided by S. Goodman or antisera raised against purified *C. crescentus* IHF-B.

**Flagellar gene expression.** The synchronizable LS107 and JG1216 *C. crescentus* strains either alone or harboring the *fliK-lacZ* reporter fusion were used in cell cycle gene expression experiments performed as described previously (38). The expression of all flagellar gene transcriptional reporters in unsynchronized cultures was determined in triplicate (on three independent cultures) by quantitative measurements of  $\beta$ -galactosidase activity as previously described (37). Immunoblots were prepared as described previously (61) and were analyzed using antiflagellin (*C. crescentus*), anti-IHF-B (*C. crescentus*), anti-IHF (*E. coli*), anti-MreB (*C. crescentus*) (16), and anti-HU (*C. crescentus*) (J. C. England and J. W. Grober, unpublished data) antisera.

## RESULTS AND DISCUSSION

**Cell cycle expression pattern of IHF.** We first determined whether the cellular concentration of IHF changed during the course of the cell cycle, since this could conceivably influence the temporal regulation of class III/IV flagellar gene transcription. Microarray experiments have indicated that the transcription of *ihfA* (*himA*), encoding the IHF  $\alpha$ -subunit, is under cell cycle control (34). Using antiserum generated against *E. coli* IHF, we observed two cross-reacting bands in an immunoblot of cell extracts that had been shown by previous experiments to correspond to *C. crescentus* IHF  $\alpha$ - and  $\beta$ -subunits (Fig. 1) (18, 19). We found that levels of both polypeptides increased gradually over the course of the cell cycle, with peak cellular concentrations occurring in predivisional cells (Fig. 1). This result is consistent with previous experiments showing that IHF was more concentrated in isolated predivisional cells than in isolated swarmer or stalked cells (18). It is interesting, however, that this temporal expression pattern does not parallel the cell cycle microarray data showing that *himA* (*ihfA*) mRNA levels peak in the swarmer cell type (33). These contrasting findings may indicate that an additional posttranscriptional mechanism is responsible for the cell cycle regulation of IHF levels.

**An IHF deficiency results in a motility defect.** In order to assess the role of IHF in the transcriptional regulation of late flagellar genes, we deleted a gene encoding one of the subunits of the IHF heterodimer. We chose to disrupt the single gene locus encoding the  $\beta$ -subunit, *ihfB* (*himD*) (CC3586), reasoning that a disruption in the *himA* locus could potentially affect expression of a downstream conserved hypothetical gene

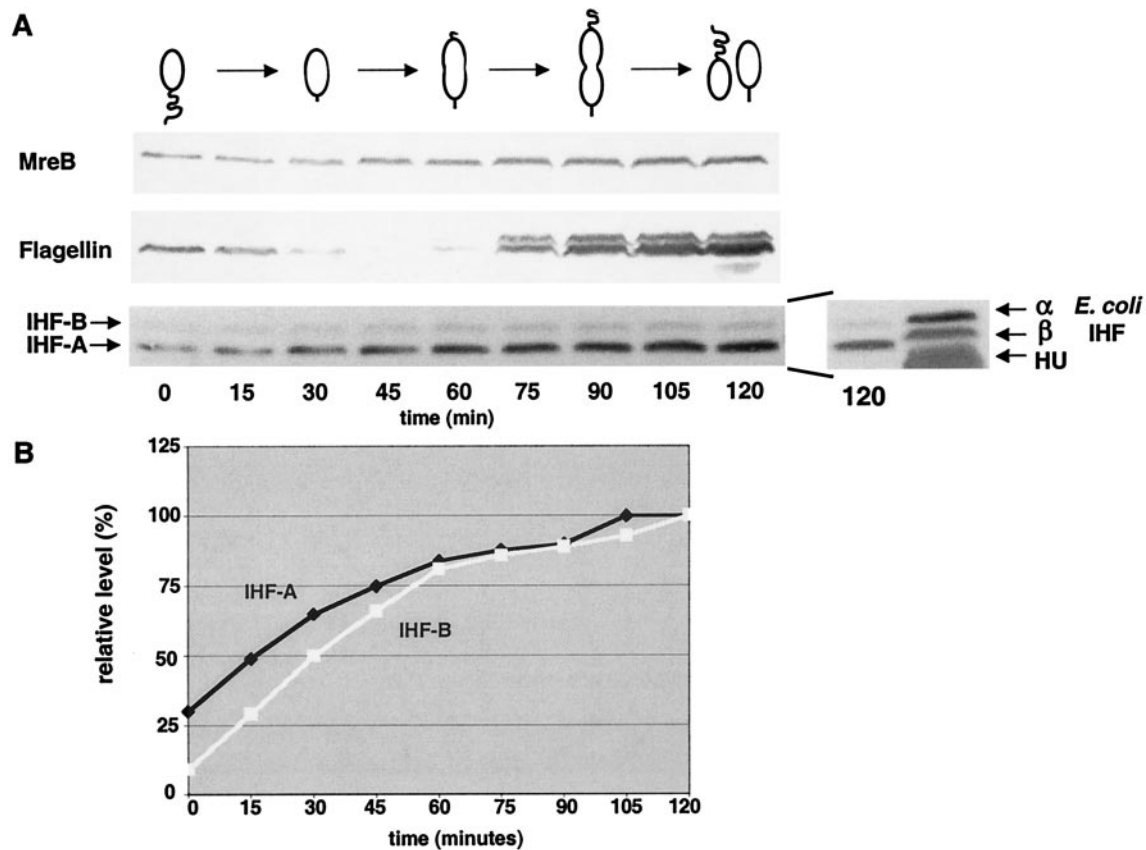


FIG. 1. Cell cycle expression of *C. crescentus* IHF. (A) Pure populations of wild-type swarmer cells were isolated (15) and allowed to progress synchronously through the cell cycle in PYE medium. At the time points indicated, cells from a 1-ml portion of culture (optical density at 600 nm, 0.35) were isolated by centrifugation. The resulting cell pellets were suspended in SDS sample buffer and boiled for 5 min, and an equal amount of each sample was subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblot analyses. A schematic of the cell types present at each time point during the cell cycle, as determined by light microscopy, is shown above the immunoblots. A sample of an *E. coli* cell extract was electrophoresed with the *C. crescentus* cell cycle for comparison. As previously demonstrated (17, 18), the *E. coli* anti-IHF antibodies cross-react with the two *C. crescentus* polypeptides that are similar in molecular mass to the *E. coli* IHF  $\alpha$ -subunit (10.5 kDa) and  $\beta$ -subunit (9.5 kDa). The *C. crescentus* IHF  $\alpha$ - and  $\beta$ -subunits (indicated as IHF-A and IHF-B, respectively) gradually increased in intensity over the course of the cell cycle. Note that the *C. crescentus* IHF  $\beta$ -subunit migrates slower than the  $\alpha$ -subunit (see below). The anti-MreB and anti-flagellin antisera served as two different cell type controls. (B) Graph depicting quantitation of IHF levels from the immunoblot shown in panel A during the course of the *C. crescentus* cell cycle. The x axis represents relative levels, with 100% representing the peak cell cycle level for each indicated subunit.

(CC1371) that together with *himA* (*ihfA*) comprises a two-gene operon. A plasmid containing *ihfB* was digested with *Ava*II and religated, generating a 200-bp internal deletion (Fig. 2A). The deleted gene was then integrated in the *C. crescentus* genome by homologous recombination. These cells were then grown in liquid medium and selected for excision of the integrated plasmid by plating onto sucrose-containing medium, followed by screening for kanamycin sensitivity (see Materials and Methods) (55). Previous experiments have demonstrated the presence of IHF binding sites upstream of at least 12 flagellar genes. Thus, we performed a secondary screen for a motility defect by inoculating the sucrose-resistant, kanamycin-sensitive colonies into semisolid motility agar. When grown in motility agar, strains in which motility defects are evident produce a halo of growth or swarm that is noticeably smaller in diameter than that produced by the wild-type strain. Surprisingly, the frequency of sucrose-resistant, kanamycin-sensitive colonies that also exhibited an unequivocal motility defect was quite low (1 out of 192 screened). The cells from this single

isolate were confirmed to have a deletion in *ihfB* when tested by PCR (data not shown), indicating a low frequency of allelic exchange via homologous recombination. This may have been a consequence of the small amount of homologous DNA on the plasmid containing the deleted *ihfB* gene (see Materials and Methods). The motility of the  $\Delta$ *ihfB* strain (JG1216) was compared to that of wild-type and nonmotile *flbD*::Tn5 cells by growth in motility agar (Fig. 2C). The  $\Delta$ *ihfB* strain appeared motile, with a swarm size approximately 50 to 60% that of wild-type cells (Fig. 2C), although liquid cultures contained significantly less motile cells than wild-type cultures when observed by phase-contrast microscopy (data not shown). We then performed a complementation test by introducing a multicopy plasmid containing the *ihfB* promoter and open reading frame (pREM85) into the  $\Delta$ *ihfB* strain. This plasmid was able to restore a wild-type proportion of motile cells to the mutant strain as judged by light microscopy (data not shown), suggesting that the motility defect displayed by the  $\Delta$ *ihfB* strain was a consequence of the absence of a functional *ihfB* gene. Both



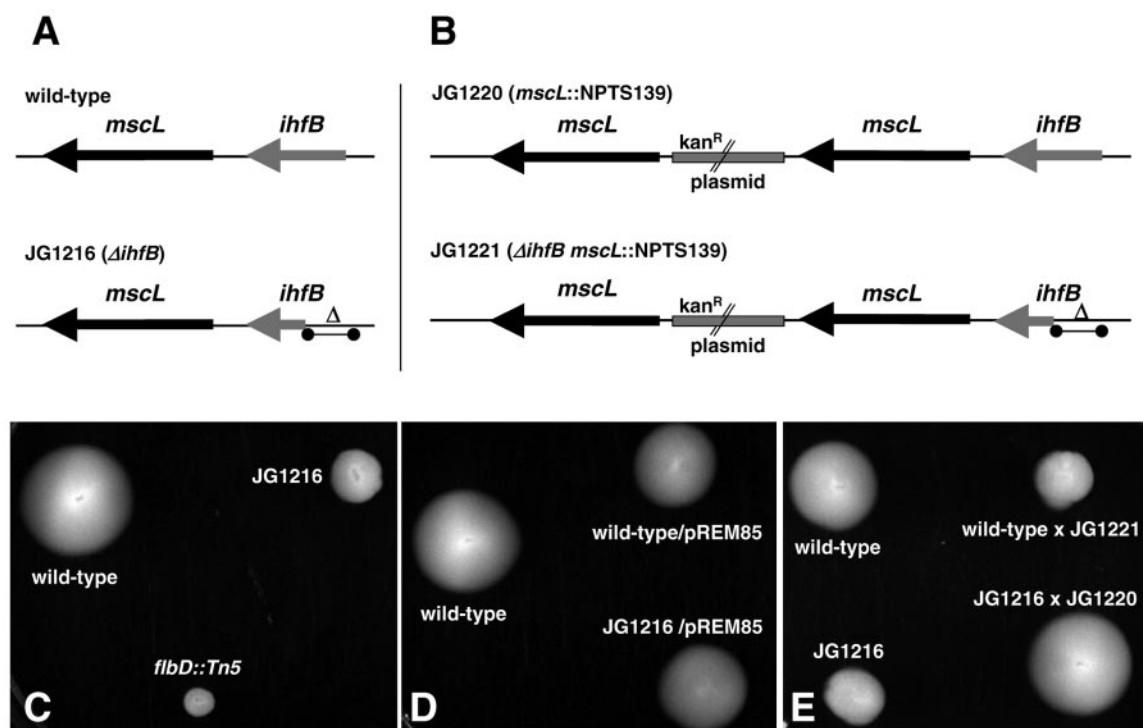


FIG. 2. The  $\Delta ihfB$  strain displays a motility defect and expresses reduced levels of the 27-kDa flagellin. (A) Schematic showing the region of the genome adjacent to *ihfB* and the introduced deletion in *ihfB* within strain JG1216. The open reading frame downstream of *ihfB*, *mscL* (CC3565), is predicted to encode a polypeptide with similarity to the large subunit of mechano-sensitive channels. (B) Schematic showing the pNPTS139 plasmid containing *mscL* integrated into either wild-type (JG1220) or  $\Delta ihfB$  (JG1221) cells. Integration of this plasmid via single-crossover, homologous recombination results in the presence of a duplicated *mscL* locus. Complementation tests were performed by transducing the kanamycin resistance marker from JG1220 or JG1221 into either wild-type or  $\Delta ihfB$  cells (see below). (C) *C. crescentus* wild-type (LS107), *flbD*::Tn5 (SC1032), and  $\Delta ihfB$  (JG1216) cells were inoculated into motility agar and grown for 4 days. The nonmotile *flbD*::Tn5 strain lacks the concentric ring of swarming cells displayed by the wild-type and  $\Delta ihfB$  strains. The  $\Delta ihfB$  strain displays a slight motility defect, demonstrated here as a motile swarm smaller in diameter (approximately 40%) than that generated by the wild-type strain. (D) The  $\Delta ihfB$  strain harboring *ihfB* on a multicopy plasmid, pREM85, generates a motile swarm of cells comparable in diameter to that generated by the wild-type strain harboring the same plasmid. (E) The integrated *mscL*::npts139 plasmid from either JG1221 ( $\Delta ihfB$ ) or JG1220 (wild-type *ihfB*) or that was transduced into the wild-type strain (wild type  $\times$  JG1221) or the  $\Delta ihfB$  strain (JG1216  $\times$  JG1220), respectively. The kanamycin-resistant transductants were then scored for a motility defect by inoculation into semisolid motility agar (see Results). Examples of strains that acquired a motility defect, in the case of wild-type recipient (wild-type  $\times$  JG1221), or a wild-type motility phenotype, in the case of  $\Delta ihfB$  recipient (JG1216  $\times$  JG1220), are shown.

wild-type and  $\Delta ihfB$  cells possessing pREM85 produced a smaller-sized swarm than wild-type cells without plasmid, which may be attributable to a slower growth rate of the plasmid-bearing cells (data not shown). The diameter of the swarm produced by both wild-type and  $\Delta ihfB$  cells containing pREM85 was identical, indicating that expression of *ihfB* from pREM85 probably complements the motility defect exhibited by the  $\Delta ihfB$  strain (Fig. 2D).

Since the swarm size of  $\Delta ihfB$  cells possessing pREM85 was significantly smaller than that exhibited by wild-type cells without plasmid, we performed an additional complementation test by transduction. We constructed strains in which a plasmid containing the adjacent *mscL* gene and a kanamycin resistance marker was integrated into either the wild-type or deleted *ihfB* gene (see Materials and Methods) (Fig. 2B). The transduction frequency of the kanamycin resistance marker into either wild-type or JG1216 cells was similar (approximately  $10^{-5}$  per PFU) whether the phage were derived from the wild-type strain or JG1216, indicating that acquisition of the deleted *ihfB* allele was not especially deleterious or required a secondary mutation. In order to determine whether the *ihfB* deletion was

responsible for the observed motility defect in the original strain, we also assessed the frequency of motility defects in each transduction experiment. Transducing phage derived from wild-type cells plated onto JG1216 resulted in a high frequency of recipients (94%) that had normal motility, thus complementing the motility defect in JG1216 (Fig. 2E). In a complementary experiment, we found that transducing phage prepared from JG1216 conferred a motility defect (Fig. 2E) to wild-type recipients at a similar frequency (95%). Thus, the integrated *mscL*-containing plasmid is 94 to 95% linked to *ihfB* by transduction. Importantly, this result shows that the low frequency of motility-deficient,  $\Delta ihfB$  cells recovered in the original screen is not a consequence of the required acquisition of second-site, compensatory mutations and that the observed motility defect is probably attributable to the deletion in *ihfB*.

**The cellular levels of the 27-kDa flagellin are reduced in an  $\Delta ihfB$  mutant strain.** Next we performed immunoblot analysis to determine whether the  $\Delta ihfB$  mutant strain was deficient in IHF. Initially, we used antiserum raised against the *E. coli* protein (Fig. 3A). As shown above (Fig. 1), this antiserum reacts with two low-molecular-weight polypeptides present in

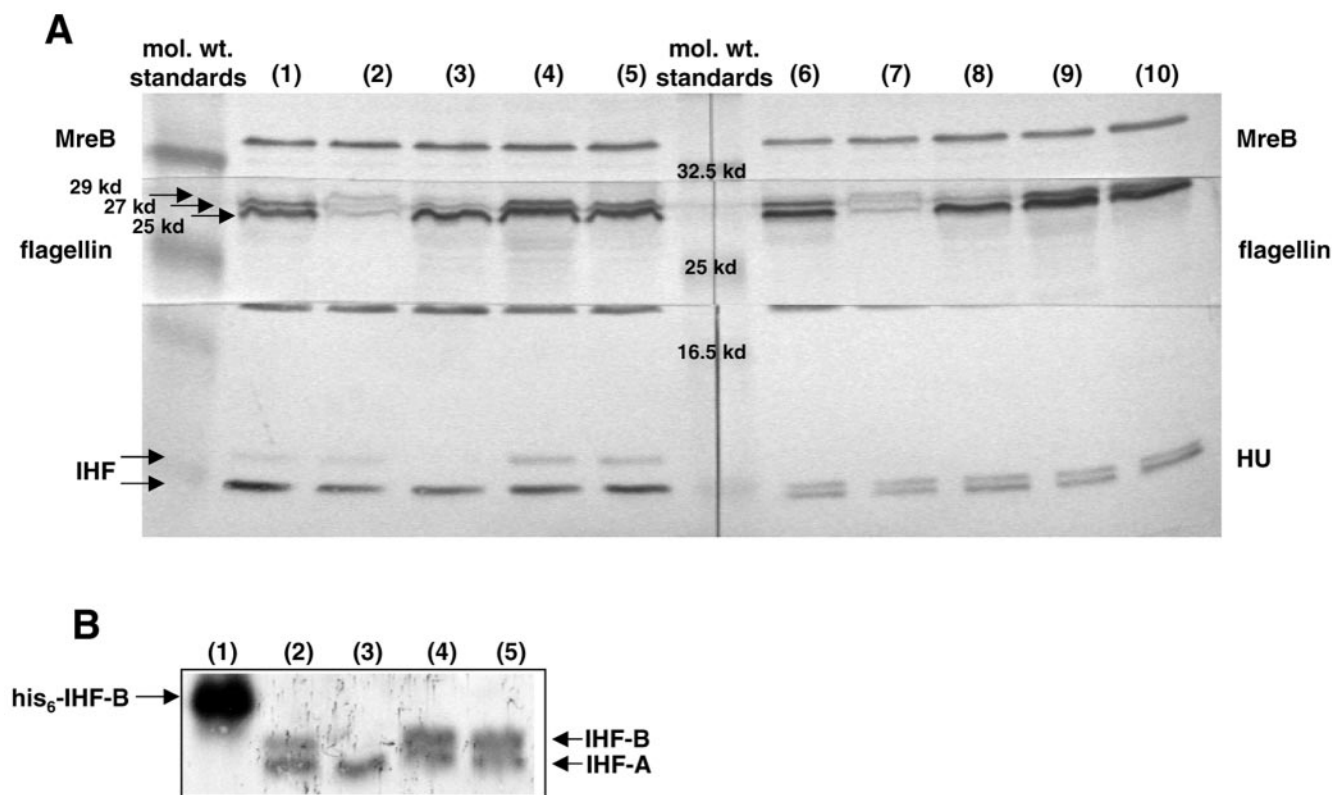


FIG. 3. The  $\Delta ihfB$  strain expresses reduced levels of the 27-kDa flagellin. (A) Crude cell extracts of LS107 (wild type) (lanes 1 and 6), SC1032 (*flbD::Tn5*) (lanes 2 and 7), JG1216 ( $\Delta ihfB$ ) (lanes 3 and 8), JG1216 containing pREM85 (lanes 4 and 9), and LS107 containing pREM85 (lanes 5 and 10) were prepared and subjected to immunoblot analyses with anti-flagellin, *E. coli* anti-IHF (lanes 1 to 5), anti-HU (lanes 6 to 10), and anti-MreB (loading control) antisera. The IHF  $\alpha$ -subunit and  $\beta$ -subunit electrophoresed as two distinct bands, with the slower-migrating band (indicated as IHF) absent in extract prepared from the  $\Delta ihfB$  strain. The  $\Delta ihfB$  strain contains reduced amounts of the 27-kDa flagellin while still possessing wild-type levels of the 25- and 29-kDa flagellins. Expression of *ihfB* from the multicopy plasmid construct pREM85 restores wild-type levels of the 27-kDa flagellin to the  $\Delta ihfB$  strain. (B) Cell extracts of LS107 (wild type) (lane 2), JG1216 ( $\Delta ihfB$ ) (lane 3), JG1216 containing pREM85 (lane 4), and LS107 containing pREM85 (lane 5) were prepared and subjected to immunoblot analysis with *C. crescentus* anti-IHF-B antibody. Purified His<sub>6</sub>-IHF-B was loaded in lane 1. The slower-migrating band is absent in the  $\Delta ihfB$  strain.

extracts of wild-type *C. crescentus* cells that presumably correspond to the  $\alpha$ - and  $\beta$ -subunits of IHF. This antiserum reacted weakly with the polypeptide of apparent higher molecular weight (Fig. 3A, lane 1) and showed an absence of this slower-migrating species in the  $\Delta ihfB$  cells (Fig. 3A, lane 3). Since the slower-migrating band disappeared in this mutant strain, we propose that this polypeptide corresponds to the  $\beta$ -subunit of *C. crescentus* IHF. This is in contrast to the behavior of IHF from *E. coli*, in which the slower-migrating species corresponds to the higher-molecular-weight IHF  $\alpha$ -subunit. In support of this idea, the introduction of an intact copy of *ihfB* on a plasmid (pREM85) restored, and increased, the level of expression of the slower-migrating polypeptide (Fig. 3A, lane 4). Since the *C. crescentus* IHF  $\beta$ -subunit and 9.6-kDa HU proteins share approximately 31% identity and the antiserum we employed in these experiments cross-reacts with *E. coli* HU (Fig. 1), we performed a control to determine whether the polypeptides observed in these immunoblots corresponded to HU. In order to accomplish this, we employed *C. crescentus* anti-HU antibody (11) in immunoblots of cell extracts prepared from each of these strains (Fig. 3A). The HU antiserum showed two distinct polypeptides, one that was of similar apparent molecular weight to the faster-migrating band observed using anti-

IHF antiserum (Fig. 3A). These polypeptides were of a molecular weight distinct from that of the slower-migrating polypeptide detected with the anti-IHF antibody. Furthermore, the levels of the HU subunits were unaffected in the  $\Delta ihfB$  strain, suggesting that the slower-migrating band is IHF.

In order to confirm that the slower-migrating polypeptide absent in the  $\Delta ihfB$  strain was in fact the  $\beta$ -subunit of *C. crescentus* IHF, we raised antibodies to purified His-tagged IHF-B from *C. crescentus*. This antiserum reacted strongly with the purified His-tagged IHF-B (Fig. 3B, lane 1), as well as with two polypeptides present in extracts from wild-type cells of approximate predicted molecular masses corresponding to either subunit of IHF (Fig. 3B, lane 2). The slower-migrating species was once again absent in extracts prepared from the  $\Delta ihfB$  strain (Fig. 3B, lane 3) and was restored by the introduction of *ihfB* on plasmid pREM85 (Fig. 3B, lane 4). This result indicates that the  $\Delta ihfB$  strain is deficient in the synthesis of the *ihfB* gene product and that this subunit of IHF migrates slower under these electrophoretic conditions than the  $\alpha$ -subunit. We speculate that the slower migration of this polypeptide may be attributable to either differences in the degree of its association with sodium dodecyl sulfate (SDS) and/or its isoelectric point.

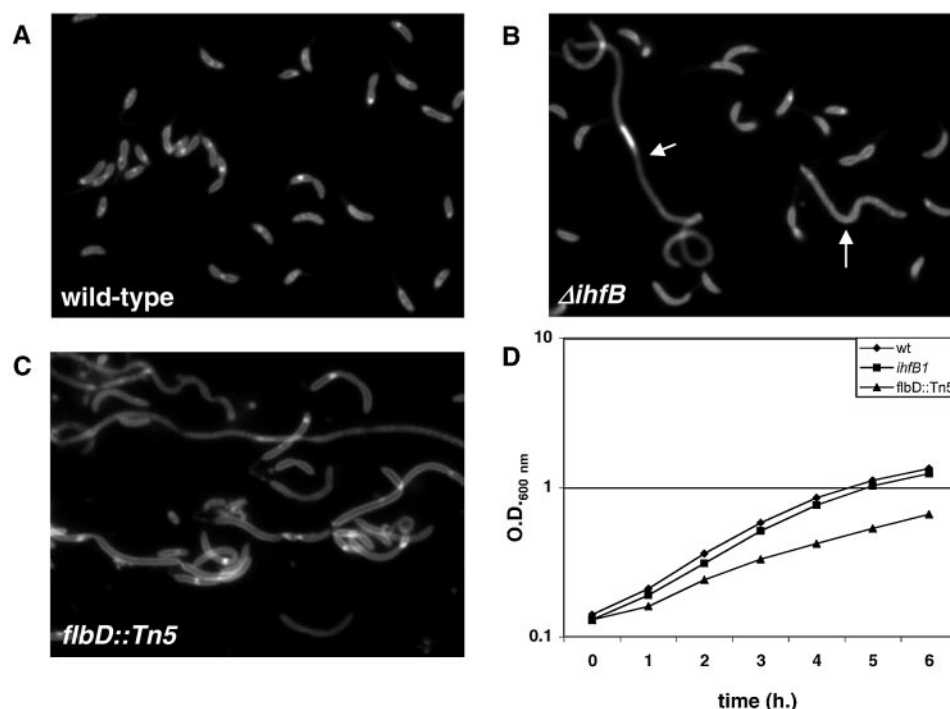


FIG. 4. Loss of IHF alters cell morphology but does not affect the growth rate of *C. crescentus* cells. Shown are phase-contrast micrographs of the wild-type (A),  $\Delta ihfB$  (B), and  $flbD::Tn5$  (C) strains grown in liquid PYE to an optical density at 600 nm of 0.5 and stained with the lipophilic fluorescent dye FM4-64. *C. crescentus* class II flagellar mutants exhibited a characteristic cell division defect, seen as an accumulation of filamentous cells during the mid- to late-log phase of growth. Cultures of  $\Delta ihfB$  cells contained some elongated and filamentous cells (indicated by arrows) (B); however, they do not resemble the cell division phenotype typical of class II flagellar mutants, such as  $flbD::Tn5$  (C). (D) Comparison of growth rates of the wild-type,  $\Delta ihfB$ , and  $flbD::Tn5$  strains grown in liquid PYE medium. The class II flagellar mutant,  $flbD::Tn5$ , grows 1.5-fold slower than either the wild-type or  $\Delta ihfB$  strain. While the  $\Delta ihfB$  strain displays a cell division defect mildly similar to that exhibited by the  $flbD::Tn5$  strain (B), it grows at a wild-type rate.

Since the  $\Delta ihfB$  mutant strain exhibited a motility defect, we next determined if the  $\Delta ihfB$  mutation affected the cellular levels of flagellin (Fig. 3A). Previous mutational analysis of flagellar promoters predicted that the IHF-deficient strain may lack significant class III flagellar gene expression, thus resulting in a partially assembled basal body-hook complex (18, 19, 38). In the absence of a completed class III-encoded hook-basal body structure, the mRNAs of class IV genes, encoding the flagellin proteins of the filament, are not translated (2, 3, 36, 37). Immunoblot analysis of cell extracts prepared from the wild-type,  $flbD::Tn5$  (the class III/IV transcriptional activator) and  $\Delta ihfB$  strains revealed that the  $\Delta ihfB$  strain expressed wild-type levels of the most abundant filament protein, the 25-kDa flagellin, that are encoded by the class IV *fljKMNO* genes (Fig. 3A). Interestingly, the 27-kDa flagellin, encoded by *fljL*, was present in significantly reduced amounts in the  $\Delta ihfB$  strain, resembling that produced in the  $flbD::Tn5$  strain. Expression of *ihfB* from pREM85 completely restored production of FljL to the  $\Delta ihfB$  strain. In fact, the amount of the 25- and 27-kDa flagellins was found to be slightly higher in both the wild-type and  $\Delta ihfB$  strains harboring pREM85 than in the wild-type strain alone (Fig. 3A).

**Cells possessing a  $\Delta ihfB$  mutation exhibit a minor cell division defect.** Class II flagellar mutants exhibit a cell division defect characterized by the presence of an abundant frequency of elongated cells when grown in PYE medium (41, 66). Likewise, such mutants typically grow at a reduced rate compared

to either wild-type or late flagellar assembly mutant strains when cultured in liquid medium (41). In contrast, class III and IV flagellar mutants are morphologically indistinguishable from wild-type cells when observed under the light microscope (41). We compared the cell morphology (Fig. 4A to C) and growth rate (Fig. 4D) of the  $\Delta ihfB$  strain to those of the wild-type and the  $flbD::Tn5$  cells grown in PYE medium. The  $\Delta ihfB$  mutant strain exhibited a relatively minor cell division defect, with the accumulation of some slightly elongated and filamentous cells (approximately 10%) in late logarithmic phase (Fig. 4B). This is in contrast to a  $flbD::Tn5$  strain (Fig. 4C) and other class II flagellar mutants, which exhibit an abundance of filamentous cells as the culture approaches stationary phase. Although the  $\Delta ihfB$  cells were less motile and possessed a minor cell division defect, they grew at a rate almost identical to that of wild-type cells, approximately 1.5-fold faster than the growth rate observed for the  $flbD::Tn5$  cells (Fig. 3D). Interestingly, recent experiments have shown that the *C. crescentus* origin of DNA replication contains IHF binding sites (56). It is hypothesized that IHF binding to these sites enhances the initiation of replication. The growth rate and cell morphology data presented here indicate that there is probably no gross cell cycle defect in this strain lacking IHF. We have also found that there is no increase in the frequency of anucleate cells in the  $\Delta ihfB$  strain (data not shown).

**Effect of  $\Delta ihfB$  on flagellar gene expression.** A role for IHF in the temporal and spatial expression of late flagellar genes in

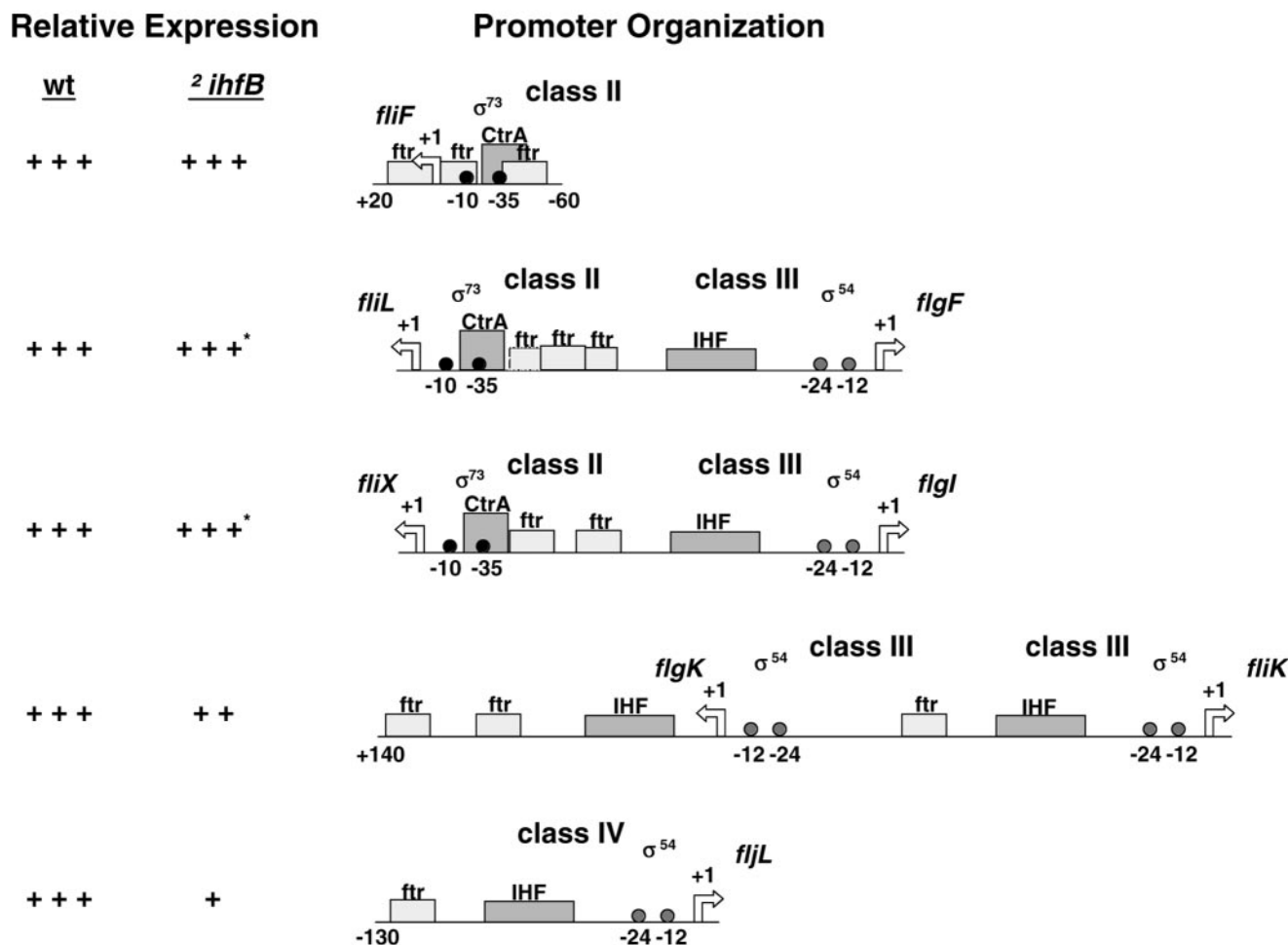


FIG. 5. *C. crescentus* class II, III, and IV flagellar promoter organization. The schematic depicts the locations of *cis*-acting regulatory elements relative to the transcription initiation site of the flagellar promoters used in this study. The consensus sequences corresponding to the  $\sigma^{54}$  and  $\sigma^{73}$  binding sites, -12 and -24 (gray circles) and -10 and -35 (black circles), respectively, are shown. A checkered box illustrates the consensus sequence for the IHF binding site, a striped box shows the consensus CtrA binding site, and a dotted box denotes the enhancer-like *ftr* (flagellar transcription regulator) site recognized by the transcription factor FlbD. To the left is a summary of the data presented in the figure. +++, expression level equal to wild-type levels; +, expression level 20% of wild type. Note, for the class II-class III-divergent promoters shown, reporter gene expression measured in the  $\Delta ihfB$  strain was marginally lower for the class II promoters and was slightly higher for the class III promoters than those measured in the wild-type strain.

*C. crescentus* had been previously suggested by assaying the expression of flagellar reporter gene fusions that possessed mutated IHF binding sites (18, 19). These initial studies focused on the transcription of the divergently transcribed class III flagellar genes, *flgK* and *fliK* (formerly *flaN* and *flbG*, respectively). Both *flgK* and *fliK* require FlbD and  $\sigma^{54}$ -containing RNA polymerase for transcription, as well as an intact IHF binding site, in order to achieve maximal expression levels (18, 19). The intergenic regulatory region shared by *flgK* and *fliK* is unique in *C. crescentus* in that it is the only flagellar promoter region that contains two divergently transcribed class III genes (14, 17) (Fig. 5). It is organized such that IHF binding and bending the DNA, in conjunction with FlbD binding, could create a higher-order nucleosome-like structure (18). The remaining class III flagellar genes are divergently transcribed from class II flagellar genes (or class II flagellar gene operons) that require  $\sigma^{73}$ -containing RNA polymerase and CtrA for expression (14, 17, 27) (Fig. 5). For example, the class III

flagellar gene *flgF*, encoding the proximal rod, is divergently transcribed from the class II gene *fliL* (Fig. 5) (67). Likewise, the class III, *flgI* operon, which also contains putative IHF and FlbD binding sites, is divergently transcribed from the class II *fliX* gene (Fig. 5). As with the *fliK* and *flgK* promoters, mutations in the IHF binding site of the *flgF* promoter have indicated that IHF is required for maximal transcription (38). In contrast, the class IV flagellin genes all exist as solitary transcription units (Fig. 5). We hypothesized that the higher-order structure induced upon IHF binding may have varied effects on the transcriptional activity of these differently organized divergent promoters. For example, does IHF binding to a class III promoter influence the CtrA-activated transcription of the divergent class II promoter? We investigated the role of IHF in transcriptional activation of late flagellar genes, including any effects on expression of the divergently transcribed early class II flagellar genes. In order to accomplish this, we assayed the expression of flagellar promoter-*lacZ* transcription fusions in



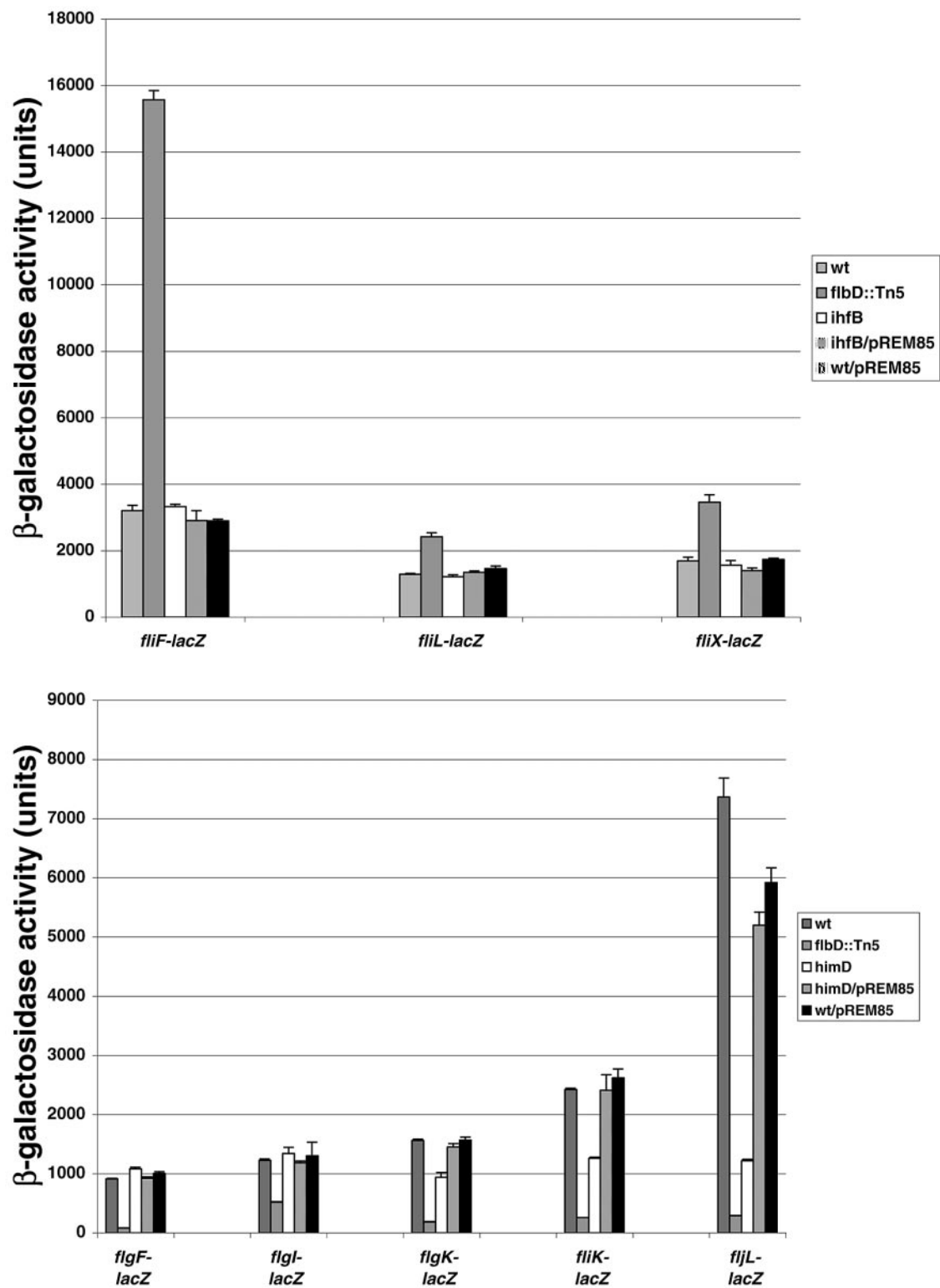


FIG. 6. Effect of IHF on expression of flagellar genes. (A) Activity of the indicated class II flagellar gene-*lacZ* transcriptional reporter fusions was assayed in strains containing the  $\Delta ihfB$  (JG1216) allele (white bars) as well as in wild-type cells (LS107) (gray bars), a strain containing *flbD*::Tn5 (SC1032) (dark gray bars), JG1216 containing *ihfB* on a multicopy plasmid (pREM85) (hatched bars), and wild-type cells containing pREM85 (black bars). Levels of reporter gene expression are shown in units of  $\beta$ -galactosidase activity. The expression of the flagellar gene transcriptional reporters in unsynchronized cultures was determined in triplicate (on three independent cultures) by quantitative measurements of  $\beta$ -galactosidase activity as previously described (37). The shown values represent the mean activity. The error bars represent the standard deviation. (B) Effect of IHF on expression of class III and IV flagellar genes. The activity of the indicated flagellar gene-*lacZ* transcriptional reporter fusions was assayed in strains containing the  $\Delta ihfB$  (JG1216) allele (white bars) as well as in wild-type cells (LS107) (gray bars), a strain



the  $\Delta ihfB$  mutant strain. The promoters in this experiment consisted of the divergent class II *fliL-lacZ* and class III *flgF-lacZ* fusions and the divergent class II *fliX-lacZ* and class III *flgI-lacZ* fusions (Fig. 5).  $\beta$ -Galactosidase activity generated from these fusions was compared with that measured in wild-type and *flbD::Tn5* cells, as well as in  $\Delta ihfB$  cells containing the complementing plasmid, pREM85. We also measured the expression of the two divergent class III promoters, using *flgK*- and *fliK-lacZ* reporter fusions, as well as the class IV, *fliJ-lacZ* fusion. A schematic of the promoters used in these assays and a summary of the results is depicted in Fig. 5.

As a control, we tested the expression of the class II *fliF-lacZ* fusion, which contains a binding site for the transcriptional activator, CtrA, but does not possess an IHF binding site (Fig. 5). Expression of *fliF-lacZ* in the  $\Delta ihfB$  strain was no different than that observed for the wild-type strain and, as previously reported, was found to be fivefold higher in the *flbD::Tn5* strain (Fig. 6A). Thus, a deletion in IHF probably does not have an adverse effect on CtrA-activated gene expression. In the  $\Delta ihfB$  strain, the divergently transcribed class II flagellar reporters, *fliL-lacZ* and *fliX-lacZ*, were expressed at essentially wild-type levels, being reduced only 5% compared to expression in the wild-type strain (Fig. 6A). The activity of both of these increased approximately twofold in the *flbD::Tn5* strain relative to wild-type, similar to what has been previously reported for these class II promoters (35). Interestingly, promoter activity of reporter gene fusions of the two genes divergently transcribed from *fliL* and *fliX*, the class III *flgF* and *flgI* genes (Fig. 6B), was slightly increased in the  $\Delta ihfB$  strain (ca. 10 to 20% increase) compared to that in wild-type cells (Fig. 6B). This was an unanticipated result, because *flgF-lacZ* reporter expression, which is governed solely by the class III promoter elements, had been previously shown to decrease when IHF binding sites were mutated (38). These results also differ from the example of the divergently transcribed  $\sigma^{54}$ -dependent *prpBCDE* operon of *Salmonella enterica* serovar Typhimurium (49). Like these two *C. crescentus* flagellar promoters, the *S. enterica prpBCDE* promoter shares a regulatory region with the divergently transcribed  $\sigma^{73}$ -governed *prpR* gene. In contrast to the results presented here, transcription of the *prpBCDE* promoter is reduced 15-fold in the absence of IHF (49).

The activity of the class III divergently transcribed promoters *flgK* and *fliK* was significantly decreased in the absence of IHF (Fig. 6B). Although this effect was not as severe as that observed in the *flbD::Tn5* strain (approximately 10% compared to wild-type cells), the *flgK-lacZ* and *fliK-lacZ* expression levels assayed in the  $\Delta ihfB$  strain were found to be 60 and 48% of wild-type, respectively (Fig. 6B). Introduction of the complementing plasmid containing *ihfB* restored wild-type reporter gene expression to the  $\Delta ihfB$  mutant (Fig. 6B). Previous experiments using *fliK* and *flgK* reporter gene fusions with

mutated IHF binding sites showed an approximate 35 to 50% reduction in  $\beta$ -galactosidase expression (18, 19). Thus, these results are consistent with a previously proposed model for IHF in the transcriptional activation of  $\sigma^{54}$  promoters, in which IHF-mediated looping of the DNA intervening between the promoter and enhancer sequences facilitates interaction between RNA polymerase and the FlbD transcriptional activator but is not absolutely required for transcriptional activation.

Since  $\Delta ihfB$  cells lack significant amounts of the 27-kDa flagellin (FljL) (Fig. 3), we next investigated the influence IHF has on transcription of the class IV *fliJ* gene (40) (Fig. 6B). At least two possible mechanisms might account for the decreased concentration of the 27-kDa flagellin in the  $\Delta ihfB$  strain. First, the observation that  $\Delta ihfB$  cells have a decrease in *fliK* and *flgK* expression may indicate an absence of a completed class III structure in a significant population of  $\Delta ihfB$  mutant cells which, in turn, inhibits the translation of the *fliJ*-encoded message (2, 36, 37). A second possibility for the decreased amount of FljL in the  $\Delta ihfB$  mutant is that transcription of *fliJ* is reduced in the absence of IHF. Consistent with this latter case, *fliJ-lacZ* expression was the most severely affected of the flagellar genes we assayed in the IHF mutant strain (Fig. 6B). The *fliJ-lacZ* reporter gene expression levels dropped to 20% of wild-type in IHF-deficient cells (Fig. 6B). Introduction of the *ihfB*-complementing plasmid restored a significant level of *fliJ-lacZ* expression to the  $\Delta ihfB$  cells (Fig. 6B). These data suggest that the observed defect in *fliJ* transcription in the  $\Delta ihfB$  strain contributes to the loss of the 27-kDa flagellin and the subsequent motility defect exhibited by the  $\Delta ihfB$  mutant. Consistent with this conclusion, the motility defect exhibited by the  $\Delta ihfB$  strain is similar to that observed when *fliJ* is deleted (40).

#### Effect of IHF on cell cycle expression of late flagellar genes.

In *C. crescentus*, coordination of the progression of flagellar assembly and cell division ensures the correct timing of expression of late flagellar genes (14, 17). A delay in class III flagellar gene transcription would yield an incomplete flagellar structure by the time of cell division, resulting in a loss of motility. One plausible possibility was that the impaired motility phenotype exhibited by the  $\Delta ihfB$  strain could be attributed to just such a delay in late flagellar gene expression. In order to address this idea, we determined whether IHF influences the temporal expression of late flagellar genes by assaying the cell cycle pattern of *fliK-lacZ* expression in the  $\Delta ihfB$  strain and compared it to that observed for wild-type cells (Fig. 7). The  $\Delta ihfB$  strain was found to possess an almost identical cell cycle-regulated pattern of *fliK-lacZ* reporter gene expression as that observed for the wild-type strain, with maximal promoter activity in the predivisional cell stage (Fig. 7). Thus, it appears that a delay in late flagellar gene expression cannot account for either the decrease in class III *fliK* transcription or impaired motility.

containing *flbD::Tn5* (SC1032) (dark gray bars), JG1216 containing *ihfB* on a multicopy plasmid (pREM85) (hatched bars), and wild-type cells containing pREM85 (black bars). Levels of reporter gene expression are shown in units of  $\beta$ -galactosidase activity. The expression of the flagellar gene transcriptional reporters in unsynchronized cultures was determined in triplicate (in three independent cultures) by quantitative measurements of  $\beta$ -galactosidase activity as previously described (37). The shown values represent the mean activity. The error bars represent the standard deviations. The deletion in *ihfB* results in a significant decrease in *flgK* (hook-associated protein), *fliK* (hook operon), and *fliJ* (27-kDa flagellin) promoter activity.

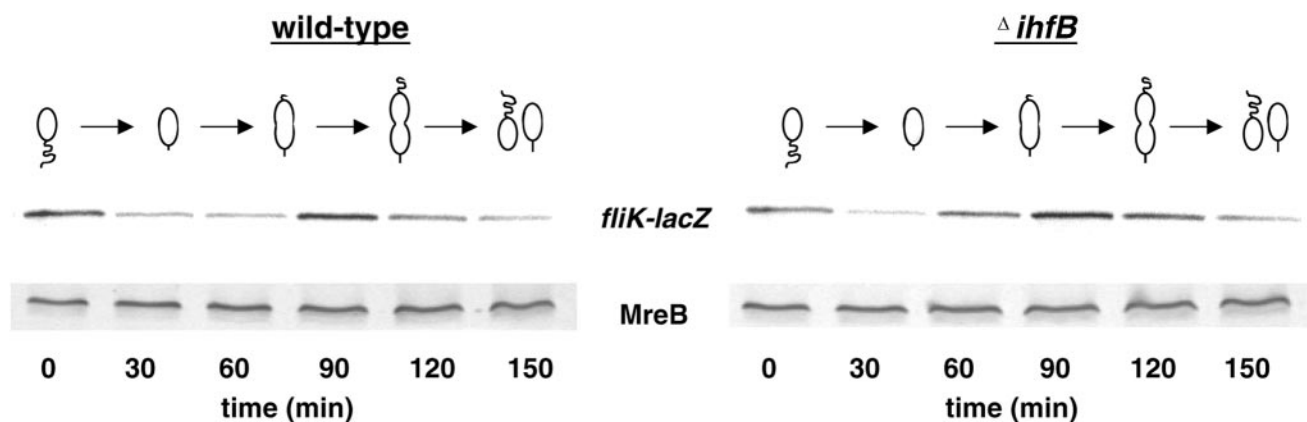


FIG. 7. Cell cycle expression of late flagellar genes is unaffected by the loss of IHF. Pure populations of wild-type and  $\Delta ihfB$  swarmer cells containing a class III, *fliK-lacZ* transcriptional reporter fusion were isolated and allowed to progress synchronously through the cell cycle in M2 minimal medium. At the indicated time points a portion of the culture was pulse-labeled with  $^{35}\text{S}$ Trans-label for 5 min followed by an ice-cold methionine chase. The labeled reporter fusion was immunoprecipitated using anti- $\beta$ -galactosidase antibody from portions of each time point sample containing equal amounts of labeled protein and separated by SDS-PAGE. Cells from a 1-ml portion of each culture were also isolated by centrifugation at the time points indicated. The resulting cell pellets were processed and subjected to immunoblot analyses. The anti-MreB (loading control) immunoblot is shown directly below the corresponding phosphorimage depicting the *fliK-lacZ* cell cycle expression pattern observed for the wild-type and  $\Delta ihfB$  strains. A schematic of the cell types present at each time point during the cell cycle, as determined by light microscopy, is shown above the phosphorimages of the dried electrophoresed gels. The  $\Delta ihfB$  strain exhibited an almost identical cell cycle pattern of *fliK-lacZ* reporter gene expression as the wild-type strain, with peak expression occurring in the predivisional cell.

**Conclusions.** The data presented here show that IHF is required for achieving maximal expression of a subset of the cell cycle-regulated, FlbD-dependent class III and IV flagellar genes and, consequently, for normal flagellar assembly and motility. These findings are analogous to those reported for an *ihfA* mutant of *P. putida* that exhibited reduced expression of the  $\sigma^{54}$ -dependent *xyl* operon and consequently was unable to degrade benzyl alcohol (9). We propose that the motility defect in the *C. crescentus ihfB* mutant strain is attributable to markedly reduced levels of the 27-kDa flagellin (FljL). This is more than likely the result of reduced transcription of *fljL* caused directly by the absence of IHF, and not the result of a flagellar assembly defect, since previous experiments have shown that flagellar assembly represses *fljL* translation but not transcription.

One surprising finding in *C. crescentus* was that an absence of IHF had varied effects on the different flagellar class III and IV promoters. We speculate that this may be attributed to the differences in the overall organization of each of the late flagellar promoter regions. Experiments in *E. coli* that examined IHF function in regulating bacteriophage Mu transcription were among the first to indicate that the ability of IHF to organize regulatory DNA sequences into stable supercoiled structures is important in influencing or controlling gene expression (24). The negative supercoils, possibly generated during transcription of the class II genes at the class II-class III divergent promoter regions, could function in place of IHF to enhance interaction of *ftr*-bound FlbD and  $\sigma^{54}$ -containing RNA polymerase at the class III regulatory region and, thus, promote transcriptional initiation. In fact, IHF function has been demonstrated to be dispensable for the  $\sigma^{54}$ -dependent expression of the NR<sub>1</sub>-regulated *glnHp2* promoter when negative supercoils are present in the expression templates and

NR<sub>1</sub>-phosphate is supplied to the transcription reaction mixture (10).

We propose that the reduced expression levels observed for the class III divergently transcribed *flgK* and *fliK* flagellar genes in the  $\Delta ihfB$  strain may be attributed to the multiple *ftr* sites present within this promoter region (Fig. 5). FlbD belongs to a class of transcriptional activators that is known to oligomerize at the enhancer DNA sequences (26, 51). Oligomerization of these transcriptional activators is thought to greatly enhance their function in stimulating the isomerization of RNA polymerase-bound closed complex to an open complex (32, 51). FlbD binding at one of the two *ftr* elements positioned within each half of the class III divergent promoter region may aid in the recruitment of additional molecules of FlbD to the regulatory region shared by the *flgK* and *fliK* flagellar genes. IHF-induced DNA bending may serve to enhance the interaction of FlbD molecules bound to these distantly spaced enhancer sequences. Interestingly, the expression of the transcription of *fljL* was markedly more sensitive than other class III or IV promoters to an absence of IHF. It is thought that the major role of IHF in the transcriptional activation of these promoters is to deliver FlbD bound at the enhancer sequences to RNA polymerase bound to the promoter region. Previous experiments using *nif* promoters have indicated that an IHF-induced bend is required when RNA polymerase binds weakly to promoter DNA sequences (26, 54). Therefore, a plausible mechanism to account for the stringent requirement for IHF in *fljL* transcriptional activation could be a reflection of weak binding of RNA polymerase and/or FlbD to the *fljL* promoter region.

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