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RESEARCH LETTER

Probing flagellar promoter occupancy in wild-type and mutant *Caulobacter crescentus* by chromatin immunoprecipitation

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Abstract

In the asymmetric predivisional cell of *Caulobacter crescentus*, TipF and TipN mark the cellular pole for future flagellar development. TipF is essential for motility and contains a cyclic-di-GMP phosphodiesterase-like (EAL) domain that is necessary for proper function. TipN is localized to the flagellar pole before TipF and is essential for the proper placement of the flagellum in *C. crescentus*. Using β-galactosidase promoter-probe assays and quantitative chromatin immunoprecipitation, we investigated the influence of the *C. crescentus* flagellar assembly regulator TipF on flagellar gene transcription. We compared the transcriptional activity of class II-*fliF-lacZ*, class III-*flgE-lacZ*, and class IV-*fljL-lacZ* fusions in a Δ*tipF* mutant with that of other flagellar mutants and the wild-type strain. We subsequently verified the *in vivo* occupancy of the *fliF*, *flgE*, and *fljL* flagellar promoters by the flagellar regulators CtrA, FlbD, and FliX in addition to RNA polymerase. We deduce that TipF contributes to proper expression of flagellar genes in *C. crescentus* by acting both within and outside of the canonical flagellar gene expression hierarchy.

Introduction

The alphaproteobacterium *Caulobacter crescentus* divides asymmetrically every cell cycle to form two dissimilar progeny: a nonmotile stalked cell and a motile, polarly flagellated swarmer cell. Assembly of the single, polar flagellum in the predivisional cell occurs with the aid of the birth scar markers TipN (Huitema *et al*., 2006; Lam *et al*., 2006) and TipF (Huitema *et al*., 2006). The latter contains an EAL domain homologous to the catalytic domain in bis-(3′-5′)-cyclic dimeric GMP (cyclic-di-GMP) phosphodiesterases (Bobrov *et al*., 2005; Schmidt *et al*., 2005; Tamayo *et al*., 2005; Huitema *et al*., 2006). Cells that lack TipF are nonmotile and impaired in the translation and secretion of the FljK flagellin, a class IV flagellar gene product and major component of the flagellar filament (Huitema *et al*., 2006). With the goal of further characterizing the flagellar assembly defect of TipF$^-$ cells, we studied flagellar gene expression in Δ*tipF* cells, comparing it with that of wild-type (*WT*) cells and other flagellar assembly mutants.

Flagellar biogenesis in *C. crescentus* requires over 50 genes organized into a regulatory hierarchy of four expression classes (Fig. 1) to link the assembly of flagellar gene expression to cell cycle progression (Minnich & Newton, 1987; Ohta *et al*., 1991; Ramakrishnan *et al*., 1994). The master cell cycle transcriptional regulator CtrA, encoded at the class I transcriptional level, accumulates and initiates the transcription of class II flagellar genes in S-phase (Quon *et al*., 1996). As class II gene products are expressed and assembled into the early (MS-ring basal body) substructure, their transcription ceases as a result of the repressive action of the σ^{54}-dependent transcriptional regulator FlbD and its interacting partner FliX (Mohr *et al*., 1998; Anderson & Gober, 2000; Gober & England, 2000) at the time of cell division. Concurrent with the repression of class II genes, FlbD/FliX and σ^{54}-containing RNA polymerase ($E\sigma^{54}$) activate the transcription of class III/IV flagellar genes that form the hook (FlgE), P-, and L-rings and the flagellar filament (Anderson & Gober, 2000). An additional layer of regulation operates on the expression of class IV (flagellin) genes, whose message stability is modulated by the negative regulator FlbT, an RNA-binding protein (Mangan *et al*., 1999), and FlaF, a protein with unknown biochemical activity (Llewellyn *et al*., 2005). Collectively, all levels of regulation ensure the accrual of gene products at the time when they are needed for the ordered expression and assembly into the growing flagellum structure.

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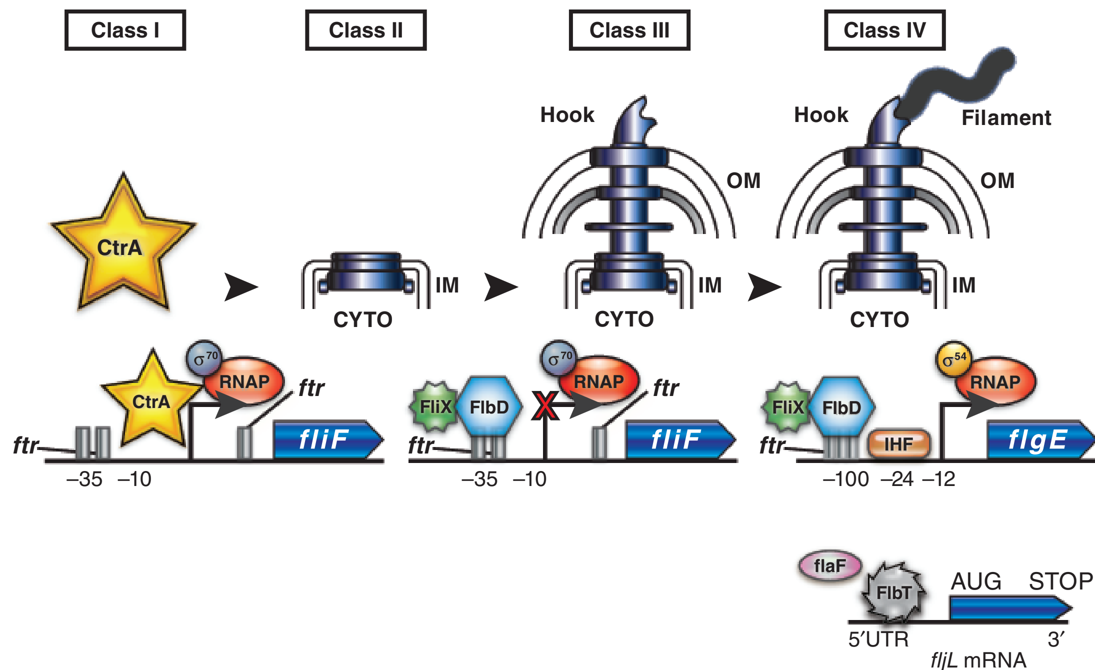

Fig. 1. Illustration of flagellar biogenesis in *Caulobacter crescentus* divided into four classes. The upper diagram depicts flagellar assembly as it proceeds from the inner cell membrane (IM) to the outer cell membrane (OM). The hook is assembled on the outside of the cell, followed by the filament. The lower section shows a schematic of representative genes (*fliF*, *flgE*, and *fljL*) from each class with the regulatory elements: FliX, FlbD, CtrA, σ^{70}-, and σ^{54}-containing RNAP, integration host factor (IHF), and flagellar transcriptional regulator (*ftr*) present at the promoters.

The transcription of flagellar genes is controlled by FlbD, a σ^{54}-dependent protein that binds the flagellar transcriptional regulation (*ftr*) sequence motif located in the −10 to −35 region of the class II promoters, and the −100 region of the class III promoters, to repress and activate transcription, respectively (Benson *et al.*, 1994; Mullin *et al.*, 1994; Wingrove & Gober, 1994; Dutton *et al.*, 2005). Direct evidence of FlbD binding to flagellar promoters *in vivo* has not been shown. FlbD activity is modulated by the *trans*-acting factor FliX that links class II flagellar assembly to class III/IV flagellar gene transcription in two ways (Wingrove & Gober, 1994; Muir *et al.*, 2001; Muir & Gober, 2004). First, FliX stimulates the activation of class III genes by FlbD during the assembly of the basal body. Second, when flagellar assembly is blocked, FliX prevents the activation of the class III gene pathway by FlbD (Muir & Gober, 2002, 2004). Genetic and biochemical studies provide evidence for FliX binding directly to FlbD (Muir & Gober, 2002, 2004) to prevent binding to *ftr* (Dutton *et al.*, 2005); yet, whether FliX associates with FlbD-dependent promoters *in vivo* remains to be determined.

TipF, a predicted 50-kDa protein with two N-terminal transmembrane domains, a coiled-coil region, and a C-terminal EAL domain, is required for flagellum biogenesis (Huitema *et al.*, 2006). TipN, a membrane-embedded landmark protein, dictates the proper localization of TipF and the flagellar structure (Huitema *et al.*, 2006; Lam *et al.*, 2006). Little is known about how TipF and TipN affect flagellar gene expression. Here, we use β-galactosidase promoter probe assays and quantitative chromatin immunoprecipitation (qChIP) analyses to explore how a Δ*tipF* mutation affects the activity of flagellar promoters when compared with *WT*, a flagellar assembly (Δ*fliG*) mutant, positioning (Δ*tipN*), and regulatory (*fliX*::Tn5 and *flbD*::Tn5) mutants. These experiments reveal, for the first time, the direct quantification of the occupancy of flagellar promoters by their cognate transcriptional regulators *in vivo*.

Materials and methods

Strains and growth conditions

Caulobacter crescentus NA1000, a synchronizable derivative of the CB15 wild-type strain (Evinger & Agabian, 1977), and derivatives were grown at 30 °C in peptone yeast extract (PYE) [2 g peptone, 1 g yeast extract, 0.2 g $MgSO_4$, and 1 mL $CaCl_2$ (0.5 M) per liter] (Poindexter, 1964; Johnson & Ely, 1977).

β-Galactosidase assays

β-Galactosidase activity (Miller, 1972) was measured at 30 °C with log-phase cultures grown in PYE–tetracycline (0.5 μg mL^{-1}). Assays were performed in triplicate, with a

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minimum of two independent cultures for each promoter construct.

Antibody production

For the generation of anti-FlbD antibodies, FlbD was overexpressed in *Escherichia coli* Rosetta (DE3)/pLysS using pET28a (Novagen) as an N-terminal His6-tagged variant and purified using Ni-NTA agarose (Qiagen). Purified proteins were cut out from a 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gel and used to immunize rabbits (Josman LLC).

qChIP

Cells (20 mL) were grown to the mid-log phase and cross-linked in 10 mM sodium phosphate (pH 7.6) and 1% formaldehyde for 10 min at room temperature and on ice for 30 min thereafter. Cells were then washed three times in phosphate-buffered saline (pH 7.4), resuspended in 500 μL of TES buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 100 mM NaCl] containing 20 μL of 250 U μL^{-1} of Ready-Lyse (Epicentre), and incubated at room temperature for 15 min. Following incubation, 500 μL of ChIP buffer [1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), and 167 mM NaCl] containing one protease inhibitor tablet (Roche) was added to the lysates and incubated at 37 °C for 10 min. The lysates were then sonicated (Sonicator 3000, Misonix Inc., Farmingdale, NY) on ice using 10 bursts of 20 s at output level 4.5 to shear DNA fragments to an average length of 300–700 basepairs and cleared by centrifugation at 10 956 ***g*** for 2 min at 4 °C. The protein content of the lysates was normalized, diluted to 1 mL in ChIP buffer with 0.01% SDS, and precleared with 100 μL of Protein-A agarose (Roche), 100 μg bovine serum albumin (BSA), and 300 μg herring sperm DNA for 1 h at 4 °C. The supernatant (10%) was removed and used as total chromatin input DNA. Antisera: anti-CtrA (2 μL) (Quon *et al.*, 1996); anti-RNA polymerase (RNAP) against RpoC subunit (2 μL) (Neoclone); anti-FlbD (1 μL); or anti-FliX (0.5 μL) (Mohr *et al.*, 1998) was added to the remaining lysate, respectively, and incubated overnight at 4 °C. After overnight incubation, the supernatant was incubated with Protein-A agarose beads (100 μL), previously incubated with BSA and herring sperm, for 2 h at 4 °C. The beads were then washed once with: low-salt buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl]; high-salt buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl], and LiCl buffer [0.25 M LiCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)], and twice with TE buffer [10 mM Tris-HCl (pH 8.1) and 1 mM EDTA]. The protein–DNA complexes were eluted from the beads by adding 500 μL of elution buffer (1% SDS, 0.1 M $NaHCO_3$) with 300 mM NaCl to the beads, and incubating them overnight at 65 °C to reverse cross-linking. The samples were then incubated with 2 μg of Proteinase K for 2 h at 45 °C in 40 mM EDTA and 40 mM Tris-HCl (pH 6.5). DNA was extracted using phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and precipitated with 100% ethanol, using glycogen (20 μg) as a carrier, and resuspended in 50 μL of water.

Quantitative real-time PCR

Real-time PCR was performed using a MyIQ single-color real-time PCR detection system (Bio-Rad, Hercules, CA) using 5% of each ChIP sample, 12.5 μL of SYBR green PCR master mix (Bio-Rad or Quanta), 10 pmol of primers, and 5.5 μL of water per reaction. A standard curve generated from the cycle threshold (C_t) value of the serially diluted chromatin input was used to calculate the percentage input value of each sample. Average values are from triplicate measurements performed per culture. The final data were generated from three independent cultures. DNA regions analyzed by real-time PCR were from nucleotides −190 to −8 relative to the start codon of *fliF*; from −176 to +23 relative to the start codon of *flgE*; and from −214 to −21 relative to the start codon of *fljL*.

Results and discussion

To examine the transcription of flagellar genes in *WT* and the Δ*tipF* mutant, we first measured β-galactosidase activity of *lacZ* transcriptional reporters fused to class II-*fliF* (MS-ring), class III-*flgE* (hook), and class IV-*fljL* (flagellin) promoters. The Δ*tipF* mutant strain was also compared with a hook basal-body mutant Δ*fliG* (lacking a component of the flagellar switch bound to the MS-ring), the flagellar placement mutant Δ*tipN*, and the transcriptional regulatory mutants, *fliX*::Tn5 and *flbD*::Tn5. Relative to *WT*, the class II-*fliF-lacZ* fusion was upregulated in Δ*tipF* (174 ± 5%) and Δ*fliG* (318 ± 4%) (Fig. 2). Because the promoter activity of class III flagellar genes is impaired in class II flagellar mutants due to an unknown regulatory mechanism imposed by the absence of the basal body, the transcription of class III-*flgE-lacZ* fusion was less active in Δ*fliG* (19 ± 1%) and Δ*tipF* (57 ± 1%) relative to *WT* (Fig. 2). Unlike the Δ*fliG* mutant (5 ± 0.5%), the class IV-*fljL-lacZ* fusion was as active in the Δ*tipF* mutant as in the *WT* background (87 ± 1%) (Fig. 2). These indirect *in vivo* assays suggest that class IV flagellar genes are efficiently transcribed in the Δ*tipF* mutant despite the absence of an assembled flagellum.

fliX::Tn5 and *flbD*::Tn5 mutant strains were included as controls, while the Δ*tipN* mutant allowed for comparison with a strain that can possess multiple flagella that are frequently misplaced (Huitema *et al.*, 2006; Lam *et al.*, 2006). Subsequently, similar to canonical class II flagellar

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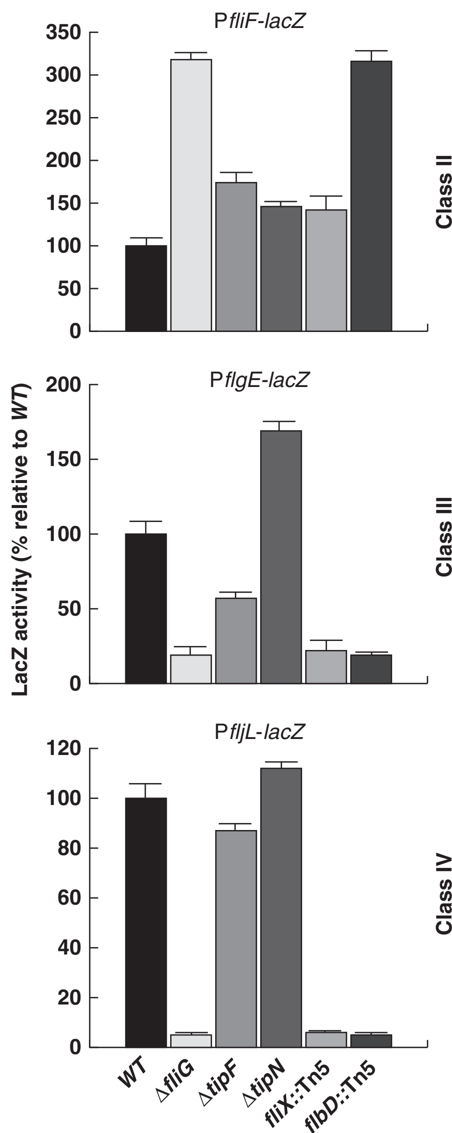

Fig. 2. β-Galactosidase promoter-probe assays. LacZ activity of class II-*fliF* (MS-ring), class III-*flgE* (hook), and class IV-*fljL* (flagellin) in *WT*, Δ*fliG*, *tipF*, *tipN*, *fliX*::Tn5, and *flbD*::Tn5 strains was quantified as a percentage relative to *WT*.

mutants, the class II-*fliF-lacZ* fusion was upregulated in the *fliX*::Tn5 (142 ± 9%) and *flbD*::Tn5 (316 ± 7%) mutants, while the class III-*flgE-lacZ* fusion (22 ± 2% and 19 ± 1%, respectively) and class IV-*fljL-lacZ* fusion (6 ± 0% and 5 ± 0%, respectively) were less active in the *fliX*::Tn5 and *flbD*::Tn5 strains when compared with the *WT* background (Fig. 2). Interestingly, the Δ*tipN* mutant transcribed class II-*fliF-lacZ* (146 ± 1%) and class III-*flgE-lacZ* (169 ± 2%) at higher levels than those observed in the *WT* background, while class IV-*fljL-lacZ* (112 ± 1%) was transcribed at levels near *WT* (Fig. 2). We speculate that the increased levels of flagellar gene transcription seen in the Δ*tipN* for class II-*fliF* and class III-*flgE* are a consequence of the multiple flagella present in the absence of TipN.

To validate the β-galactosidase promoter-probe assays, we relied on qChIP experiments to directly measure the *in vivo* occupancy of the transcriptional factors CtrA, FlbD, FliX, and RNAP at the *fliF*, *flgE*, and *fljL* promoters using polyclonal antibodies to CtrA, FlbD, and FliX, and a monoclonal antibody to the RpoC subunit of RNAP. The occupancy of flagellar promoters in Δ*tipF* was compared with *WT*, Δ*fliG*, Δ*tipN*, *fliX*::Tn5, and *flbD*::Tn5 mutants, with minor modifications (Radhakrishnan *et al.*, 2008). Measurement of RNAP occupancy at the *fliF* promoter by qChIP corroborated the β-galactosidase results, with comparable trends being observed (i.e. elevated levels in Δ*fliG*) (Fig. 3). Notably, qChIP experiments revealed that CtrA occupied the *fliF* promoter at similar levels in Δ*fliG* and Δ*tipF* (99 ± 4% and 80 ± 6% relative to *WT*, respectively) (Fig. 3), indicating that the increase in class II flagellar gene transcription in

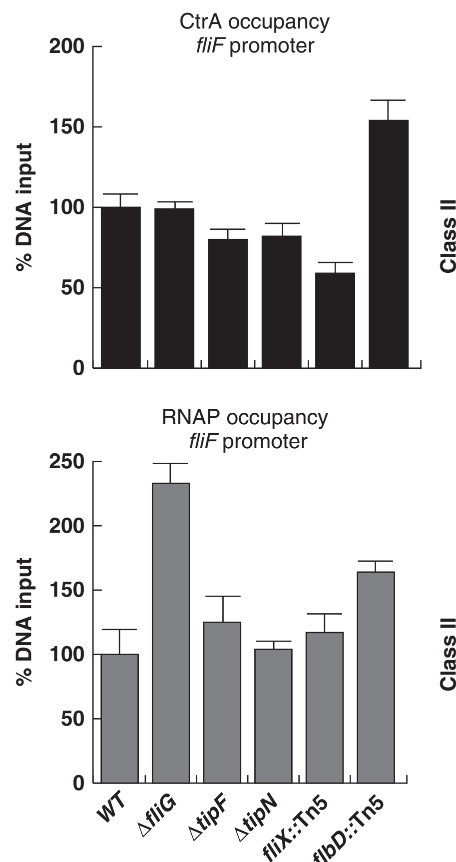

Fig. 3. qChIP assays. The occupancy of the class II-*fliF* flagellar promoter by flagellar regulators CtrA and RNAP *in vivo* is shown for *WT*, *fliG*, *tipF*, *tipN*, *fliX*::Tn5, and *flbD*::Tn5 strains. Promoter regions were immunoprecipitated with a polyclonal antibody to CtrA or a monoclonal antibody to the RpoC subunit of RNAP before analyses by quantitative real-time PCR. Results demonstrate the percentage of promoter occupancy by CtrA and RNAP relative to *WT*.

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Δ*fliG* and Δ*tipF* mutants is not due to an elevated occupancy of CtrA at the promoter(s). Consistent with *fliF* upregulation seen in Δ*fliG* and Δ*tipF* by the β-galactosidase assay, qChIP revealed that the occupancy of FlbD (repressing class II genes) was decreased at the *fliF* promoter in the Δ*fliG* (45 ± 1%) and Δ*tipF* (51 ± 8%) strains (Fig. 4a). FliX, the regulatory factor that links the status of flagellar assembly to FlbD activity (Muir & Gober, 2005), was present at the class II promoters, at higher levels than *WT*, in Δ*fliG* (170 ± 7%) and Δ*tipF* (144 ± 4%), consistent with the decreased levels of FlbD at the *fliF* promoter (Fig. 4a). FliX has been shown to interact with FlbD and block its access to enhancer DNA sequences *in vitro* (Dutton *et al*., 2005), and this new qChIP-based approach further suggests that FliX occupies the promoters to modulate FlbD activity at the class II-*fliF* promoter *in vivo*.

Next, we determined the presence of FlbD and FliX at the class III-*flgE* and class IV-*fljL* promoters. qChIP showed that FlbD occupancy at the class III-*flgE* promoter was reduced in Δ*fliG* (68 ± 5%) and Δ*tipF* strains (75 ± 10%) (Fig. 4b), while that of FliX was elevated (155 ± 5% in Δ*fliG* and 227 ± 9% in Δ*tipF*) (Fig. 4b). These data demonstrate that the Δ*tipF* strain is similar to the Δ*fliG* mutant strain with regard to the occurrence of FlbD and FliX at the *flgE*

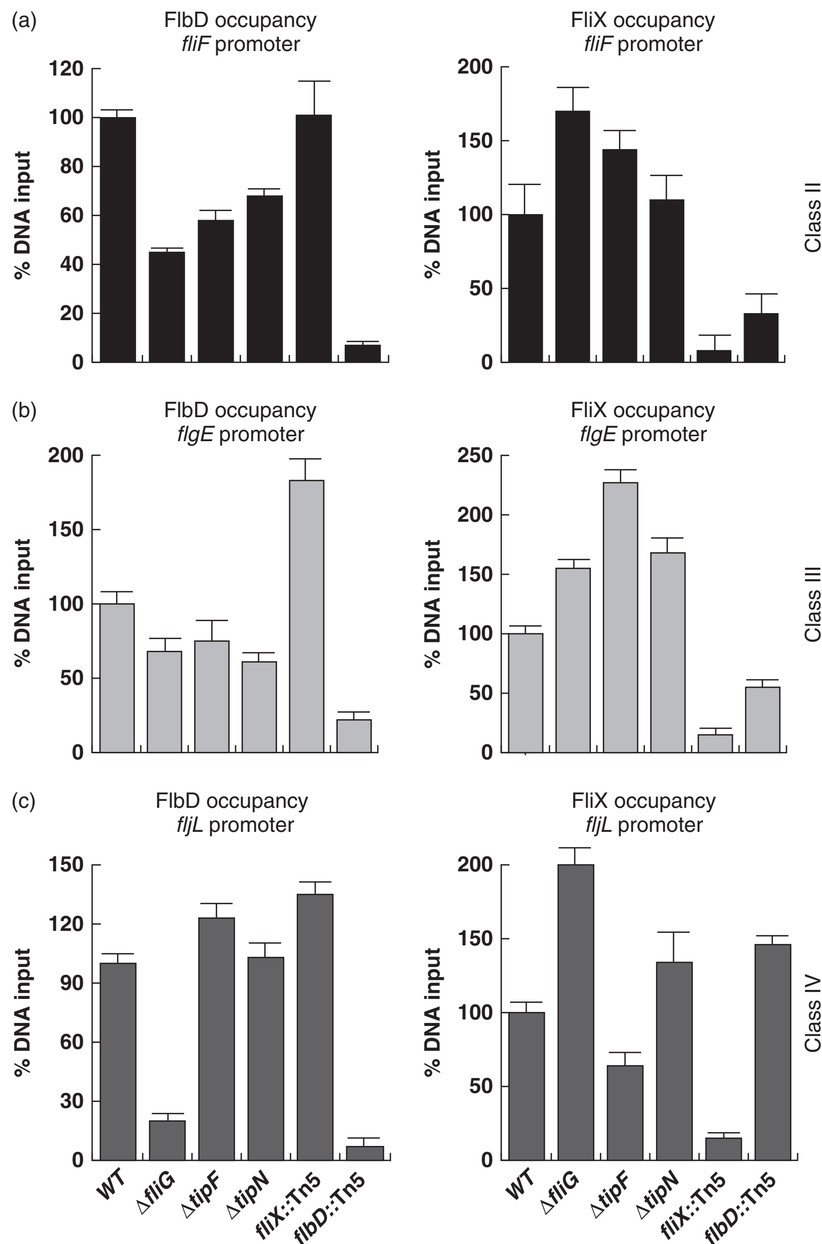

Fig. 4. qChIP assays. The occupancy of flagellar promoters by flagellar regulators FlbD and FliX *in vivo* for *WT*, *fliG*, *tipF*, *tipN*, *fliX*::Tn5, and *flbD*::Tn5 strains. (a) Class II-*fliF* (MS-ring). (b) Class III-*flgE* (hook). (c) Class IV-*fljL* (flagellin). Promoter regions were immunoprecipitated with polyclonal antibodies to FlbD or FliX, respectively, before analyses by quantitative real-time PCR. Results demonstrate the percentage of promoter occupancy by FlbD and FliX relative to *WT*.

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promoter. It is further consistent with the view that FliX is also present at class III promoters to block FlbD access. The class IV-*fljL* promoter, however, had an abundance of FlbD similar to *WT* ($123 \pm 8\%$) and decreased levels of FliX ($64 \pm 7\%$) in Δ*tipF*, while the Δ*fliG* mutant had decreased FlbD ($20 \pm 2\%$) and increased FliX ($200 \pm 9\%$) (Fig. 4c). These results, also supported by the β-galactosidase promoter-probe assays (Fig. 2), suggest that, unlike FliG, TipF is not necessary to confer the transcription of class IV flagellar genes.

Both *flbD*::Tn5 and *fliX*::Tn5 mutant strains were included as controls. Accordingly, FlbD was considerably decreased at the *fliF* ($7 \pm 1\%$), *flgE* ($22 \pm 3\%$), and *fljL* ($7 \pm 1\%$) promoters in the *flbD*::Tn5 mutant compared with *WT* (Fig. 4a–c). Similarly, the *fliX*::Tn5 mutant had decreased levels of FliX at the *fliF* ($8 \pm 2\%$), *flgE* ($15 \pm 1\%$), and *fljL* ($15 \pm 1\%$) promoters (Fig. 4a–c).

The Δ*tipN* mutant possessed lowered levels of FlbD at the *fliF* ($69 \pm 5\%$) and *flgE* ($57 \pm 3\%$) promoters, while *fljL* ($103 \pm 9\%$) was near *WT* levels (Fig. 4a–c). FliX was present at the *fliF* ($109 \pm 8\%$), *flgE* ($166 \pm 9\%$), and *fljL* ($129 \pm 25\%$) promoters in the Δ*tipN* mutant relative to *WT*. Because the Δ*tipN* mutant frequently possesses multiple flagella that are often misplaced (Huitema *et al*., 2006; Lam *et al*., 2006), these results suggest that the levels of FlbD present at the class II/III promoters are sufficient to allow for flagellar development in the absence of TipN.

Our data demonstrate the *in vivo* occupancy of *fliF*, *flgE*, and *fljL* flagellar promoters by the transcriptional regulators CtrA, FlbD, and FliX of the *C. crescentus WT* and flagellar mutants for the first time, thus providing direct *in vivo* evidence for the previously proposed hierarchical scheme in the negative and positive transcriptional regulation of flagellar genes. While FlbD and FliX have been shown to interact, an inverse correlation was observed here between FlbD and FliX at the site of flagellar promoters *in vivo*, consistent with the hypothesis that FliX blocks FlbD access to flagellar promoters to regulate flagellar gene transcription.

The results from the transcriptional activity and promoter occupancy of flagellar regulators in the Δ*tipF* mutant suggest that *tipF* does not conform to the canonical flagellar hierarchy, akin to *flgBC-fliE* (Boyd & Gober, 2001) and *fljK* (Muir & Gober, 2005). We speculate that the transcriptional data presented point to hitherto unknown coupling mechanisms or interactions of TipF with regulatory components of the flagellar gene expression hierarchy, the cell cycle, and/or organizers of the flagellum assembly.

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