FlbD of *Caulobacter crescentus* is a homologue of the NtrC (NR_I) protein and activates σ^{54} -dependent flagellar gene promoters

(transcription/cell cycle regulation)

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ABSTRACT The periodic transcription of flagellar genes in the *Caulobacter crescentus* cell cycle is controlled, in part, by their organization in a regulatory hierarchy. The *flbG* (hook operon), *flaN*, and flagellin gene operons, which are at the lowest levels of the hierarchy and expressed late in the cell cycle, contain Ntr-like promoters. We report that *flbD*, one of the early genes required in trans for expression of these operons, codes for a 52-kDa protein homologous to the transcriptional activators NtrC (NR_I), NifA, DctD, HydG, and XylR. Our results show that in *Escherichia coli* *flbD* partially complements *glnG* (*ntrC*) mutations and stimulates transcription of the *C. crescentus* σ^{54} RNA polymerase-dependent *flbG* gene. Additionally, the sequence predicts that FlbD protein, along with NtrC, DctD, and HydG proteins, is structurally related at the amino-terminal domain to a larger family of response regulators that mediate cellular responses to environmental stimuli. FlbD may be a singular member of this large protein family in that its function is tied to an internal cell-cycle signal. FlbD is also unusual in that its amino-terminal domain contains only one of the three residues conserved in previously described members of this family of response regulators.

Differentiation in the dimorphic bacterium *Caulobacter crescentus* results from a sequence of discontinuous, stage-specific events that lead to the production of a new swarmer cell after each asymmetric cell division of the stalked cell. The best understood of these events is formation of the polar flagellum, which occurs late in the cell cycle and requires the products of >30 flagellar (*fla*, *flb*, *flg*) genes. These *fla* genes are periodically transcribed in the cell cycle, generally at the times of gene-product assembly. A question central to morphogenesis is how the sequential, stage-specific expression of this large *fla* gene family is controlled in the cell cycle (for reviews, see refs. 1 and 2).

The temporal pattern of *fla* gene expression in *C. crescentus* depends, in part, on the organization of these genes in a regulatory hierarchy, in which genes at each level are required for expression of genes at lower levels (for summary, see refs. 1 and 3). Analysis of the 5' regulatory sequences has suggested that the resulting transcriptional cascade is mediated by the sequential synthesis of transcription activators and the use of alternative σ factors (4, 5). *fla* genes at the two lowest levels of the hierarchy, including *flbG* and *flaN* of the hook gene cluster, contain nucleotide sequence elements at -12 and -24 (4, 6) that are strikingly similar to the *ntr/nif* promoters in enteric bacteria recognized by the specialized σ^{54} (NtrA) RNA polymerase (7, 8). Results of site-specific mutagenesis (9) and *in vitro* transcription experiments (5) have suggested that *flaN* and *flbG* promoters, but not promoters above them in the hierarchy, are also recognized by a σ^{54} RNA polymerase.

Transcription by σ^{54} RNA polymerase in other systems requires specific transcriptional activators, some of which depend on a second protein for activation (for reviews, see refs. 10–13). Expression from the σ^{54} -dependent *glnAp2* promoter in enteric bacteria requires NtrC (NR_I) that is activated by phosphorylation by NtrB (NR_{II}) in response to ammonia starvation and that binds to an upstream enhancer element (14–16). Similarly, transcription of *nif* promoters in *Klebsiella pneumoniae* requires a homologous transcriptional activator NifA (17). The requirement of the enhancer-like sequence element *ftr* (flagellar gene transcription regulation; refs. 4 and 9) for expression of the *C. crescentus* σ^{54} *flaN* and *flbG* promoters suggested that their expression might depend on an NtrC-like transcriptional activator.

Several *fla* genes, including those in the adjacent *flaO* and *flbF* operons, are required in trans for expression from the *flaN* and *flbG* promoters (1, 3). We report that *flbD*,[†] the last gene in the *flaO* operon, codes for a protein homologous to the σ^{54} -specific transcriptional activators NtrC, NifA, DctD, HydG, and XylR (17–20) and that in *Escherichia coli*, *flbD* stimulates transcription from the *flbG* promoter. We also show that FlbD, the synthesis and function of which in *C. crescentus* is controlled by an internal cell-cycle clock (1), has an amino-terminal domain that is structurally conserved in a large family of response regulators; in other systems these response regulators respond to environmental stimuli.

MATERIALS AND METHODS

Strains. *C. crescentus* strains are derived from strain CB15 (ATCC 19089). *E. coli* strain JM107 (21) was used as host for rescuing phage from Bluescript clones. Strain HB101 harboring pRK2013 was used as a mobilizing strain in triparental matings into *C. crescentus* (22). YMC9 and its *gln* derivatives (23, 24) are described in Table 1.

Media. *C. crescentus* cells were routinely grown in Pye (peptone-yeast extract) medium, and M2 minimal salts medium was used for selecting transconjugants from triparental matings (22). Media were supplemented with isopropyl β -D-thiogalactopyranoside (IPTG) (2 mM), tetracycline (2 μ g/ml for *C. crescentus*; 15 μ g/ml for *E. coli*), ampicillin (50 μ g/ml in liquid medium; 100 μ g/ml in plates for *E. coli*), and glutamine (0.2%), as indicated.

Enzyme Assays. Glutamine synthetase levels were assayed after growth in nitrogen-limiting medium with or without ammonia, as described (25). Cells grown under these same conditions were assayed for β -galactosidase, according to Miller (26).

DNA Sequencing. Nucleotide sequences were determined in Bluescript(+) and Bluescript(-) vectors (Stratagene) by using restriction fragments, Bal-31-generated deletions, and

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Abbreviation: IPTG, isopropyl β -D-thiogalactopyranoside.

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[†]The sequence reported in this paper has been deposited in the GenBank data base (accession no. M32065).

synthetic oligonucleotides. Reactions were carried out either with Klenow enzyme and a modified reaction mixture (9) or by the recommended procedure with Sequenase (United States Biochemical); 7-deaza-dGTP was used in place of dGTP.

Labeling of Proteins. Minicells were isolated from strain P678-54 (27) carrying the plasmids indicated by using discontinuous sucrose-gradient centrifugation and labeled, as described (28). After induction with 2 mM IPTG for 1 hr, [³⁵S]methionine (50 μ Ci/ml; 1 Ci = 37 GBq) was added, and the minicells were labeled for 1 hr, centrifuged, and analyzed by NaDodSO₄/PAGE and autoradiography.

RESULTS

Defining the *flbD* Gene. The hook gene cluster of *C. crescentus* contains the four transcription units *flaN*, *flbG*, *flaO*, and *flbF* (Fig. 1A). Previous results (22) showed that the 7-kilobase (kb) *Bam*HI(a)–*Bam*HI (b) DNA fragment subcloned in plasmid S1505 contained the complete *flaO* operon because it complemented all *flaO* and *flbD* mutations examined (Fig. 1B). Limits of the *flaO* operon can be defined (i) at the 5' end by location of the *flaO* transcription start site, which is within the 285-base-pair (bp) *Bam*HI(a)–*Hind*III fragment (4) and (ii) at the 3' end by the adjacent *flbF* transcription start site, which is $\approx$ 250 bp upstream of the *Eco*RI(c) site (Fig. 1B; G.R. and A.N., unpublished results).

The location of *flbD* was determined by making internal deletions within the 7-kb DNA insert of plasmid S1505 to generate plasmids pGIR314 (*Hind*III–*Bgl* II) and pGIR315 (*Hind*III–*Pst* I). Both plasmids, which contain the intact *flaO* promoter, complemented the motility defect of *flbD* mutations *flbD198::Tn5* and *flbD609::Tn5* (22, Fig. 1B); pGIR307, which carries the same DNA insertion as pGIR314, but not the *flaO* promoter, did not complement *flbD198::Tn5*. Thus, the DNA sequence downstream from the *Pst* I site must contain the intact *flbD* gene.

FIG. 1. Location of the *flbD* gene. (A) Physical and genetic map of the hook gene cluster. (B) Complementation analysis. Triangles indicate the insertion mutations *flaO172::Tn5*, *flaO607::Tn5*, *flaO188::Tn5*, *flbD198::Tn5*, and *flbD609::Tn5* described elsewhere (22); DNA fragments in plasmid vector pRK290 were tested for complementation as described (22). + and –, Presence or absence, respectively, of motility. Relevant restriction sites are indicated: B, *Bam*HI; Bgl, *Bgl* II; E, *Eco*RI; H, *Hind*III; P, *Pst* I; Sm, *Sma* I.

Nucleotide Sequence of *flbD*. The 2.2-kb *Eco*RI(b)–*Eco*RI(c) DNA fragment containing *flbD* (Fig. 1B) was sequenced on both strands. A single long open reading frame was identified (Fig. 2). Although translation could initiate at two possible ATG triplets separated by 20 codons, the sequence analysis (see below) indicated that the upstream codon at nucleotide 114 is the translational start. This ATG is preceded by a Shine–Dalgarno (GGAGA) sequence, and the reading frame terminates at TAA within an *Afl* II site just upstream from *flbF*. The predicted *FlbD* protein contains 455-amino acid residues with a molecular mass of 51,138 Da. A possible hairpin loop after the open reading frame (indicated in Fig. 2) may function as a transcription terminator.

FlbD displays a codon usage characteristic of other G+C-rich genomes and *C. crescentus* genes examined (refs. 29–31; D. Mullin and A.N., unpublished results). Guanine- and cytosine-rich codons are employed preferentially, and bias is strong toward guanine and cytosine in the third position (data not shown).

Homology of *FlbD* to Response Regulators. Analysis by the method of Lipman and Pearson (32) showed that the central part of the translated *flbD* sequence (residues 145–342) has strong identity with the central domain conserved among

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CCCGGGGCGAGTCTGCTGCTGCGAGGCGGCTGGGGGTGACCATG
48  ACGGAATCATCAAGGACGGGACGAGGCTGACGCGGCTGCTGCTT
96  CGAGAGGAGAGTAAGAGATCGGGTCTGCTGCTGGAAAAGTGAAC
    M R L L V V G K L N
144  GGGCAGCTCTCGGTCGGGTCGAAGATGGGATGAACGGGGCGGAGG
    G Q L S V A V K M A N A G A K
192  GTCTCGACGTCGAAACGAGGAGCGGAGCAACCGGCTGGGGGG
    V S H V E T T E Q A T N A L R A
240  GGGCAGGGCGGTGACCTTCTGATGTCGATGTCGTCGACATCGG
    G Q G A D L L M V D Y V L D I A
288  GGTCTGATCGGGCGCAAGGAGCGGAGTGGGTCGCGTGGTGGT
    G L I A A N E A E R M R V V V
336  GCGTCGGGGTCGAGCGGATCGATCGCGCGGCGGCAAGCGATCA
    A C G V D A D P M R A A N A I K
384  GCGGGCGGAGGAGTTCATCGGCTCGCGGAGCGGAGCGGAGT
    A G A F E F I P L P D A E L I
432  GCGGGCTGCTGCGGCGGTCGAGGAGCGGAGCGGAGCGGAGT
    A A V I A A V T D D E K F M V V
480  CCGAGCGGCGGAGTTCGAGGAGTCAAGCTGGCGGAGCGGAGT
    R D P A M E Q V I K L A D Q V A
528  CGCTCGAAGCTGCTGCTGATCAGCGGAGCGGAGCGGAGT
    P S E A S I L I T G E S G S G K
576  GAGTTCATCGGCGGTCAGTTCAGCGGAGCGGAGCGGAGT
    E V M A R Y V H G K S R R A K A
624  CGCTTCATCAGGCTCAAGTTCGCGGAGCGGAGCGGAGT
    P F I S V N C A A I P E N L L E
672  AGCGAGCTGTCGCGGAGCGGAGCGGAGTTCGCGGAGCGGAGT
    S E L F G H E K G A F T G A M A
720  CGCGGAGTTCGCGGAGCGGAGTTCGCGGAGCGGAGTTCGCGG
    R R I G K F E E A D G G T L L R
768  GACGAAATCAGGAAATGAGCTGCGGAGCGGAGTTCGCGGAG
    D E I S E M D V R L Q A K L L R
816  GCGTTCAGGAGCGGAGTTCGCGGAGCGGAGTTCGCGGAGT
    A I Q E R E I D R V G G S K P V
864  AAGGTCAATTCGCGGAGTTCGCGGAGCGGAGTTCGCGGAG
    K V N I R I L A T S N R D L A Q
912  CGCGTGAAGGAGCGGAGTTCGCGGAGCGGAGTTCGCGGAG
    A V K D G T F R E D L L Y R L N
960  GTGTCGAACTGCGGAGTTCGCGGAGCGGAGTTCGCGGAGT
    V V N L R L P P L R E R P A D V
1008  ATCAGGCTGTCGCGGAGTTCGCGGAGCGGAGTTCGCGGAG
    I S L C E F F V K K Y S A A N G
1056  ATCAGGAGGAGCGGAGTTCGCGGAGCGGAGTTCGCGGAG
    H R W P G N V R E L E N A M H R
1104  CACGCTGCGGAGCGGAGTTCGCGGAGCGGAGTTCGCGGAG
    S T V A E V E Q Q L I I D T L E
1152  CGGTCGCTGCTGTCGCGGAGCGGAGTTCGCGGAGTTCGCGG
    A V L L S A G P E I E E F A I R
1200  CTGCGGAGCGGAGCGGAGTTCGCGGAGCGGAGTTCGCGGAG
    L P D G Q P M A P A P D V A V A
1248  CGCGGCGGAGTTCGCGGAGCGGAGTTCGCGGAGTTCGCGG
    R G A Q M A A D A A S R A F V G
1296  TCGACCGTTCGCGGAGTTCGCGGAGCGGAGTTCGCGGAG
    S T V A E V E Q Q L I I D T L E
1344  CACTGCTGGGCAACCGGAGTTCGCGGAGCGGAGTTCGCGG
    H C L G N R T H A A N I L G I S
1392  ATCCGACCGTTCGCGGAGTTCGCGGAGCGGAGTTCGCGGAG
    I R T L R N K L K E Y S D A G V
    A F I I I
1440  CAGTCCGCGGCGGAGGCGGAGTTCGCGGAGCGGAGTTCG
    Q V P P P Q G G V G A A A *
1488  GCGTTCGAGTTCGCGGAGCGGAGTTCGCGGAGTTCGCGGAG
    G T S A G G T T T C T A T G C T G G C G G G C T T C A T T G C T G C T A C C C T G A
1536  G T S A G G T T T C T A T G C T G G C G G G C T T C A T T G C T G C T A C C C T G A
1584  G C C T G T C G A A G G G C G A G C A C G C A A G C T 1614

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FIG. 2. Nucleotide sequence of *flbD* and translated amino acid sequence (in one-letter code). The *flbD* sequence is shown starting at the *Sma* I site (Fig. 1B). The putative Shine–Dalgarno sequence preceding the ATG start codon is underlined. Inverted repeat sequences after the termination codon are indicated by arrows.

transcriptional activators of σ^{54} promoters. This ATP-binding domain in NtrC has been implicated in open complex formation by the σ^{54} RNA polymerase (10). Fig. 3 compares this central domain of FlbD to the translated sequences of the *K. pneumoniae* ntrC and nifA genes (17), the *Rhizobium meliloti* dctD gene (18), the *E. coli* hydG gene (19), and the xylR gene of the *Pseudomonas putida* TOL plasmid (20). Within this 198-residue central domain FlbD has 55% identity with *K. pneumoniae* NtrC, and 56 of the residues are conserved in all six proteins analyzed (Fig. 3, boxed residues); a greater degree of similarity is seen when conservative substitutions are considered.

The amino-terminal sequence of FlbD (residues 1–114) and those of NtrC, DctD, and HydG (Fig. 3) contain conserved sequences of hydrophobic residues also found in CheY (33), as indicated by the shaded residues in Fig. 3. This common sequence motif identifies these proteins as members of a larger family of response regulatory proteins (for review, see ref. 13), and they may share common structural elements with CheY (33). Interestingly, of the three residues conserved in other members of this family (arrowheads in Fig. 3), FlbD contains only the residue corresponding to Asp-57 of CheY and lacks the residues corresponding to Asp-13 and Lys-109 (ref. 33; see Fig. 3 and Discussion). This structural domain has been suggested to regulate activity of the protein by interacting with a cognate protein kinase (for review, see ref. 13). The amino-terminal sequences of NifA and XylR proteins do not contain these characteristic hydrophobic sequence motifs, which probably reflects different roles of these domains in regulating protein function.

The carboxyl terminus of FlbD contains a sequence similar to the helix-turn helix present in many DNA-binding proteins (ref. 34; Fig. 3), and using the statistical weight matrix developed by Dodd and Egan (35), we estimate the proba-

bility of this domain (residues 416–435) being Cro-like at $\approx 60\%$. Some residues important for DNA-binding activity in both *K. pneumoniae* NtrC (36) and NifA (37) are also conserved in FlbD, including Arg-428 and Lys-433.

Identification of the FlbD Protein. To identify the FlbD protein, the *Pst* I–*Eco*RI(c) fragment (Fig. 1B) and the shorter *Sma* I–*Eco*RI fragment that contains the entire *flbD* gene, as determined by DNA sequencing (Fig. 2), were cloned behind the inducible *lppp*–*lacZ* promoters of the pINIII (lpp-5)A3 vector (38). A protein of ≈ 52 kDa was detected when minicells containing the resulting plasmids pGIR165 and pGIR166 were labeled with [35 S]methionine (Fig. 4, lanes C–F) but were not detected in minicells containing the parent pINIII(lpp-5)A3 vector (lanes A and B). Level of the 52-kDa protein was increased by induction with IPTG (Fig. 4, lanes D and F). The size of the 35 S-labeled protein agrees closely with 51,138 Da predicted from the nucleotide sequence (Fig. 3).

We verified that the protein expressed in the minicells was, indeed, FlbD by examining the ability of the promoter fusion in pGIR166 to complement *flbD* mutations. Plasmid pGIR323 was constructed by fusing plasmid pGIR166 and the broad-host-range plasmid pRK290 (39); Fig. 1B shows that this chimeric plasmid complemented both *flbD198::Tn5* and *flbD609::Tn5*.

flbD Complements *E. coli* glnG Mutations and Activates Transcription of *flbG*. Given the close sequence relationship between FlbD and NtrC, we examined the possibility that the *C. crescentus* *flbD* gene can complement *E. coli* mutations in *glnG*, the structural gene for NtrC, and restore *glnAp2* expression, as reported for *K. pneumoniae* nifA (24). Plasmid pGIR166, which carries the inducible *flbD* gene, was introduced into the *gln*⁺ strain YMC9 and *glnG::Tn5* derivative YMC12, and glutamine synthetase activity was assayed.

FIG. 3. Sequence alignment of FlbD with response regulator proteins; sequences are described and referenced in text. Shaded residues in the amino-terminal domains indicate conserved, hydrophobic motifs present in CheY and related proteins (see Discussion; ref. 13). Arrows indicate the conserved residues corresponding to Asp-13, Asp-57, and Lys-109 in CheY protein (32). Potential DNA-binding domains are indicated by asterisks, and shading denotes those residues conserved among a large number of DNA-binding proteins (33). Residues identical in all aligned sequences are boxed.

FIG. 4. Expression of *flbD* in minicells. Minicells of strain P678-54 carrying plasmid vector pINIII(1pp-5)A3 (lanes A and B), *flbD*⁺ *Pst* I-EcoRI subclone pGIR165 (lanes C and D), and *flbD*⁺ *Sma* I-EcoRI subclone pGIR166 (lanes E and F) were induced with IPTG (lanes B, D, and F), labeled with [³⁵S]methionine, and analyzed by PAGE on a 10% gel. Bla, β -lactamase. Numbers at left represent kDa.

Glutamine synthetase levels in YMC12 were elevated substantially after induction of pGIR166 by IPTG; this increase depended on ammonia starvation and presence of the *glnF* (*ntrA*) gene (Table 1).

The ability of pGIR166 to activate transcription of the *C. crescentus flbG* promoter in *E. coli* was also tested. The *lacZ* gene fused to the *flbG* promoter in plasmid pANZ405 (3) was not transcribed in YMC9 or in any *gln* derivatives of *E. coli*, even under conditions of ammonia starvation (Table 1). When plasmid pGIR166, containing the *flbD* gene, was also present in the strain, however, β -galactosidase was synthesized after induction with IPTG. Comparable levels of β -galactosidase activity were expressed from the *flbGp-lacZ* fusion in the *glnG::Tn5* strain YMC12 (data not shown). *flbG* activation by *flbD* did not depend on ammonia starvation,

and *flbG* expression was also seen in strain YMC11, in which the entire *glnALG* locus is deleted (Table 1). Failure to detect elevated levels of *lacZ* expression in strain TH1 indicates that transcription of *flbG* depends on the *glnF* (*ntrA*) gene (Table 1) and that the *flbD* gene product acts directly to stimulate transcription from the σ^{54} -dependent promoter of *flbG*.

DISCUSSION

fla genes at the bottom of the *C. crescentus* regulatory hierarchy are transcribed from promoters with highly conserved -12 and -24 sequence elements that in other bacteria are transcribed by a σ^{54} RNA polymerase. Our results suggest that the periodic transcription of *flbG* and presumably other σ^{54} promoters is activated by the product of *flbD*, which is near the top of the regulatory hierarchy (3). Analysis of the FlbD sequence shows that this protein belongs to a large family of response regulators that in other systems mediate responses to a variety of environmental stimuli (11-13). By contrast, FlbD in *C. crescentus* appears to regulate *fla* gene expression in response to a cell-cycle signal.

The site of *flbD* action has not been defined, but genetic experiments have shown that, in addition to the positive control of *flbG* expression, *flbD* also has strong negative autoregulatory effects on its own promoter, *flaOp* (3, 4). Because transcription of *flbG* requires the upstream activator element *ftr* (9) and the same sequence element is adjacent to the *flaO* promoter (4), it is tempting to speculate that FlbD can act at *ftr* or another conserved sequence as both an activator of *flbG* and a repressor of *flaO*.

The NtrC family of transcriptional activators to which FlbD is related contains three domains. A conserved central domain is required for activation of transcription by σ^{54} RNA polymerase and a carboxyl-terminal domain contains a DNA-binding sequence (10, 17). Except for NifA and XylR, these proteins also contain hydrophobic sequence motifs at the amino termini that are conserved in a larger family of response regulatory proteins that includes CheY, the structure of which has been recently published (13, 33). Sequence elements common to these proteins are present in FlbD (Fig.

Table 1. Complementation and transcriptional activation by *flbD* in *E. coli*

Strain	Genotype	Glutamine synthetase*				β-Galactosidase†			
		+ NH ₄ ⁺		- NH ₄ ⁺		+ NH ₄ ⁺		- NH ₄ ⁺	
		+ IPTG	- IPTG	+ IPTG	- IPTG	+ IPTG	- IPTG	+ IPTG	- IPTG
<i>glnAp2</i> expression									
YMC9/pINIII	<i>lacΔ glnG⁺/flbD⁻</i>	200		2100					
YMC9/pGIR166	<i>lacΔ glnG⁺/flbD⁺</i>	240		2000					
YMC12/pINIII	<i>lacΔ glnG::Tn5/flbD⁻</i>	395		230					
YMC12/pGIR166	<i>lacΔ glnG::Tn5/flbD⁺</i>	400	400	765	260				
TH1/pINIII	<i>lacΔ glnFΔ/flbD⁻</i>	170		94					
TH1/pGIR166	<i>lacΔ glnFΔ/flbD⁺</i>	145		150					
<i>flbGp</i> expression									
YMC9/pANZ405, pINIII	<i>lacΔ glnG⁺/flbGp-lac, flbD⁻</i>					0.65		0.21	
YMC9/pANZ405, pGIR166	<i>lacΔ glnG⁺/flbGp-lac, flbD⁺</i>					15.5		20	
YMC11/pANZ405, pINIII	<i>lacΔ glnALGΔ/flbGp-lac, flbD⁻</i>					0.6		1.4	
YMC11/pANZ405, pGIR166	<i>lacΔ glnALGΔ/flbGp-lac, flbD⁺</i>					36	6.4	50	6.2
TH1/pANZ405, pINIII	<i>lacΔ glnFΔ/flbGp-lac, flbD⁻</i>					0.75		1.1	
TH1/pANZ405, pGIR166	<i>lacΔ glnFΔ/flbGp-lac, flbD⁺</i>					2		2.5	

Strains carrying pINIII(1pp-5)A3 (pINIII) and pGIR166 were grown with ampicillin; strains that also carried plasmid pANZ405 were grown in medium containing ampicillin and tetracycline. IPTG was present except where indicated.

*Glutamine synthetase activity is expressed in nmol of glutamylhydroxamate per min/mg of protein (36).

† β -Galactosidase activity is expressed in Miller units (37).

3) at residues 3–6, 48–51, and 74–77, which correspond to clusters of hydrophobic residues in the three central β -strands, and at residues 13–21, 60–66, and 106–114, which correspond to the hydrophobic residues on three adjacent α -helices in the center of the CheY structure. Stock *et al.* (13) have suggested that these sequence elements reflect a common α/β structure seen in CheY.

Proteins sharing the CheY sequence motif contain three conserved residues at positions corresponding to Asp-13, Asp-57, and Lys-109 (13). These residues are located at the carboxyl-terminal ends of β -strands forming an acidic pocket where aspartyl phosphorylation by the cognate histidine kinase occurs (12, 13, 34). Although FlbD shares the conserved hydrophobic sequence motif of these proteins, it contains lysine in place of Asp-13 and leucine in place of Lys-109 (Fig. 3). Despite these differences, *flbD* partially complements *glnG* mutations and restores glutamine synthetase levels to $\approx 36\%$ of wild-type level on ammonia starvation and in the presence of *glnF* (*ntrA*; Table 1). This regulation by nitrogen raises the possibility that FlbD function depends on the protein kinase NtrB (*glnL* product) in *E. coli*. It is interesting to note that the one conserved aspartyl residue in FlbD corresponds to Asp-57, which is reported to be phosphorylated in phospho-CheY (40). At the same time, activation of the *C. crescentus flbG-lacZ* fusion by *flbD* in *E. coli* did not require nitrogen starvation or the presence of NtrB (Table 1). Thus, the apparent requirement of phosphorylation for FlbD activity in *E. coli* must be examined in greater detail.

These findings raise the question of how FlbD function is regulated in *C. crescentus*. *flbD* is transcribed during a very brief time in the cell cycle just before the periodic expression of *flbG* and its other target genes (N. Ohta, L.-S. Chen, D. Mallin, and A. N., unpublished work). Although this temporal pattern of *flbD* expression can account for the timed activation of the *flbG* promoter, FlbD function may also be modulated by a cognate protein kinase, as discussed above. A number of other *fla* genes are needed to activate σ^{54} promoters *flbG* and *flaN* in addition to *flbD* (3). These genes include *flaO*, *flbF*, and *flaW*, which are also in the hook gene cluster (Fig. 1A), and *flaS* and *flaB*, which map elsewhere on the chromosome. Although one or more of these genes could function to modulate FlbD activity, a specific FlbD kinase has not yet been identified.

In summary, the DNA sequence analysis presented here shows that the *C. crescentus flbD* gene product is a homologue of NtrC. S. Van Way and D. Mullin have also sequenced the *flbD* gene and arrived at a similar conclusion (personal communication). Our results show further that FlbD behaves as a functional homologue of NtrC and that it stimulates transcription of the σ^{54} -dependent *flbG* gene in *E. coli*. Analysis of the FlbD protein sequence places it within a large family of response regulators that in other systems mediates the cellular response to environmental stimuli. Although a protein like FlbD could respond to a nutritional or other environmental signal, flagellar gene expression in *C. crescentus* is under tight cell-cycle regulation; it seems likely that the signal for FlbD function and *fla* gene activation in *C. crescentus* is generated by DNA replication (41) or some other internal cell-cycle signal (1).

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