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Caulobacter FliQ and FliR Membrane Proteins, Required for Flagellar Biogenesis and Cell Division, Belong to a Family of Virulence Factor Export Proteins

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The *Caulobacter crescentus fliQ* and *fliR* genes encode membrane proteins that have a role in an early step of flagellar biogenesis and belong to a family of proteins implicated in the export of virulence factors. These include the MopD and MopE proteins from *Erwinia carotovora*, the Spa9 and Spa29 proteins from *Shigella flexneri*, and the YscS protein from *Yersinia pestis*. Inclusion in this family of proteins suggests that FliQ and FliR may participate in an export pathway required for flagellum assembly. In addition, mutations in either *fliQ* or *fliR* exhibit defects in cell division and thus may participate directly or indirectly in the division process. *fliQ* and *fliR* are class II flagellar genes residing near the top of the regulatory hierarchy that determines the order of flagellar gene transcription. The promoter sequence of the *fliQR* operon differs from most known bacterial promoter sequences but is similar to other *Caulobacter* class II flagellar gene promoter sequences. The conserved nucleotides in the promoter region are clustered in the −10, −20 to −30, and −35 regions. The importance of the conserved bases for promoter activity was demonstrated by mutational analysis. Transcription of the *fliQR* operon is initiated at a specific time in the cell cycle, and deletion analysis revealed that the minimal sequence required for transcriptional activation resides within 59 bp of the start site.

Each *Caulobacter crescentus* cell division generates distinct progeny cells: a stalked cell which is competent for DNA replication, and a motile swarmer cell which initiates DNA replication only after it sheds its flagellum and differentiates into a stalked cell later in the cell cycle. Following the initiation of DNA replication, the flagellar transcriptional hierarchy is activated, culminating in the assembly of a single flagellum at the cell pole opposite that bearing the stalk. The biogenesis of the flagellum is a landmark in the establishment of asymmetry in the predivisional cell (Fig. 1).

A large number of the flagellar structural and regulatory genes have been grouped into classes (Fig. 1) that form a regulatory hierarchy (7, 9, 11, 43, 62). We have preliminary evidence that the class I genes respond to cell cycle cues (44a). The class II flagellar genes are transcribed early in the cell cycle and are required for the transcription of genes in classes III and IV. Two class II genes, *rpoN* and *flbD*, encode a sigma factor (σ^{54}) (6), and a transcriptional activator, FlbD (45, 60), respectively, which are required directly for the transcription of class III and class IV genes. Other class II genes encode proteins required for flagellar assembly and function (24, 45a, 65). The order of flagellar gene expression approximates the order of assembly of the gene products into the nascent structure (11, 13, 17, 43, 62). In *C. crescentus*, *Salmonella typhimurium*, and *Escherichia coli*, if flagellar assembly is aborted, transcription of the remaining flagellar genes is blocked, suggesting that the two processes are coupled (19, 23, 24, 31).

We reported previously that a nonmotile mutant with a deletion in the *flaS* locus not only diminished or abolished the transcription of class III and class IV genes but also exhibited a cell division defect (13). To understand the role of this class II *flaS* locus in the assembly of the flagellum and in cell divi-

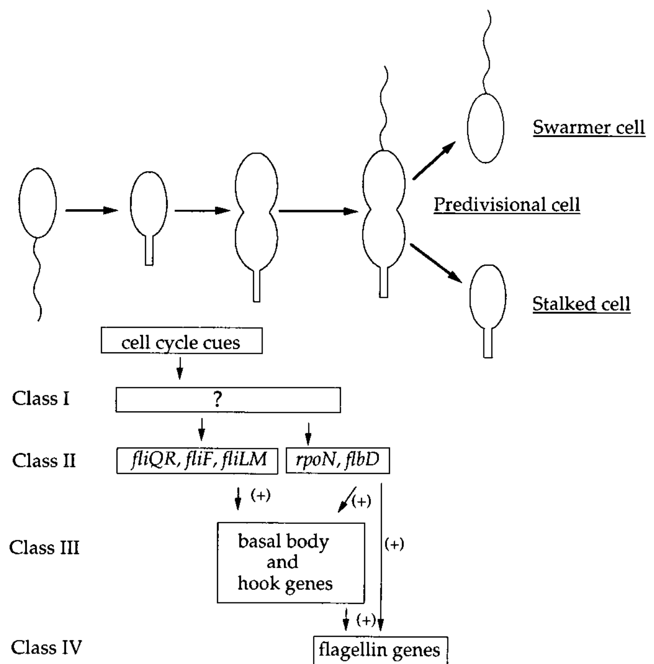

FIG. 1. Schematic of the *C. crescentus* cell cycle and the flagellar regulatory hierarchy. At each division, a predivisional cell gives rise to two different progeny, a stalked cell and a motile swarmer cell. As the cell cycle proceeds, the swarmer cell sheds its flagellum and assembles a stalk at the previously flagellated pole. The new stalked cell begins to divide, assembling a new flagellum at the pole opposite the stalk. The flagellar genes are expressed in an order that reflects their positions in the regulatory hierarchy and the order of assembly of their protein products into the flagellum (7, 42). Arrows indicate positive regulation, such that transcription of each class of genes requires the expression of the gene products of the preceding class. Both *rpoN* and *flbD* encode transcription proteins (6, 45), whereas other class II genes appear to encode proteins involved in the assembly, structure, and function of the flagellum (24, 65).

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TABLE 1. *C. crescentus* strains and plasmids used

Strain or plasmid	Genotype or description	Reference(s) or source
Strains		
NA1000	CB 15N, synchronizable derivative of wild-type *C. crescentus* CB15	14
SC508	*flaS153*, a spontaneous deletion within the *flaS* locus	13, 25
LS253	CB 15N *fliR* insertion	This study
LS254	CB 15N *fliQ* deletion	This study
Plasmids		
pJAMY 30, pJAMY 31	Km^r ColE1 replicon, β-lactamase translational fusion vector	1
pRSZ3	Tet^r RK2 derivative, *lacZ* transcriptional fusion vector	1
pRSZ6	Tet^r RK2 derivative, *lacZ* and *gusA* transcriptional fusion vector	1
pCS98	*flgF-gusA* and *fliLM-lacZ* transcriptional fusions in pRSZ6	50a
pRK290/20R	Tet^r RK2 derivative vector	17
pRK290lac	Tet^r RK2 derivative, *lacZ* transcriptional fusion vector	17
pWZ 20	1.7-kb *Sma*I-*Bam*HI fragment of the *fliQR* operon	This study
pWZ 35	pRK290/20R + *fliQR* (−268 to +1077)	This study
pWZ 56	pRSZ3 + *fliQR* (−388 to +378)	This study
pWZ 57	pRSZ3 + *fliQR* (−268 to +378)	This study
pWZ 72	pRSZ3 + *fliQR* (−27 to +378)	This study
pWZ 83	pRSZ3 + *fliQR* (−59 to +378)	This study
pWZ 162	pRK290lac + *fliQR* (−268 to +378)	This study
pWZ 165	pRK290/20R + *fliQR* (−268 to 1077) with a 2-kb Ω cassette inserted at the *Ava*I site in *fliR*	This study
pWZ 166	pWZ 162 with a 12-mer insertion at −27	This study
pWZ 192	pWZ 162 with a 3-base substitution at −9 to −11	This study
pWZ 193	pWZ 162 with a 2-base substitution at −36 and −35	This study
pWZ 194	pWZ 162 with a 2-base substitution at −26 and −27	This study
pWZ 196	pWZ 162 with a 4-base substitution at −28 to −25	This study
pWZ 218	pWZ 162 with a 2-base substitution at −30/−31	This study
pWZ 220	pWZ 162 with a base substitution at −27	This study
pWZ 223	pWZ 162 with a base substitution at −28	This study
pWZ 106	pJAMY 30 + *fliQ* translational fusion	This study
pWZ 113	pJAMY 31 + *fliR* translational fusion	This study
pGM52758	pJAMY31 + *hem* translational fusion	37

sion, we have characterized this locus and shown it to be an operon composed of two genes that are homologs of the *E. coli* (35) and *B. subtilis* (4, 8) *fliQ* and *fliR* genes. The *Caulobacter fliQ* and *fliR* genes encode membrane proteins that, like the *B. subtilis* FliQ and FliR proteins (8), show significant homology to the *Shigella flexneri* Spa9 and Spa29 proteins (49) and to the *Erwinia carotovora* MopD and MopE proteins (40). FliQ is also homologous to the *Yersinia pestis* YscS protein. The Spa9, Spa29, and YscS proteins belong to a family of proteins that are involved in the export of bacterial virulence factors, suggesting that the *Caulobacter* FliQ and FliR proteins may be involved in the unique export pathway (32) that mediates flagellar assembly.

The *fliQR* operon is activated early in the cell cycle (13), and studies were initiated to define the *cis*-acting sites that might contribute to its temporal regulation. Mutational analysis has revealed that the *cis*-acting sites that mediate the temporal activation of *fliQR* transcription reside within 59 bp of the transcription start site. Within this region, three unique sequence elements at −10, −20 to −30, and −35, which are conserved in the promoter regions of other class II operons (52, 55), are found to be important for transcriptional activity.

MATERIALS AND METHODS

Materials. Oligonucleotides were obtained from Operon Technologies. Restriction endonucleases were obtained from Boehringer Mannheim and New England Biolabs. Calf intestine alkaline phosphatase, polynucleotide kinase, DNA polymerase I, and Klenow polymerase were obtained from Boehringer Mannheim. [α-^{35}S]dATP was from Amersham Life Science, and [^{35}S]methionine Trans-label was obtained from ICN Biomedicals, Inc.

Bacterial strains and growth media. Bacterial strains and plasmids are described in Table 1. *C. crescentus* wild-type strain NA1000 and mutant strains were grown at 32°C in either PYE medium (44) or minimal M-2 glucose medium (25). Cultures containing plasmid pRSZ3 (1) or pRK290lac (17) or derivatives were supplemented with 1 μg of tetracycline per ml for plasmid maintenance. *E. coli* strains were grown at 37°C in either LB broth or superbroth (47).

Expression of *fliQR* as a function of the cell cycle. *C. crescentus* NA1000 containing a *fliQR* transcriptional fusion to *lacZ* was grown in minimal M-2 glucose medium to an optical density at 600 nm of 0.8 to 1.0. Cells were synchronized by Ludox density centrifugation as described by Evinger and Agabian (14). Isolated swarmer cells were allowed to proceed through the cell cycle, and periodically culture samples were removed and pulse-labeled with 15 μCi of [^{35}S]methionine for 5 min. The labeled cells were then lysed, and proteins were immunoprecipitated with antibodies to β-galactosidase or flagellins, as indicated. The immunoprecipitated proteins were separated on a sodium dodecyl sulfate–10% polyacrylamide gel and visualized by autoradiography.

DNA sequencing. A 1.7-kb DNA fragment (Fig. 2A) that complements the *flaS* mutant strain SC508 was cloned into either pBluescript(II)KS(+) or pBluescript (II)SK(+) (Stratagene Cloning Systems). Deletions of the insert were generated using a double-stranded nested deletion kit from Pharmacia. Each deletion was transformed into *E. coli* TG1 cells, from which single-stranded DNA templates were prepared for sequence analysis. DNA sequences of both strands were determined by the dideoxynucleotide chain termination method (48) and analyzed by using the Genetics Computer Group package (10).

Amplification of a DNA fragment containing a portion of the *C. crescentus fliQR* operon. To determine the location of the deletion in strain SC508, a DNA fragment that spans the breakpoint of the deletion was amplified by PCR. Reactions were carried out in 50 μl of reaction buffer containing 100 pmol of each oligonucleotide primer, 50 ng of *C. crescentus* chromosomal DNA from strain SC508 or NA1000, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% gelatin, 0.5 to 2.5 mM $MgCl_2$, 200 mM each deoxynucleoside triphosphate, and 2 U of *Taq* polymerase (Perkin-Elmer Cetus). After denaturation at 94°C for 4 min, reactions were carried out for 40 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min. The products were then analyzed by agarose gel electrophoresis. A fragment of the expected size was purified from an agarose gel and subsequently cloned into the pBluescript(II)KS(+) vector.

Site-directed mutagenesis. Site-directed mutagenesis was done with an Altered Sites kit (Promega). A portion of the *fliQR* sequence (nucleotides 320 to 983 as in Fig. 2B) was cloned into the *Kpn*I and *Hin*dIII sites of the pSelect vector. Mutagenesis was performed according to the manufacturer's protocol, using designed oligonucleotides, and the identity of each mutation was confirmed

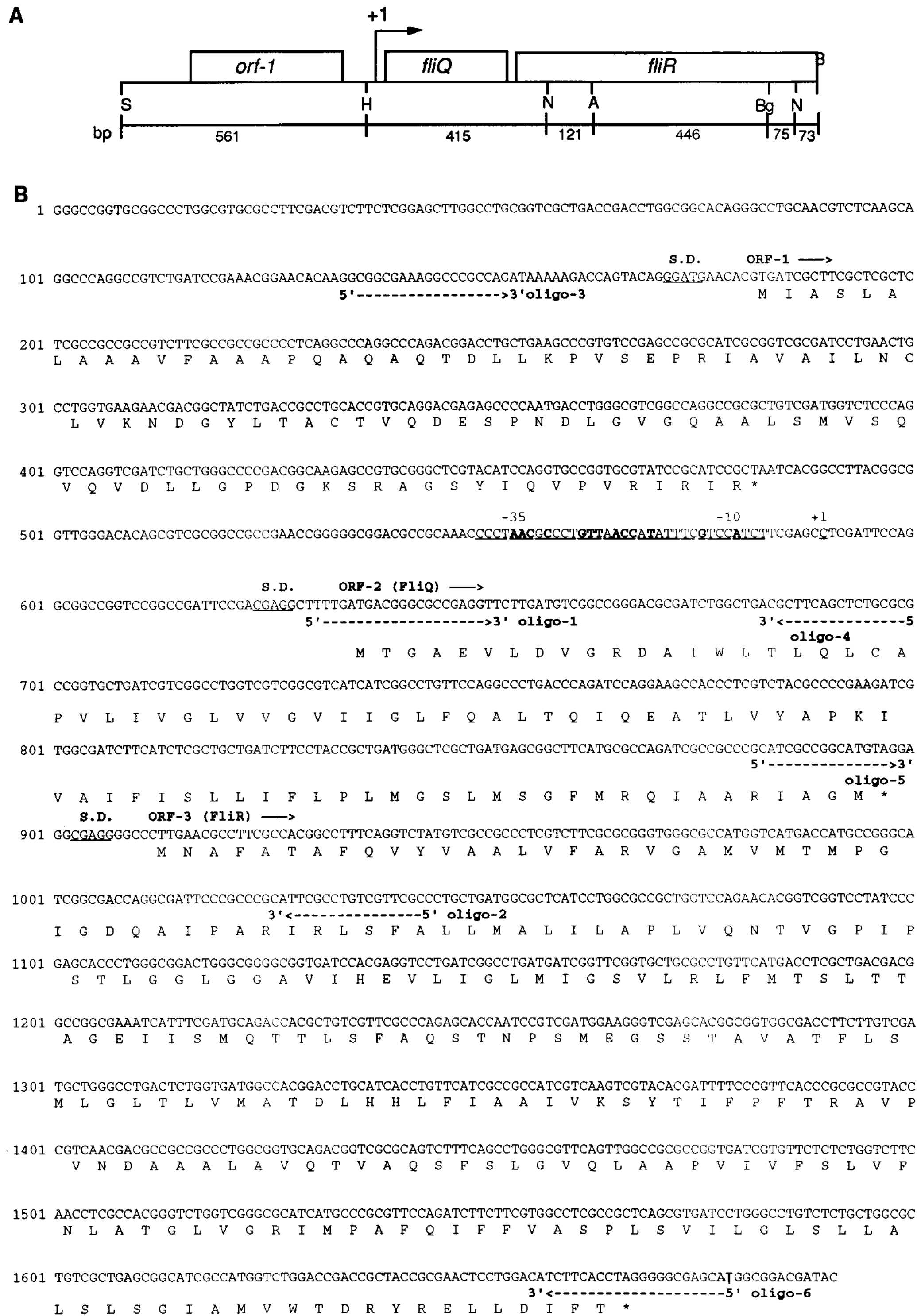


FIG. 2. Nucleotide sequence and predicted amino acid sequence of the *flaS* locus. (A) Map of the *flaS* locus. S, *Sma*I; H, *Hpa*I; N, *Nco*I; A, *Ava*I; Bg, *Bgl*II; B, *Bam*HI. (B) Nucleotide sequence of the *flaS* locus, containing three potential ORFs: ORF-1, ORF-2 (*fliQ*), and ORF-3 (*fliR*). The three ORFs are predicted to encode proteins of 100, 89, and 251 amino acids, respectively. S.D. represents the Shine-Dalgarno sequences preceding the initiation codons of the ORFs. The promoter sequence of the *fliQR* operon is underlined, and the highlighted nucleotides in the promoter region are conserved among the class II *fliQR* (13), *fliLM* (52), and *fliF* (55) promoters. Primers used for PCRs depicted in Fig. 6 are indicated as dashed lines (oligo-1 through oligo-6).

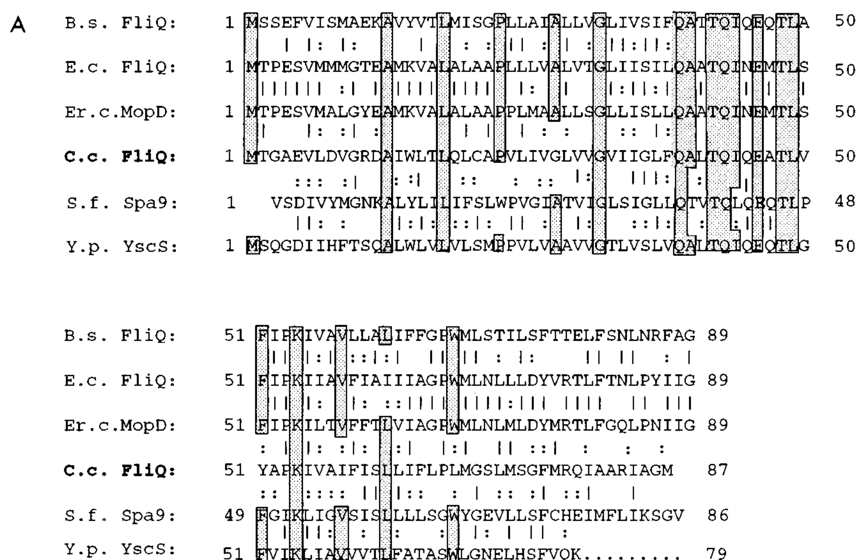


FIG. 3. Comparison of the predicted *C. crescentus* FliQ and FliR protein sequences with their homologs. Sequences were aligned by using the GAP program from the Genetics Computer Group package (10). Lines between the sequences represent identical residues; colons represent similar residues. Residues conserved in at least 75% of the peptide sequences are shaded. Gaps are shown as dots interrupting the sequence. Gaps without dots were introduce to accommodate gaps in other sequences. Numbers indicate amino acids. (A) Comparison of the *C. crescentus* (C.c.) FliQ protein with its homologs. (B) Comparison of the *Caulobacter* FliR protein with its homologs. B.s., *B. subtilis*; Er.c., *Erwinia carotovora*; E.c., *E. coli*; S.f., *S. flexneri*; Y.s., *Y. pestis.*

by DNA sequencing. The mutagenized *fliQR* fragments were then transferred from pSelect to plasmid pRK290lac (17), generating transcriptional fusions to *lacZ*. These constructs were transformed into *E. coli* S17-1 by electroporation and then conjugated into *C. crescentus* NA1000 (16).

Construction of *fliQR* promoter deletions. The 1.7-kb *Sma*I-to-*Bam*HI DNA fragment (Fig. 2A) was cloned into plasmid pBluescript(II)KS(+). Deletions were made in the promoter region by using a double-stranded nested deletion kit from Pharmacia, and the precise position of each deletion was determined by sequence analysis. To create a convenient site for cloning, a *Hin*dIII linker (New England Biolabs) was inserted at the *Nco*I site near the 5′ end of the *fliR* coding region (see Fig. 10). The *Bam*HI-*Hin*dIII fragment containing a deleted promoter region of the *fliQR* operon was then transferred to plasmid pRSZ3 (1) for further analysis.

Analysis of FliQ– and FliR–β-lactamase fusion proteins. *C. crescentus* NA1000 carrying a portion of either *hemE* (encoding amino acids 131 to 452) (37), *fliQ* (encoding amino acids 1 to 84), or *fliR* (encoding amino acids 1 to 178) fused to the *bla* gene encoding β-lactamase was harvested at an optical density at 600 nm of 0.8 to 1.0. Cells were lysed with a French press, and the lysate was then separated into membrane and cytosolic fractions as described by Shaw et al. (50). Equal amounts of protein from each fraction were analyzed by immunoblotting using antibody against β-lactamase (19).

Electron microscopy. Cells were placed onto carbon-coated copper grids, stained with 1% uranyl acetate, washed with distilled water, and air dried (61). The stained cells were examined and photographed on a Philips 301 electron microscope at 60 kV.

Construction of a *fliQ* in-frame deletion. DNA fragments flanking the *fliQ* coding region were amplified by PCR and ligated, such that an in-frame deletion was generated (amino acids 23 to 79 were deleted). The two pairs of oligonucleotides used in the PCRs are indicated as oligo-3, oligo-4, oligo-5, and oligo-6 in Fig. 2B.

RESULTS

Identification of the genes in the *flaS* locus. The *flaS* locus was initially defined by a single allele in *C. crescentus* SC508 (13). The nonflagellated phenotype of SC508 is fully complemented in *trans* by a 1.7-kb *Sma*I-to-*Bam*HI DNA fragment (13). The nucleotide sequence of this DNA fragment was determined (Fig. 2). On the basis of G:C bias at the third position of each codon, we identified three open reading frames (ORFs) which represented likely protein-coding regions in this DNA fragment (Fig. 2A). ORF-1 encodes a predicted protein of 100 amino acids with a calculated molecular mass of 11.0 kDa. ORF-2, 150 bp downstream from the ORF-1 stop codon, encodes a putative 87-amino-acid protein with a predicted molecular mass of 9.6 kDa. ORF-3, located 14 bp downstream from ORF-2, appears to utilize TTG as the start codon and to encode a predicted protein of 251 amino acids of approximately 27.6 kDa. A more conventional start codon, ATG, appears 64 bp downstream from the TTG codon in the same reading frame. Both the TTG and the ATG are preceded by a relatively conserved ribosome binding site. However, because the ATG codon is located within a conserved region of the coding sequence (discussed below), we propose that the TTG is the start codon.

A search of the GenBank database revealed several potential homologs of the predicted proteins encoded by each ORF (Fig. 3). The predicted ORF-1 peptide sequence showed 27% identity and 50% similarity to the sequence of the MopB protein in *Erwinia carotovora* (40). ORF-2 was found to be 39% identical (75% similar) to the *B. subtilis* FliQ protein (4), 41% identical (71% similar) to the *E. coli* FliQ protein (35), 39% identical (68% similar) to the *Erwinia carotovora* MopD protein (40), 29% identical (66% similar) to the *S. flexneri* Spa9 protein (49), and 34% identical (65% similar) to the *Y. pestis* YscS protein (unpublished; GenBank accession number L22459). Similarly, ORF-3 was 26% identical (58% similar) to the *B. subtilis* FliR protein (8), 28% identical (57% similar) to the *Erwinia carotovora* MopE protein (40), 29% identical (60% similar) to the *E. coli* FliR protein (35), and 21% identical (57% similar) to the *S. flexneri* Spa29 protein (49). Because of the strong similarity between *C. crescentus* ORF-2 and ORF-3 and the *E. coli* and *B. subtilis* FliQ and FliR proteins, we designated ORF-2 as FliQ and ORF-3 as FliR.

The *E. coli fliQ* and *fliR* genes are located in the *fliL* operon, which is required for flagellar biogenesis (63, 64). This operon consists of seven genes, *fliL*, *fliM*, *fliN*, *fliO*, *fliP*, *fliQ*, and *fliR*, among which the gene products of *fliN* and *fliP* have been proposed to be involved in flagellum-specific export (35, 58).

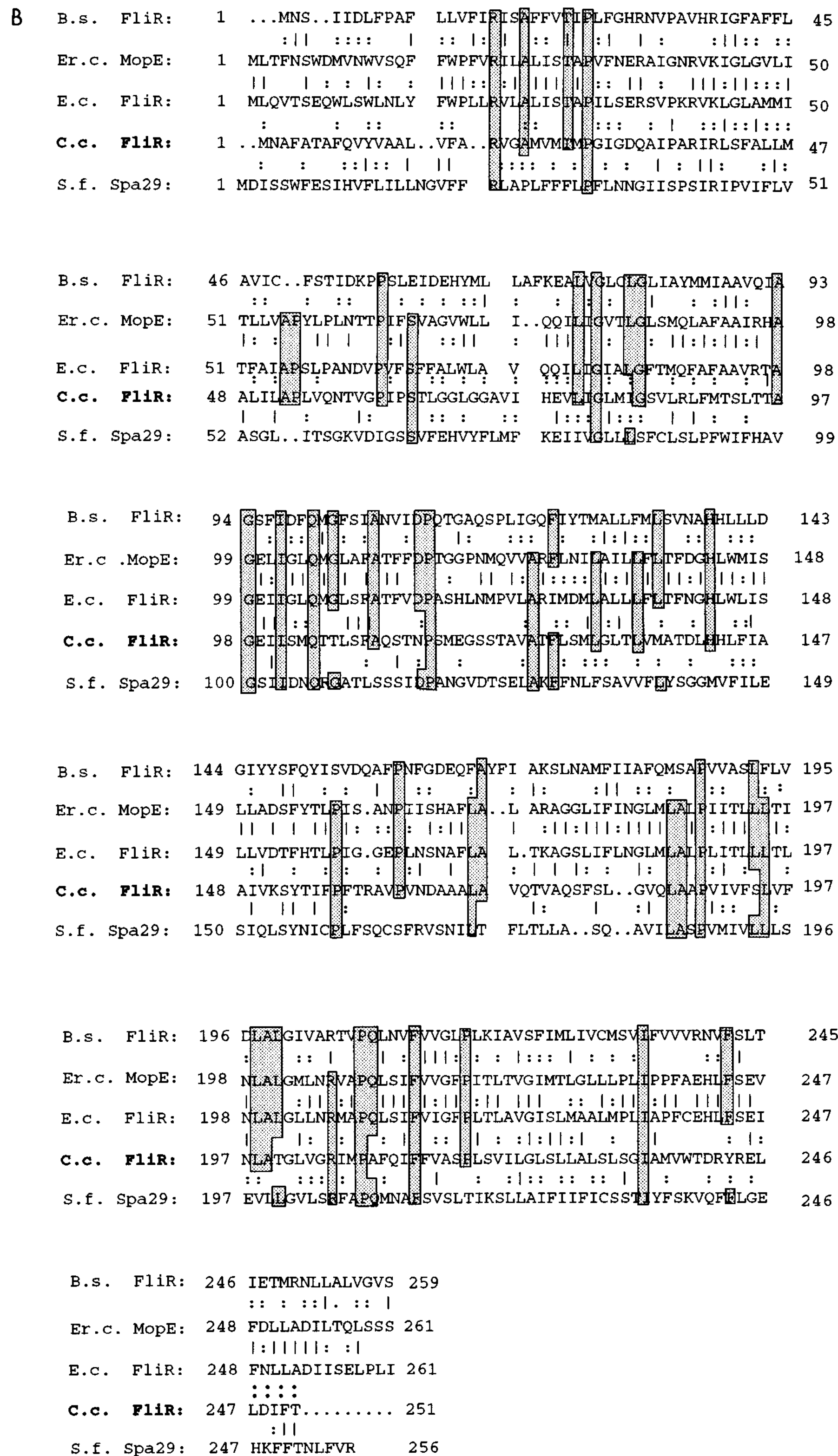


FIG. 3—*Continued.*

The *Erwinia carotovora mopD* and *mopE* genes are in the same operon as the *mopB* gene. Mutations in the *Erwinia carotovora mop* operon exhibit reduced virulence in plants. In addition, a mutation in the *mopE* gene results in a nonflagellated phenotype (40). The *S. flexneri spa9* and *spa29* genes reside in an operon which encodes several genes, most of which, including *spa9* and *spa29*, are essential for invasion of host cells and surface presentation of the virulence proteins IpaB, IpaC, and

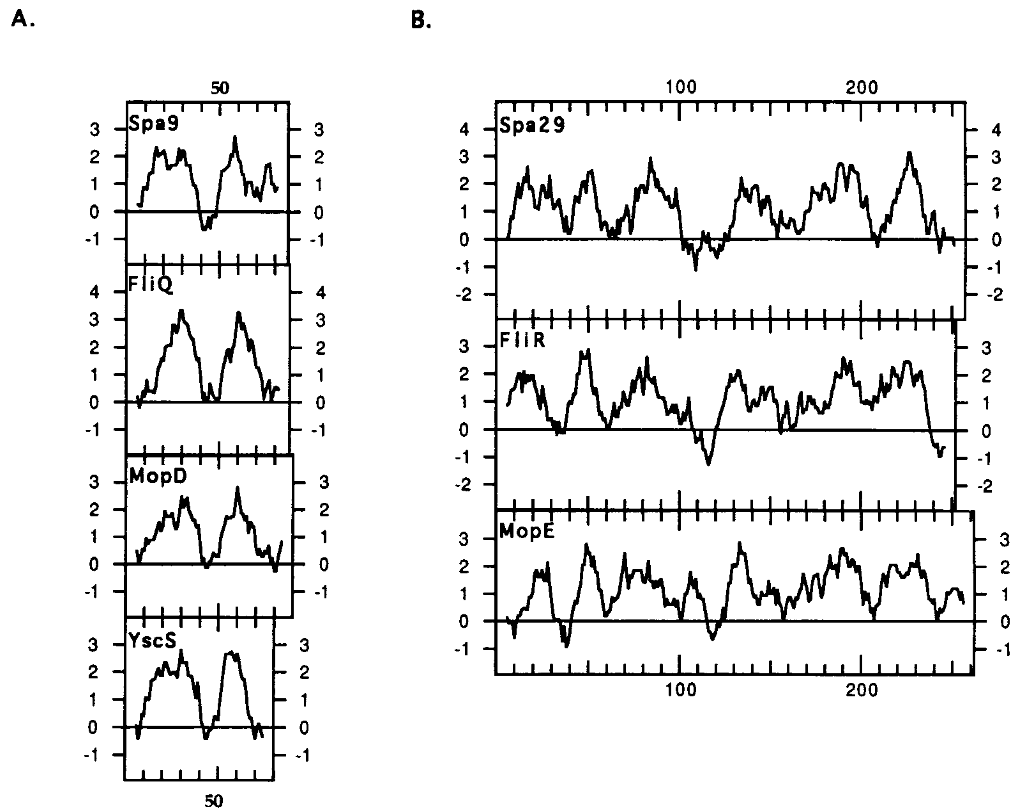


FIG. 4. Hydrophobicity plots of the FliQ and FliR proteins and their homologs. The graph was generated by the Hydrophobicity Plot program in the DNA Strider package. Negative values indicate hydrophilic regions, and positive values indicate hydrophobic regions. (A) Hydrophobicity plots of the *Caulobacter* FliQ protein and its homologs, the *S. flexneri* Spa9 protein, the *Erwinia carotovora* MopD protein, and the *Y. pestis* YscS protein. (B) Hydrophobicity plots of the *Caulobacter* FliR protein and its homologs, the *S. flexneri* Spa29 protein and the *Erwinia carotovora* MopE protein.

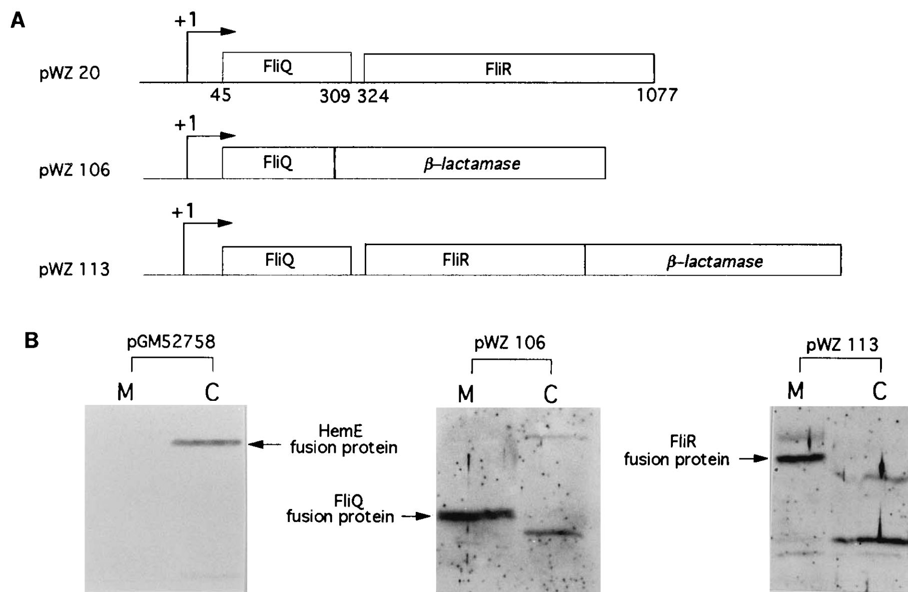


FIG. 5. Analysis of membrane association of FliQ and FliR fused to β-lactamase. (A) Schematic of the intact *fliQ* and *fliR* genes in pWZ 20 and fusions of the *bla* (β-lactamase) reporter gene to *fliQ* in pWZ 106 and to *fliR* in pWZ 113. As a control, a portion of the cytosolic protein HemE (from amino acids 131 to 452) (37) was also fused to the *bla* reporter gene. (B) Immunoblots of *C. crescentus* protein extracts, using antibody to β-lactamase. Cell extracts were prepared from strains carrying one of the plasmids integrated into the *Caulobacter* chromosome and subsequently fractionated into membrane (M) and cytosolic (C) fractions by differential centrifugation as reported previously (50).

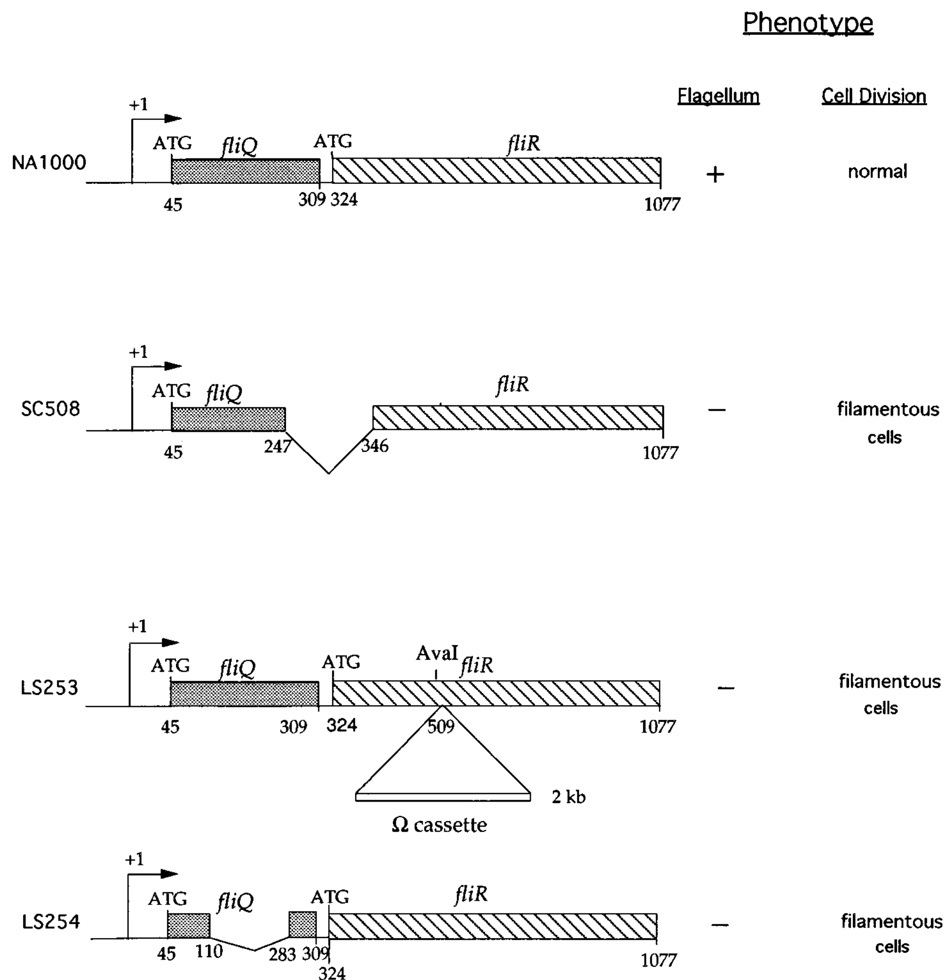


FIG. 6. Phenotypes of mutant alleles of the *fliQR* operon. A schematic of the *fliQR* operon is shown, and positions of the mutations are marked as nucleotide numbers below the diagram. Strain NA1000 contains the wild-type allele, while strains SC508, LS253, and LS254 contain chromosomal mutant alleles that are either deletions or insertions in the *fliQR* operon, as indicated. The presence (+) or absence (−) of a flagellum in each mutant strain examined by electron microscopy is indicated, and cells unable to divide normally are referred to as filamentous cells.

IpaD (56). Although the function of *yscS* in *Y. pestis* has not been reported, it is known that the homologous *yscS* gene of *Yersinia pseudotuberculosis* is essential for export of Yop proteins (5).

FliQ and FliR are membrane proteins. The *C. crescentus* FliQ and FliR proteins are very hydrophobic throughout almost the entire polypeptide sequence, suggesting that they are membrane proteins. The hydrophobicity plots of these proteins are similar to those of their homologs, MopD, MopE, Spa9, Spa29, and YscS (Fig. 4), even though FliQ and FliR are only modestly similar to some of the homologs at the amino acid level (Fig. 3). Thus, these peptides are likely to fold into similar protein structures and may well serve similar functions. To test whether the *Caulobacter* FliQ and FliR proteins in fact reside in the cell membrane, a fusion of the FliQ or FliR protein to a fragment of β-lactamase which lacks its signal peptide for membrane translocation was constructed (Fig. 5A). As a control, a β-lactamase protein fusion to a portion of the *Caulobacter* cytosolic protein, HemE, was also constructed (37). Extracts of *Caulobacter* strains bearing each protein fusion integrated into the chromosome were separated into membrane and cytosolic fractions by centrifugation as described by Shaw et al. (50). The relative amount of fusion protein in each fraction was analyzed by immunoblotting using antibodies to β-lactamase. The HemE fusion protein was recovered in the cytosolic fraction, whereas both FliQ and FliR fusion proteins were predominantly located in the membrane fraction, indicating that they are membrane associated (Fig. 5B).

Characterization of mutant alleles of *fliQ* and *fliR*. Strain SC508 carries a deletion in the *flaS* locus (13). To characterize the nature of this deletion, we determined its precise location by using PCR to amplify the relevant region of the *flaS* locus from SC508. The primers used in the PCRs are indicated in Fig. 2B as oligo-1 and oligo-2. As a control, the locus was also amplified from the wild-type strain NA1000 with the same primers. Analysis of the PCR fragments by gel electrophoresis confirmed the existence of a deletion in the SC508 chromosome (data not shown). The PCR products were cloned and sequenced as described in Materials and Methods. The location of the 100-bp deletion in SC508 is shown in Fig. 6. The deletion begins near the C terminus of the *fliQ* coding region and ends within the *fliR* coding region (from nucleotides 247 to 346 relative to the transcription start site). This deletion results in the fusion of the FliQ and FliR proteins without altering the

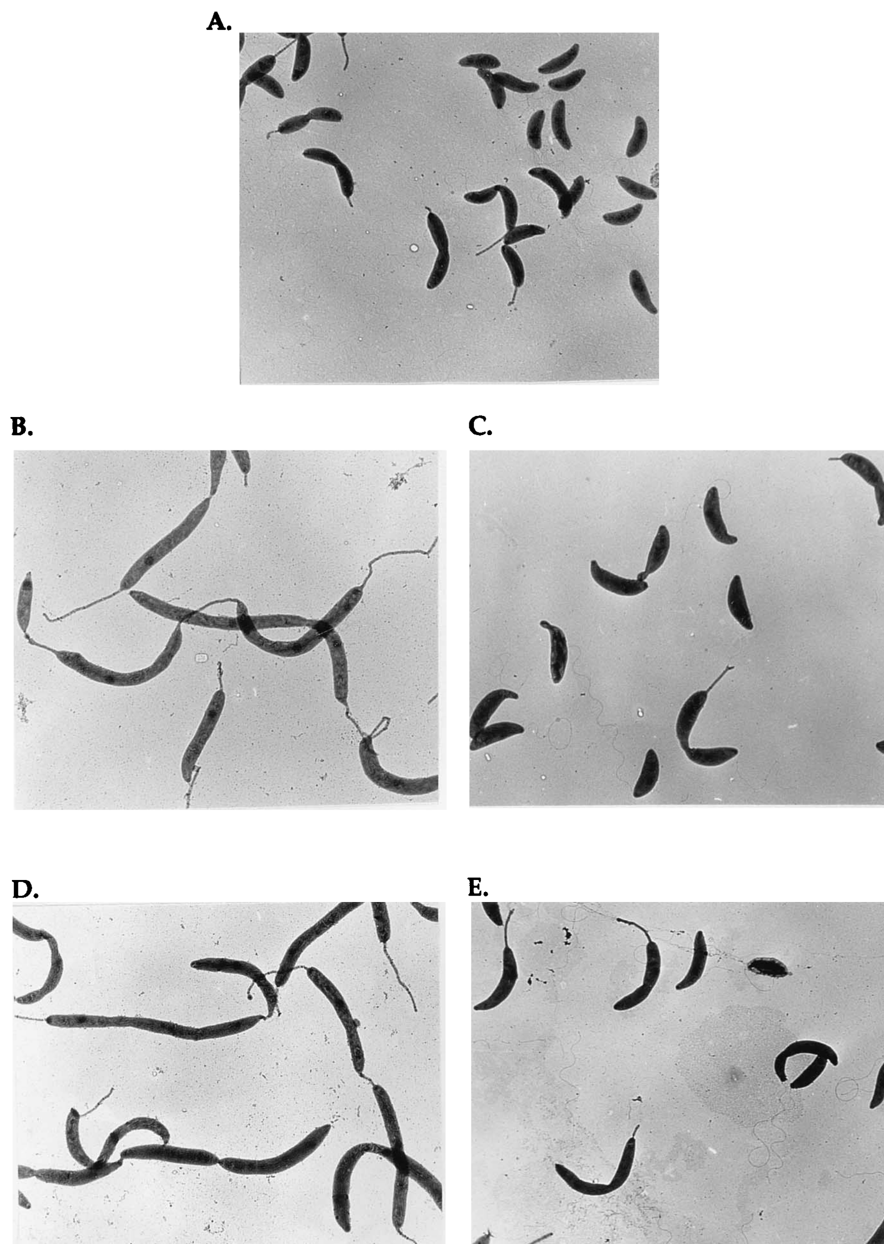
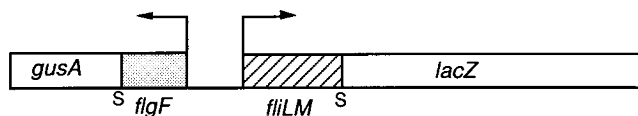


FIG. 7. Electron micrographs of the *C. crescentus* wild-type and mutant alleles of the *fliQR* operon. *fliQ* (LS254) and *fliR* (LS253) mutant strains lacked a flagellum and formed filamentous cells (B and D). In both mutant strains, the phenotypes were fully restored by a complementing plasmid (C and E). A wild-type strain is shown in panel A.

reading frame of FliR (Fig. 6, SC508). As a result, the functions of both FliQ and FliR might be impaired in this strain.

To investigate the independent functions of *fliQ* and *fliR*, mutations were generated in each gene. An in-frame deletion (amino acids 23 to 72; Fig. 2B) was generated in the *fliQ* gene and integrated into the chromosome, replacing the wild-type copy of the gene, using the method described by Jenal et al. (24). The resulting strain (LS254) appeared nonmotile by light microscopy and formed small colonies on 0.3% soft agar plates (data not shown). Electron microscopy revealed that these cells lacked a flagellum. In addition, both light and electron microscopy showed that this strain had defects in cell division (Fig. 7B). All of these phenotypes were complemented in *trans* by plasmid pWZ 35, which carries a wild-type copy of the *fliQR* operon (Fig. 7C), and by plasmid pWZ 165, which contains an intact *fliQ* gene and a truncated *fliR* gene (data not shown). A mutation in the *fliR* gene was generated by inserting a 2-kb DNA fragment containing stop codons in all three reading frames into the *fliR* coding region at the *Ava*I site (Fig. 2A). Upon integration into the chromosome to generate strain LS253 (Fig. 6), this *fliR* mutant allele yielded the same phenotypes as strain LS254: lack of motility, lack of a flagellum, and appearance of filamentous cells (Fig. 7D). The mutant phenotypes were fully restored by plasmid pWZ 35 (Fig. 7E).

We have previously reported that the time of expression and promoter sequence of the *flaS* locus are consistent with it



Strain	% of Activity P_{fliL}	P_{flgF}
NA100	100	100
LS254 (*fliQ*⁻)	202	27
LS253 (*fliR*⁻)	368	18

FIG. 8. Effects of the *fliQ* and *fliR* mutants on class II and class III flagellar gene transcription. A 1.4-kb *Sal*I-*Sal*I DNA fragment containing the promoter region of the *fliLM* and *flgF* genes (11) was cloned into plasmid pRSZ6. In this plasmid, the class II promoter, *fliLM*, was fused to *lacZ* and the class III promoter, *flgF*, was fused to *gusA*. In wild-type strain NA1000, *fliL* and *flgF* gave rise to 769 and 41 Miller units (39), respectively. The background activities of these two reporter genes are 140 and 5 Miller units, respectively (1). S, *Sal*I.

belonging to the class II genes in the flagellar regulatory hierarchy (13). To determine if both *fliQ* and *fliR* function as class II genes, we performed epistasis experiments. A plasmid in which a class II promoter (*fliLM* operon) and a class III promoter (*flgF* operon) were fused to the reporter genes *lacZ* and *gusA*, respectively (50a), was used to determine the effects of mutations in *fliQ* and *fliR* on class II and class III promoter activity (Fig. 8). Mutations in either the *fliQ* or the *fliR* gene diminished the transcription of the class III *flgF* promoter but elevated the expression of the class II *fliL* promoter. These results are consistent with the predicted behavior of class II flagellar genes (43, 62).

Analysis of the *fliQR* promoter. We previously identified the transcription start site of the *flaS* locus 45 bp upstream of a putative ORF and showed that a single transcript spans the *Hpa*I-*Ava*I region (see Fig. 2A) (13), suggesting that the ORFs within this region form an operon. To confirm this, we determined if a single promoter was used for the transcription of both *fliQ* and *fliR*. A DNA fragment containing a portion of *fliR* and the entire *fliQ* gene except for the preceding promoter was fused to *lacZ* and assayed for β-galactosidase activity (pWZ 72; see Fig. 11). This transcriptional fusion showed background levels of β-galactosidase activity (see Fig. 11), indicating the absence of a promoter between *fliQ* and *fliR*. We conclude that *fliQ* and *fliR* are organized in an operon, which is transcribed from the promoter preceding *fliQ*.

The promoter regions of class II operons, including *fliQR*, *fliLM*, and *fliF*, have extensive sequence homology (13, 52, 55, 65) but differ from bacterial promoter sequences recognized by known sigma factors. Most notably, the conserved nucleotides are highly concentrated in the region between −20 and −30 (Fig. 9A) and the −35 region; the −10 region is weakly conserved. In vitro mutagenesis of the *fliQR* promoter region was carried out to determine if the conserved nucleotides are important for promoter activity (Fig. 9B). Several multiple base pair mutations were generated in the conserved −35, −20 to −30, and −10 regions, and the resulting promoters were fused to *lacZ* to determine their effects on promoter activity (Fig. 9B). Multiple base pair changes in each of these regions severely reduced transcriptional activity (Fig. 9B). A 12-bp insertion at −26 (pWZ 166) also abolished transcriptional activity. In addition to these down mutations, an up mutation was obtained by a CC→AT change at positions −31 and −30 (pWZ 218; Fig. 9B), which increased transcription about twofold. This change was not in the bases conserved among the class II promoters (Fig. 9A).

These multiple base pair substitutions confirmed the importance of these regions for *fliQR* promoter activity. Several point mutations were also generated in the −35 and −20 to −30 regions (Fig. 10). The number below each mutation indicates its promoter activity relative to the wild-type promoter, defined as 1.0. In the −35 region, a single base pair deletion (deletion 7) at a conserved position reduced the activity by 60%, as did the substitution of a T for C at position −34 (substitution 6). In the −20 to −30 region, three of five substitutions (substitutions 1, 2, and 3) significantly impaired promoter activity (<60%), despite the fact that substitution 2 changed a nonconserved base pair. Substitution of a C residue for the conserved G residue at −28 (substitution 4) had no effect on transcription activity. Substitution of an A residue for a nonconserved C residue at −30 (substitution 5) retained 70% of wild-type activity. Mutational analysis thus suggests that the *fliQR* promoter is functionally similar to that used by two other class II operons, *fliLM* (52) and *fliF* (55).

Temporal regulation of the *fliQR* operon. Transcriptional activation of the *flaS* locus is cell cycle regulated and occurs prior to the activation of class III and class IV genes (13). To determine the minimum sequence necessary for the temporal activation of the *fliQR* promoter, a series of deletions in the 5′ region was constructed. Each deletion, which has the same 3′ end, was fused to a promoterless *lacZ* reporter gene on the low-copy-number plasmid pRK290lac (three to five copies per cell) (Fig. 11). Promoter function was assayed by measuring β-galactosidase activity. As shown in Fig. 11, deletions up to −59 relative to the transcriptional start site (pWZ 83) retained full promoter activity. In contrast, deletion to −27 (pWZ 72) sharply reduced promoter activity. To determine if the deletion to −59 (pWZ 83) retained temporal control of *fliQR* transcription, the synthesis of β-galactosidase from the *lacZ* fusion was measured as a function of the cell cycle (Fig. 11B). As a control, cell cycle expression of the wild-type promoter region in pWZ 56 was also determined. Strains bearing pWZ 56 or pWZ 83 exhibited the same temporal control of *lacZ* expression (Fig. 11B), which is indistinguishable from that previously observed for the wild-type chromosomal allele (13). Thus, sequences within −59 bp of the transcription start site are sufficient for normal transcriptional activity and correct temporal expression of the *fliQR* operon.

Promoters with base substitutions in conserved residues that either did not affect (Fig. 10, 4), partially reduced (Fig. 10, 3), or increased (Fig. 9, pWZ 218) *fliQR* promoter activity were assayed in synchronized cultures to determine if these mutations affected temporal expression. All three mutant promoters were temporally regulated and exhibited peak activity at 0.4 to 0.5 division unit, as is observed with the wild-type promoter (data not shown). We conclude that these mutant alleles in the −20 to −30 region, which retained some level of promoter activity, do not affect the temporal pattern of expression.

DISCUSSION

Possible roles of the FliQ and FliR proteins. The bacterial flagellum consists of three distinct structures: the basal body complex, the hook, and the filament. The basal body complex contains the transmembrane basal body (composed of several rings threaded on a rod), a switch complex, and a proposed

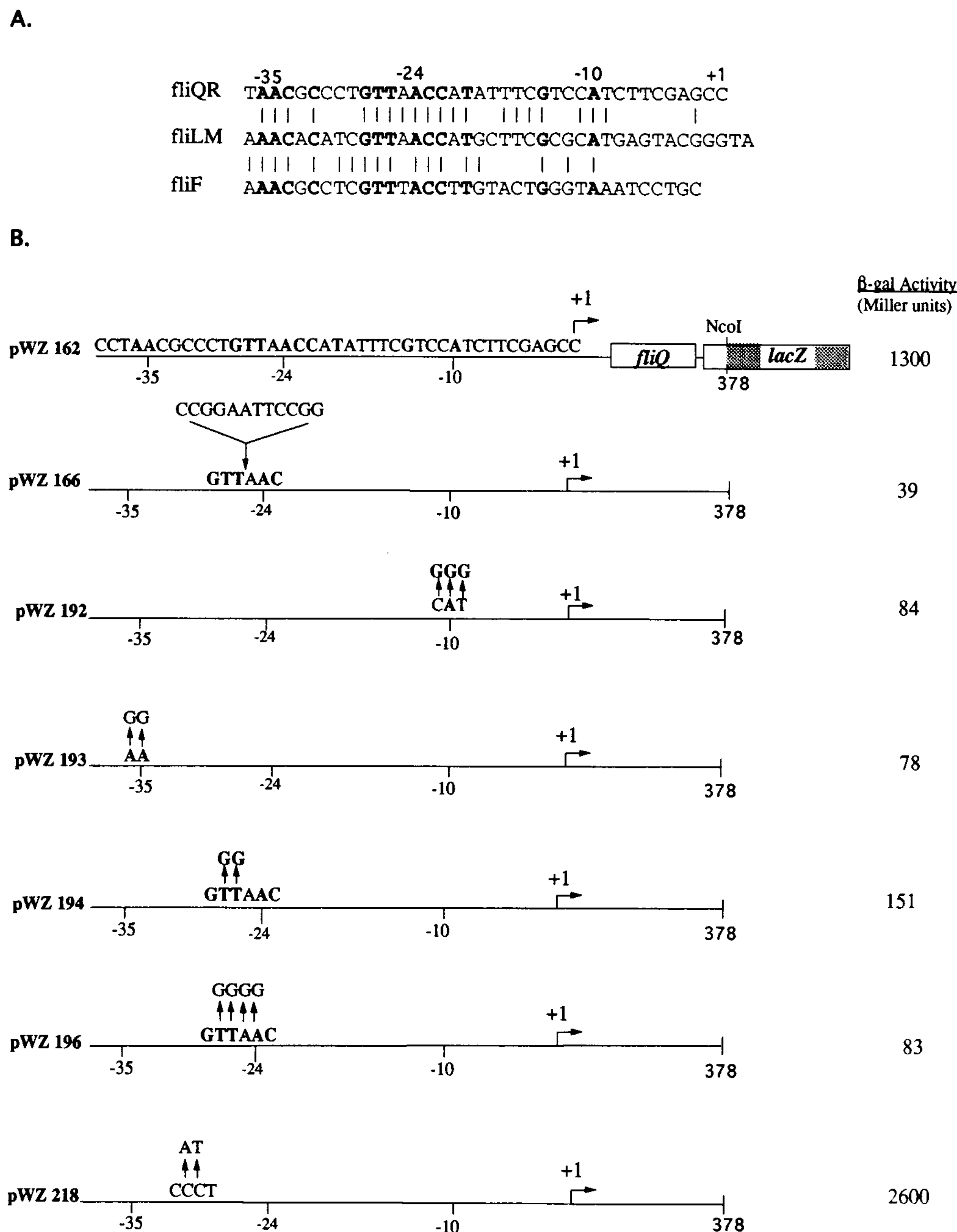


FIG. 9. Effects of multiple base changes in the 5′ region on *fliQR* promoter activity. (A) Alignment of the promoter region of the three class II genes *fliQR* (13), *fliLM* (65), and *fliF* (55). Residues in boldface letters are conserved among all three genes. (B) Base pair substitutions were introduced by site-directed mutagenesis. The promoter activities of *lacZ* transcription fusions in strains bearing one of the *lacZ* fusions on a plasmid were assayed by measuring β-galactosidase (β-gal) activity (39). pWZ 162 carries a wild-type *fliQR* promoter, whereas other plasmids carry mutations in the 5′ region of *fliQR*, as shown.

flagellum-specific export apparatus (for a review, see reference 34). The switch complex, which controls the direction of flagellar rotation, lies below the basal body at the cytoplasmic face of the inner membrane, some portion of which interacts with the chemotaxis signal transduction cascade. The flagellum is assembled sequentially from the most cell-proximal structure, the basal body, to the most cell-distal structure, the filament (18, 27, 30, 53, 54). The first step of the assembly process is believed to be the insertion of the M ring into the cytoplasmic membrane, on which the rest of the basal body is built (30). Two distinct export mechanisms are thought to be used for the assembly of the flagellum (33, 34). One is used for flagellum-specific export of the axial proteins (rod, hook, hook-associated proteins, and flagellins) (20, 28, 32). The other, which is a more general *secA*-dependent pathway, is used for the export of the ring proteins to the periplasm (21, 22, 26). The flagellum-specific export apparatus is presumably located at or near the basal body interface with the cell membrane and selects and exports the axial proteins through a central channel in the growing structure (41, 46). The tunneling of the axial proteins

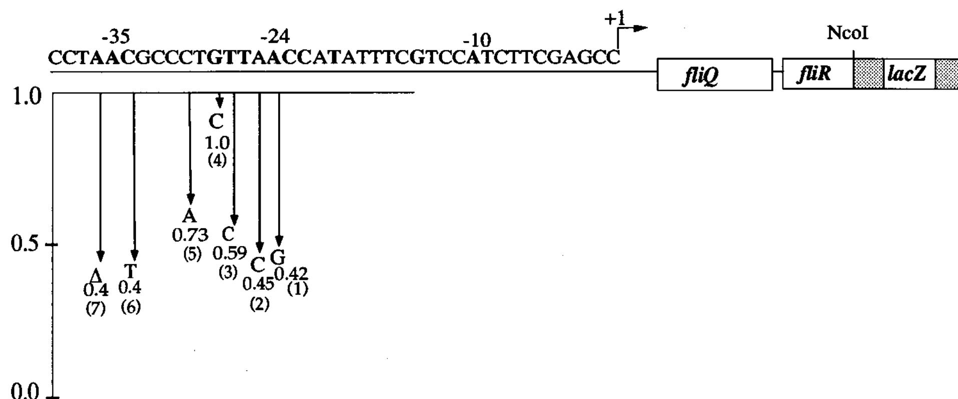


FIG. 10. Effects of point mutations in the promoter region of *fliQR* on promoter activity. The point mutations were generated by site-directed mutagenesis as described in Materials and Methods. Each mutant promoter fragment was cloned into pRK290lac, generating a transcriptional fusion to the *lacZ* gene. Promoter activity is presented as the percentage of the wild-type promoter activity (1,300 U). Each mutant is indicated by a number in parentheses.

from the cytoplasm to the surface structure eliminates the need to negotiate the hydrophobic membrane. It has been demonstrated that several axial proteins contain conserved domains in the N-terminal and C-terminal regions (11, 20) which may play a role in the export assembly process. In the case of *C. crescentus*, flagellum-specific export proteins are likely to be localized at the pole bearing the flagellum.

In this study, we have identified two *Caulobacter* genes that encode homologs of the *E. coli* FliQ and FliR proteins, which are possible components of the flagellum-specific export pathway. This possibility is based on several observations. First, it has been shown that in *E. coli* and *Salmonella fliQ* and *fliR* mutant strains, the M ring is inserted into the membrane, but no further assembly of the basal body occurs (27, 30, 53, 54). Second, the *C. crescentus* and *E. coli* FliQ and FliR proteins appear to be membrane proteins that belong to a family of export proteins, including Spa9 and Spa29 from *S. flexneri* and YscS from *Y. pestis*, all of which are involved in the specific export of virulence factors. Mutations in the *Shigella spa9* and *spa29* genes impair the surface presentation of virulence proteins such as IpaB, IpaC, and IpaD (56). The YscS homolog from *Y. pseudotuberculosis* is also involved in the export of Yop proteins (5). A distinct feature of these export pathways is that the virulence proteins selected for export lack cleavable signal peptide sequences (2, 15, 38, 57). Similarly, a hallmark of the flagellar export pathway is that the axial proteins, which must be transported through the pore of the growing flagellum, lack cleavable signal sequences (20, 34), whereas the ring proteins have potential cleavable signal peptides at their amino termini (12, 18, 19, 20, 26, 29).

The MopD and MopE proteins of *Erwinia carotovora*, a plant bacterial pathogen, may also be involved in virulence factor export (40), as judged from their homology to the *Shigella* Spa9 and Spa29 virulence factor excretion proteins (56). Furthermore, strains bearing a *mopE* mutant allele are unable to assemble flagella (40), suggesting that a virulence factor export pathway may also be used for the export of flagellar components. In addition to the similarity of the MopD and MopE proteins to FliQ and FliR, respectively, other genes in the *mop* locus also encode homologs of flagellar proteins. For example, *mopA* and *mopC* encode homologs of FliN from *E. coli* and *Salmonella typhimurium* (36) and FliP from *B. subtilis* (4), respectively. FliN is part of the flagellar switch complex and has been implicated in the export process as well (58). FliP is required for the assembly of the basal body and may also play a role in the transport of flagellar proteins (35). To rationalize the coincidence of reduced virulence and the inability to assemble a flagellum in an *Erwinia mopE* mutant allele, as well as the extensive similarity between flagellar genes and *mop* genes, it has been proposed that the Mop proteins, which are similar to the FliN, FliP, FliQ, and FliR proteins, are part of the flagellum-specific export pathway, which is exploited by the virulence factors (40).

We have observed that *fliQ* and *fliR* mutations disrupt not only flagellar biogenesis but also normal cell division, as is observed in mutants of *fliLM*, another class II operon (65). The initiation of the flagellar hierarchy by activation of class II genes is likely to share, at least partially, its regulatory pathway with other cell cycle events. The class II flagellar genes have been shown to respond to a DNA replication checkpoint (13, 52), in that disruption of DNA replication results in the inhibition of class II flagellar gene transcription. In addition, it has recently been shown that the gene encoding a temporally controlled DNA methyltransferase, which is required for the normal progression of the *Caulobacter* cell cycle, also responds to a DNA replication checkpoint and has the same promoter structural and functional features as the promoter regions of the class II flagellar genes (51).

Transcriptional regulation of the *fliQR* operon. The *fliQ* and *fliR* genes are expressed at the same time in the cell cycle as other class II genes, *fliLM* (65) and *fliF* (55). All three class II operons have a conserved promoter sequence that has not been found in other bacterial promoters. Analysis of individual mutations in the *fliQR* 5′ region revealed that the *fliQR* promoter shares the essential sequence elements present in the *fliLM* promoter (52) and to a lesser extent reflects those of the *fliF* promoter (55). One significant difference between the *fliF* promoter and either the *fliQR* or *fliLM* promoter is the presence of a *ftr* binding site, located at −8 in the *fliF* promoter region (55), which serves as a site for repression of the *fliF* operon (3, 59). Nevertheless, all three class II flagellar promoters show a high degree of sequence conservation in the −20 to −30 region. Furthermore, the class II genes are activated at a specific time in the cell cycle. We have shown here that a minimal promoter within the 59 bp upstream of the transcription start site of *fliQR* is sufficient for the temporal control of transcription. We speculate that a regulatory protein binds to the conserved −20 to −30 region and that this protein

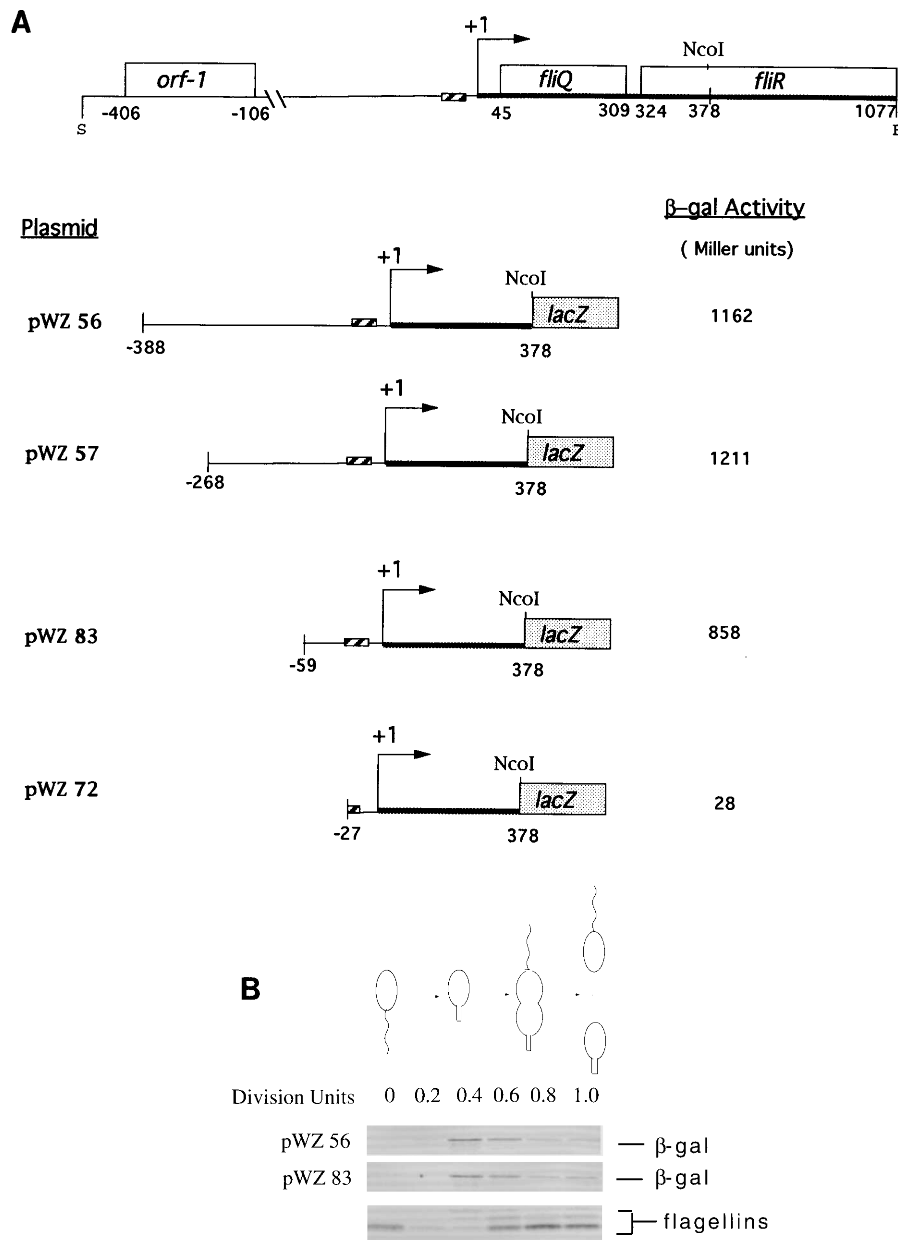


FIG. 11. Promoter activities and temporal expression of *fliQR* 5′ deletions. (A) Fragments of the *fliQR* operon containing deletions in the 5′ region were fused to a promoterless *lacZ* gene, and the β-galactosidase (β-gal) activity generated by each construct was measured and is shown in Miller units (39). Each value is the mean of at least three independent measurements. The striped box indicates the position of the conserved sequence shown in Fig. 10A. (B) Temporal expression of the transcriptional activity of the wild-type promoter in pWZ 56 and the minimal promoter in pWZ 83 is shown as a function of the cell cycle. *C. crescentus* NA1000 bearing either construction was synchronized and pulse-labeled with [^{35}S]methionine. The results are presented as a function of cell division units, which was calculated according to the duration of the cell cycle of each synchronized culture. The duration of the cell cycle in both experiments was approximately 150 min, and progression of the cell cycle was monitored by the expression pattern of flagellins (bottom panel). Swarmer cells were isolated at time zero and allowed to proceed through the cell cycle. Cell division occurred at 1.0 division unit.

is involved in transcriptional regulation. Preliminary gel shift experiments showed sequence-specific binding of proteins in crude extracts to the *fliQR* promoter region. However, the precise binding site and the possible role in transcription regulation remain to be determined.

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REFERENCES

1. **Alley, M. R. K.** Unpublished data.
1a. **Alley, M. R. K., S. L. Gomes, W. Alexander, and L. Shapiro.** 1991. Genetic analysis of a temporally transcribed chemotaxis genes cluster in *Caulobacter crescentus*. Genetics **129:**333–342.
2. **Baudry, B., A. T. Maurelli, P. Clerc, J. G. Sadoff, and P. J. Sansonetti.** 1987. Localization of plasmid loci necessary for the entry of *Shigella flexneri* into

HeLa cells, and characterization of one locus encoding four immunogenic polypeptides. J. Gen. Microbiol. **133:**3403–3413.
3. **Benson, A. K., G. Ramakrishnan, N. Ohta, J. Feng, A. J. Ninfa, and A. Newton.** 1994. The *Caulobacter crescentus* FlbD protein acts at *ftr* sequence elements both to activate and to repress transcription of cell cycle-regulated flagellar genes. Proc. Natl. Acad. Sci. USA **91:**4989–4993.
4. **Bischoff, D. S., M. D. Weinreich, and G. W. Ordal.** 1992. Nucleotide sequences of *Bacillus subtilis* flagellar biosynthetic genes *fliP* and *fliQ* and identification of a novel flagellar gene, *fliZ*. J. Bacteriol. **174:**4017–4025.
5. **Bergman, T., K. Erickson, E. Galyov, C. Persson, and H. Wolf-Watz.** 1994. The *lcrB* (*yscN/U*) gene cluster of *Yersinia pseudotuberculosis* is involved in Yop secretion and shows high homology to the *spa* genes cluster of *Shigella flexneri*. J. Bacteriol. **176:**2619–2626.
6. **Brun, Y. V., and L. Shapiro.** 1992. A temporally controlled σ factor is required for polar morphogenesis and normal cell division in *Caulobacter*. Genes Dev. **6:**2395–2408.
7. **Bryan, R., D. Glaser, and L. Shapiro.** 1990. A genetic regulatory hierarchy in *Caulobacter* development, p. 1–31. *In* J. G. Scandalios and T. R. F. Wright (ed.), A genetic regulatory hierarchy in *Caulobacter* development, vol. 27. Academic Press Ltd., London.
8. **Carpenter, P., A. R. Zuberi, and G. W. Odal.** 1993. *Bacillus subtilis* flagellar proteins FliP, FliQ, FliR and FlhB are related to *Shigella flexneri* virulence factors. Gene **137:**243–245.
9. **Champer, R., A. Dingwall, and L. Shapiro.** 1987. Cascade regulation of *Caulobacter* flagellar and chemotaxis genes. J. Mol. Biol. **194:**71–80.
10. **Devereux, D., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis program for the Vax. Nucleic Acids Res. **12:**387–395.
11. **Dingwall, A., D. Garman, and L. Shapiro.** 1992. Organization and ordered expression of *Caulobacter* genes encoding flagellar basal body rod and ring proteins. J. Mol. Biol. **228:**1147–1162.
12. **Dingwall, A., J. W. Gober, and L. Shapiro.** 1990. Identification of a *Caulobacter* basal body structural gene and a *cis*-acting site required for activation of transcription. J. Bacteriol. **172:**6066–6076.
13. **Dingwall, A., W. Zhuang, K. Quon, and L. Shapiro.** 1992. Expression of an early gene in the flagellar regulatory hierarchy is sensitive to an interruption in DNA replication. J. Bacteriol. **174:**1760–1768.
14. **Evinger, M., and N. Agabian.** 1977. Envelope-associated nucleoid from *Caulobacter crescentus* stalked and swarmer cells. J. Bacteriol. **132:**294–301.
15. **Forsberg, A., and H. Wolf-Watz.** 1988. The virulence protein Yop5 of *Yersinia pseudotuberculosis* is regulated at transcriptional level by plasmid-pIB1-encoded *trans*-acting elements controlled by temperature and calcium. Mol. Microbiol. **2:**121–133.
16. **Gilchrist, A., and J. Smit.** 1991. Transformation of freshwater and marine caulobacters by electroporation. J. Bacteriol. **173:**921–925.
17. **Gober, J. W., and L. Shapiro.** 1992. A developmentally regulated *Caulobacter* flagellar promoter is activated by 3′ enhancer and IHF binding elements. Mol. Biol. Cell **3:**913–926.
18. **Hahnenberger, K., and L. Shapiro.** 1987. Identification of a gene cluster involved in flagellar basal body biogenesis in *Caulobacter crescentus*. J. Mol. Biol. **194:**91–103.
19. **Hahnenberger, K., and L. Shapiro.** 1988. Organization and temporal expression of a flagellar basal body gene in *Caulobacter crescentus*. J. Bacteriol. **170:**4119–4124.
20. **Homma, M., D. J. DeRosier, and R. M. Macnab.** 1990. Flagellar hook and hook-associated proteins of *Salmonella typhimurium* and their relationship to other axial components of the flagellum. J. Mol. Biol. **213:**819–832.
21. **Homma, M., Y. Komeda, T. Iino, and R. M. Macnab.** 1987. The *flaFIX* gene product of *Salmonella typhimurium* is a flagellar basal body component with a signal peptide for export. J. Bacteriol. **169:**1493–1498.
22. **Homma, M., K. Ohnishi, T. Iino, and R. M. Macnab.** 1987. Identification of a flagellar hook and basal body gene products (FlaFV, FlaFVI, FlaFVII, and FlaFVIII) in *Salmonella typhimurium*. J. Bacteriol. **169:**3617–3624.
23. **Hughes, K. T., K. L. Gillen, M. J. Semon, and J. E. Karlinsey.** 1993. Sensing structural intermediates in bacterial flagellar assembly by export of a negative regulator. Science **262:**1277–1280.
24. **Jenal, U., J. White, and L. Shapiro.** 1994. *Caulobacter* flagellar function, but not assembly, requires FliL: a non-polarly localized membrane protein present in all cell types. J. Mol. Biol. **243:**227–244.
25. **Johnson, R. C., and B. Ely.** 1977. Analysis of nonmotile mutants of the dimorphic bacterium *Caulobacter crescentus*. J. Bacteriol. **137:**627–634.
26. **Jones, C. J., M. Homma, and R. M. Macnab.** 1989. L-, P-, and M-ring proteins of the flagellar basal body of *Salmonella typhimurium*: gene sequenced and deduced protein sequences. J. Bacteriol. **171:**3890–3900.
27. **Jones, C. J., and R. M. Macnab.** 1990. Flagellar assembly in *Salmonella typhimurium* temperature-sensitive mutants. J. Bacteriol. **172:**1327–1339.
28. **Joys, T. M.** 1985. The covalent structure of the phase-1 flagellar filament protein of *Salmonella typhimurium* and its comparison with other flagellins. J. Biol. Chem. **260:**15758–15761.
29. **Khambaty, F., and B. Ely.** 1992. Molecular genetics of the *flgI* region and its role in flagellum biogenesis in *Caulobacter crescentus.* J. Bacteriol. **174:**4101–4109.
30. **Kubori, T., N. Shimamoto, S. Yamaguchi, K. Namba, and S.-I. Aizawa.** 1992. Morphological pathway of flagellar assembly in *Salmonella typhimurium*. J. Mol. Biol. **226:**433–446.
31. **Kutsukake, K.** 1992. Abstr. 1C2-13. *In* Abstracts of the 15th Annual Meeting of the Japanese Society of Molecular Biology.
32. **Kuwajima, G., I. Kawagishi, M. Homma, J.-I. Asaka, E. Kondo, and R. M. Macnab.** 1989. Export of an N-terminal fragment of *Escherichia coli* flagellin by a flagellum-specific pathway. Proc. Natl. Acad. Sci. USA **86:**4953–4957.
33. **Macnab, R. M.** 1990. Genetics, structure and assembly of the bacterial flagellum. Symp. Soc. Gen. Microbiol. **46:**77–106.
34. **Macnab, R. M.** 1992. Genetics and biogenesis of bacterial flagella. Annu. Rev. Genet. **26:**131–158.
35. **Malakooti, J., B. Ely, and P. Matsumura.** 1993. Molecular characterization, nucleotide sequence and expression of the *fliO*, *fliP*, *fliQ*, and *fliR* genes of *Escherichia coli*. J. Bacteriol. **176:**189–197.
36. **Malakooti, J., Y. Komeda, and P. Matsumura.** 1989. DNA sequence analysis, gene product identification and localization of flagellar motor components of *Escherichia coli*. J. Bacteriol. **171:**2728–2734.
37. **Marczynski, G., K. Lentine, and L. Shapiro.** A developmentally regulated chromosomal origin of replication uses essential transcription elements. Submitted for publication.
38. **Michiels, T., P. Wattiau, R. Brasseur, J.-M. Ruysschaert, and G. R. Cornelis.** 1990. Secretion of Yop proteins by yersinae. Infect. Immun. **58:**2840–2849.
39. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
40. **Mulholland, V., J. C. D. Hinton, J. Sidebotham, I. K. Toth, L. J. Hyman, M. C. M. Perombelon, P. J. Reeves, and G. P. C. Salmond.** 1993. A pleiotropic reduced virulence (Rvi$^-$) mutant of *Erwinia carotovora* subspecies *atroseptica* is defective in flagella assembly proteins that are conserved in plant and animal bacterial pathogens. Mol. Microbiol. **9:**343–356.
41. **Namba, K., I. Yamashita, and F. Vonderviszt.** 1989. Structure of the core and the central channel of bacterial flagella. Nature (London) **324:**648–654.
42. **Newton, A., and N. Ohta.** 1990. Regulation of the cell division cycle and differentiation in bacteria. Annu. Rev. Microbiol. **44:**689–719.
43. **Newton, A., N. Ohta, G. Ramakrishnan, D. A. Mullin, and G. Raymond.** 1989. Genetic switching in the flagellar gene hierarchy requires both positive and negative regulation of transcription. Proc. Natl. Acad. Sci. USA **86:** 6651–6655.
44. **Poindexter, J. S.** 1964. Biological properties and classification of the *Caulobacter* group. Bacteriol. Rev. **28:**231–295.
44a. **Quon, K., and L. Shapiro.** Unpublished data.
45. **Ramakrishnan, G., and A. Newton.** 1990. FlbD of *Caulobacter crescentus* is a homologue of NtrC and activates σ^{54} dependent flagellar gene promoters. Proc. Natl. Acad. Sci. USA **87:**2369–2373.
45a. **Ramakrishnan, G., and A. Newton.** Unpublished data.
46. **Ruiz, T., N. R. Francis, D. G. Morgan, and D. J. DeRosier.** 1993. The size of the export channel in the flagellar filament of *Salmonella typhimurium*. Ultramicroscopy **49:**417–425.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
48. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
49. **Sasakawa, C., K. Komatsu, T. Tobe, T. Suzuki, and M. Yoshikawa.** 1993. Eight genes in region 5 that form an operon are essential for invasion of epithelial cells by *Shigella flexneri* 2a. J. Bacteriol. **175:**2334–2346.
50. **Shaw, P., S. L. Gomes, K. Sweeny, B. Ely, and L. Shapiro.** 1983. Methylation involved in chemotaxis is regulated during *Caulobacter* differentiation. Proc. Natl. Acad. Sci. USA **80:**5261–5265.
50a. **Stephens, C.** Unpublished data.
51. **Stephens, C., G. Zweig, and L. Shapiro.** Coordinate cell cycle control of a *Caulobacter* DNA methyltransferase and the flagellar genetic hierarchy. Submitted for publication.
52. **Stephens, C., and L. Shapiro.** 1993. An unusual promoter controls cell-cycle regulation and dependence on DNA replication of the *Caulobacter fliLM* early flagellar operon. Mol. Microbiol. **9(6):**1169–1179.
53. **Suzuki, T., T. Iino, T. Horiguchi, and S. Yamaguchi.** 1978. Incomplete flagellar structures in nonflagellated mutants of *Salmonella typhimurium*. J. Bacteriol. **33:**904–915.
54. **Suzuki, T., and Y. Komeda.** 1981. Incomplete flagellar structures in *Escherichia coli* mutants. J. Bacteriol. **145:**1036–1041.
55. **Van Way, S., A. Newton, A. Mullin, and D. Mullin.** 1993. Identification of the promoter and negative regulatory element, *ftr4*, that is needed for cell cycle timing of *fliF* operon expression in *Caulobacter crescentus*. J. Bacteriol. **175:** 367–376.
56. **Venkatesan, M. M., J. M. Buys, and E. V. Oaks.** 1992. Surface presentation of *Shigella flexneri* invasion plasmid antigens requires the products of the *spa* locus. J. Bacteriol. **174:**1990–2001.
57. **Venkatesan, M. M., J. M. Buysse, and D. J. Kopecko.** 1988. Characterization of invasion plasmid antigen genes (*ipaBCD*) from *Shigella flexneri*. Proc. Natl. Acad. Sci. USA **85:**9317–9321.
58. **Volger, A. P., M. Homma, V. M. Irikura, and R. M. Macnab.** 1991. *Salmonella typhimurium* mutants defective in flagellar filament regrowth and sequence similarity of FliI to F_0F_1, vacuolar, and archaebacterial ATPase subunits. J. Bacteriol. **173:**3564–3572.

59. **Wingrove, J., and J. W. Gober.** 1994. A σ^{54} transcriptional activator also functions as a pole-specific repressor in *Caulobacter*. Genes Dev. **8:**1839–1852.
60. **Wingrove, J. A., E. K. Mangan, and J. W. Gober.** 1993. Spatial and temporal phosphorylation of a transcriptional activator regulates pole-specific gene expression in *Caulobacter*. Genes Dev. **7:**1979–1992.
61. **Wright, R., and J. Rine.** 1989. Transmission electron microscopy and immunocytochemical studies of yeast: analysis of HMG-CoA reductase overproduction by electron microscopy. Methods Cell Biol. **31:**473.
62. **Xu, H., A. Dingwall, and L. Shapiro.** 1989. Negative transcriptional regulation in the *Caulobacter* flagellar regulatory hierarchy. Proc. Natl. Acad. Sci. USA **86:**6656–6660.
63. **Yamaguchi, S., S.-I. Aizawa, M. Kihara, M. Isomura, C. J. Jones, and R. M. Macnab.** 1986. Genetic evidence for a switching and energy-transducing complex in the flagellar motor of *Salmonella typhimurium*. J. Bacteriol. **168:**1172–1179.
64. **Yamaguchi, S., H. Fugita, A. Ishihara, S.-I. Aizawa, and R. M. Macnab.** 1986. Subdivision of flagellar genes in *Salmonella typhimurium* into regions responsible for assembly, rotation, and switching. J. Bacteriol. **166:** 187–193.
65. **Yu, J., and L. Shapiro.** 1992. Early *Caulobacter crescentus* genes *fliL* and *fliM* are required for flagellar gene expression and normal cell division. J. Bacteriol. **174:**3327–3338.