JOURNAL OF BACTERIOLOGY, Sept. 1988, p. 3953–3960
0021-9193/88/093953-08$02.00/0

Vol. 170, No. 9

Role of the 25-, 27-, and 29-Kilodalton Flagellins in *Caulobacter crescentus* Cell Motility: Method for Construction of Deletion and Tn*5* Insertion Mutants by Gene Replacement

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Received 2 March 1988/Accepted 14 June 1988

Caulobacter crescentus **incorporates two distinct, but related proteins into the polar flagellar filament: a 27-kilodalton (kDa) flagellin is assembled proximal to the hook and a 25-kDa flagellin forms the distal end of the filament. These two proteins and a third, related flagellin protein of 29 kDa are encoded by three tandem genes (alpha-flagellin cluster) in the *flaEY* gene cluster (S. A. Minnich and A. Newton, Proc. Natl. Acad. Sci. USA 84:1142–1146, 1987). Since point mutations in flagellin genes had not been isolated their requirement for flagellum function and *fla* gene expression was not known. To address these questions, we developed a gene replacement protocol that uses cloned flagellin genes mutagenized by either Tn*5* transposons in vivo or the replacement of specific DNA fragments in vitro by the antibiotic resistance omega cassette. Analysis of gene replacement mutants constructed by this procedure led to several conclusions. (i) Mutations in any of the three flagellin genes do not cause complete loss of motility. (ii) Tn*5* insertions in the 27-kDa flagellin gene and a deletion mutant of this gene do not synthesize the 27-kDa flagellin, but they do synthesize wild-type levels of the 25-kDa flagellin, which implies that the 27-kDa flagellin is not required for expression and assembly of the 25-kDa flagellin; these mutants show slightly impaired motility on swarm plates. (iii) Mutant PC7810, which is deleted for the three flagellin genes in the *flaEY* cluster, does not synthesize the 27- or 29-kDa flagellin, and it is significantly more impaired for motility on swarm plates than mutants with defects in only the 27-kDa flagellin gene. The synthesis of essentially normal levels of 25-kDa flagellin by strain PC7810 confirms that additional copies of the 25-kDa flagellin gene map outside the *flaEY* cluster (beta-flagellin cluster) and that these flagellin genes are active. Thus, while the 29- and 27-kDa flagellins are not absolutely essential for motility in *C. crescentus*, their assembly into the flagellar structure is necessary for normal flagellar function.**

Caulobacter crescentus presents unique features for the study of differentiation because of the well-defined sequence of morphological and biosynthetic changes that occur during the cell cycle. Asymmetric cell division of these gram-negative bacteria produces two morphologically different cell types, a nonmotile stalked cell and a flagellated swarmer cell, and each cell follows a distinct developmental program. The stalked cell divides repeatedly, much like a stem cell, to generate the parental stalked cell plus a new swarmer cell that carries a flagellum, DNA bacteriophage receptor sites, and pili at one cell pole. The swarmer cell, by contrast, loses motility, sheds the flagellum, and finally forms a cellular stalk at the former site of flagellum attachment before it enters the stalked cell division cycle described above. All these developmental events are under strict temporal and spatial control, with each of the surface structures formed in a stage-specific manner at only one of the cell poles (for reviews, see references 18 and 28).

Studies of morphogenesis in *C. crescentus* have focused primarily on the control of flagellum formation. Approximately 30 flagellar (*fla*) genes are required for biosynthesis of the flagellum (7), and the results of radioimmunoassays (15, 23, 30) and S1 nuclease mapping (4, 17, 21) have shown that a number of these genes are expressed periodically in the cell cycle at the time of flagellum assembly. Genetic analysis has suggested that the complex patterns of *fla* gene expression observed in *C. crescentus* are controlled in part by the organization of these genes in a regulatory hierarchy (21, 28, 30) similar to that proposed for *Escherichia coli* (13). The flagellin genes are positioned at the bottom of this hierarchy, and they are transcribed (17) and translated (15) in the order of product assembly. Thus, the 27-kilodalton (kDa) flagellin gene product, which is assembled proximal to the hook (14), is expressed first, followed by transcription and translation of the 25-kDa flagellin, which is assembled on the distal end of the filament (14, 33). These two genes are located in a tandem array (alpha-flagellin cluster) in the *flaEY* cluster along with the 29-kDa flagellin gene, whose transcript appears before either the 27- or the 25-kDa flagellin gene transcript in the cell cycle (17). Although 29-kDa flagellin was not originally found in purified, assembled flagellar filaments (29), there is recent immunological evidence that some 29-kDa flagellin is assembled at the junction of the hook and flagellar filament (L. Shapiro, personal communication).

The question of why *C. crescentus* has multiple flagellin genes has not been addressed because specific mutations in the 25-, 27- and 29-kDa flagellin genes have not been isolated; the only mutations reported previously also delete into flanking *fla* genes (3, 26). A variety of genetic techniques including generalized transduction (8), transposon mutagenesis (6), complementation with broad-host-range plasmids (3, 22), and conjugation (1, 19) are available in *C. crescentus*, but a general method for site-directed mutagenesis that could be used to construct flagellin gene mutations has not been described. As a consequence, we developed such a method

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TABLE 1. Bacterial strains and plasmids

Strain	Genotype or construction	Source
Caulobacter crescentus		
CB15	Wild type	ATCC 19089
SC259	*flaW120*	B. Ely (11)
SC1062	*flaE*::Tn*5*	B. Ely (26)
SC1391	*hexB116 rif-151*	B. Ely (5)
PC7301	*hexB*$^+$ *rif-151 zcg-301*::Tn*5*	This study
PC7302	*hexB*$^+$ *rif-151 flgK302*::Tn*5*	This study
PC7303	*hexB*$^+$ *rif-151 flgK303*::Tn*5*	This study
PC7312	*hexB*$^+$ *rif-151 flgL312*::Tn*5*	This study
PC7313	*hexB*$^+$ *rif-151 flgL313*::Tn*5*	This study
PC7314	*hexB*$^+$ *rif-151 flgL*$^+$ *flgL312*::Tn*5* Tc^r	This study
PC7315	*hexB*$^+$ *rif-151 flgL*$^+$ *flgL313*::Tn*5* Tc^r	This study
PC7321	*zcg-301*::Tn*5*; Φ(PC7301) × CB15	This study
PC7328	*flgK302*::Tn*5*; Φ(PC7302) × CB15	This study
PC7330	*flgL312*::Tn*5*; Φ(PC7312) × CB15	This study
PC7331	*flgL313*::Tn*5*; Φ(PC7313) × CB15	This study
PC7334	*zcg-301*::Tn*5*; Φ(PC7301) × SC259	This study
PC7801	*hexB*$^+$ *rif-151 flgLK*(Δ1.1, +Ω)	This study
PC7802	*flgLK*(Δ1.1, +Ω); Φ(PC7801) × CB15	This study
PC7809	*flgLKJ*(Δ1.1, +Ω, Δ2.2); Φ(PC7810) × CB15	This study
PC7810	*hexB*$^+$ *rif-151 flgLKJ*(Δ1.1, +Ω, Δ2.2)	This study
PC7811	Sp^r; omega insertion in the transcription unit II.1 (22)	N. Ohta (unpublished data)
Escherichia coli		
S17-1	*pro hsdR str mob*$^+$ Tp^r	Simon et al. (31)
HB101	*hsdR hsdM pro leu thr lacY endI recA str*	
Plasmids		
pBR322	Tc^r Amp^r	
pHC79	Tc^r Amp^r	Hohn and Collins (10)
pCN100	pHC79 containing ca. 40 kb of DNA from *flaEY* region	This study
pCN200	pBR322 with 6.6-kb *Hin*dIII fragment	This study
pSUP202	Tct^r Amp^r Cm^r	Simon et al. (31)
pSUP10.6	pSUP202 with 6.6-kb *Hin*dIII fragment	This study
pSPU301	Derivative of pSUP202 with *Eco*RI(pBR322 site)-*Eco*RI(b) fragment from pCN200 containing *zcg-301*::Tn*5*	This study
pHP45	Amp^r Sm^r-Sp^r	Prentki and Krisch (25)
pSUP1.1	Derivative of pSUP10.6 in which 1.1-kb *Eco*RI(a)-*Eco*RI(b) is replaced by omega cassette	This study
pSUP3.3	Derivative of pSUP1.1 from which 2.2-kb *Sal*I(a)-*Sal*I(b) has been deleted	This study

with the goal of investigating how these genes are regulated and the function of the individual flagellins in flagellum activity. This replacement technique may have general application to other nonenteric, gram-negative organisms.

In this report, we describe the construction of Tn*5* insertion mutations in the 25- and 27-kDa flagellin genes and of deletions of the three flagellin genes by gene replacement with DNA sequences carrying the streptomycin-spectinomycin omega cassette (25). Although all flagellin gene mutants were motile, the 27- and 29-kDa flagellin gene mutants were partially defective in motility on swarm plates. The 25-kDa flagellin gene mutants were fully motile, and like the all mutants examined, they synthesized almost normal levels of 25-kDa flagellin. Taken together, these results confirm the presence of 25-kDa flagellin genes outside the *flaEY* gene cluster (beta-flagellin cluster [16]), and they indicate that the 27- and 29-kDa flagellins are required to assemble a fully functional flagellum. The partial motility of all flagellin mutants examined may also explain the previous failure to isolate Tn*5* insertions or point mutations in the flagellin genes of *C. crescentus*.

MATERIALS AND METHODS

Media and strains. All *C. crescentus* and *E. coli* strains used are listed in Table 1. *E. coli* was grown in LB broth, and *C. crescentus* was grown in either PYE (peptone, yeast extract) or M2 minimal medium with 0.2% glucose (24).

Cloning of flagellin genes. The cosmid clone pCN100, which contains approximately 40 kilobases (kb) of contiguous DNA from the *flaEY* region, was obtained as follows. Chromosomal DNA from *C. crescentus* SC1062 (26), a Tn*5* insertion mutant of *flaE*, was partially digested with *Sau*3A to obtain fragments in excess of 40 kb and ligated to the *Bam*HI-digested cosmid vector pHC79 (10). Clones resistant to kanamycin (Km^r) were selected after transfection of packaged hybrid cosmids into *E. coli* cells. The restriction map of the DNA insert in pCN100 was compared with genomic restriction patterns by a series of Southern hybridizations with cloned fragments as probes to confirm the continuity of the cloned fragment. A 6.6-kb *Hin*dIII-*Hin*dIII fragment from the pCN100 insert was shown to contain the 25-, 27-, and 29-kDa flagellin genes (see Results [17]). This 6.6-kb fragment was subcloned in both pBR322 (designated

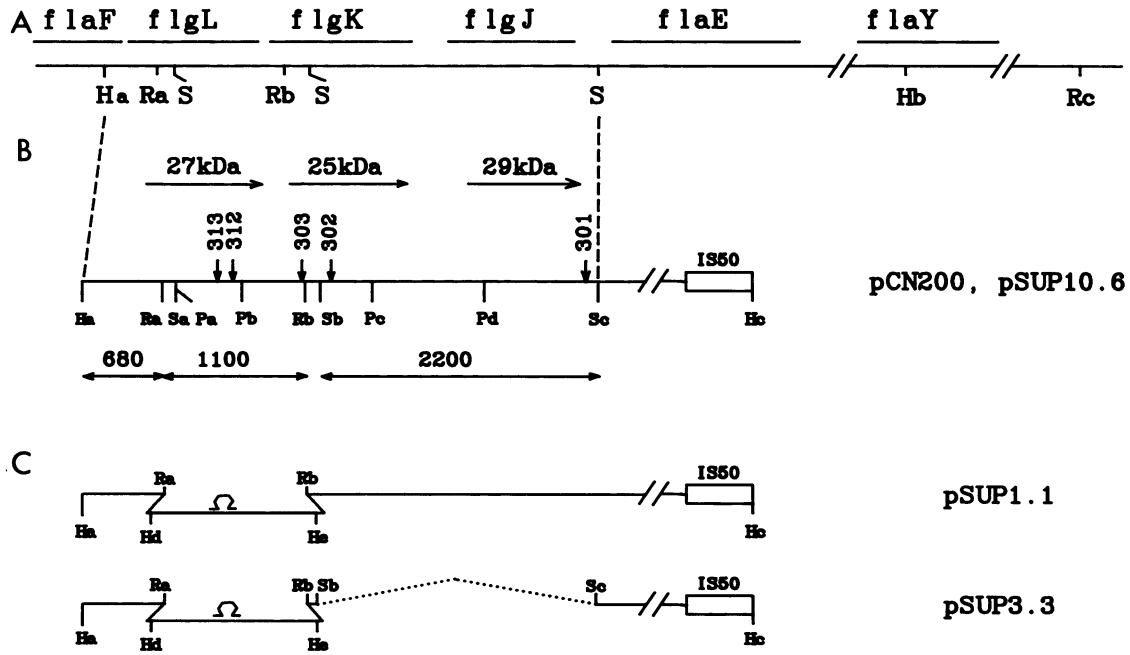

FIG. 1. Genetic, transcription, and restriction maps of the *flaEY* flagellin gene cluster. (A) Genetic organization of genes in the *flaEY* region of wild-type CB15 with abbreviated restriction map. Distances are: Ra to Rb, 1.1 kb; Rb to Rc, 10 kb; and Ha to Hb, 8 kb. (B) More detailed restriction map of the insert in pCN200 and pSUP10.6, extending from *Hin*dIII(a) to *Hin*dIII(c) located in the IS*50* element in the Tn*5* in strain SC1062 (26). The start sites and direction of transcription of the three flagellin genes are indicated by long arrows. The vertical arrows indicate the positions of Tn*5* insertions. Distances are given below in base pairs: 680 base pairs for Ha to Ra, 1,100 base pairs for Ra to Rb, and 2,200 base pairs for Sa to Sb. (C) Construction of two deletion mutation plasmids, pSUP1.1 and pSUP3.3, marked with the omega cassette. pSUP1.1 was used to construct PC7801, and pSUP3.3 was used to construct PC7810 (Table 1). Restriction sites are: H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; and S, *Sal*I.

pCN200) and the mobilizable vector pSUP202 (31), which was designated pSUP10.6 and used subsequently for mutagenesis of the *fla* genes.

Tn*5* mutagenesis. The 6.6-kb *Hin*dIII-*Hin*dIII fragment was mutagenized with lambda::Tn*5* defective in the *O* and *P* genes as described previously (2, 27). *E. coli* HB101 carrying pCN200 or pSUP10.6 was infected with lambda::Tn*5*, and Km^r colonies were pooled and plasmid DNA was isolated. The DNA was then used to transform *E. coli* S17-1, a lambda-resistant strain, and Km^r colonies were isolated to identify the plasmid insertions. Selection for insertions within the 6.6-kb *Hin*dIII cloned fragment was enhanced by selecting on plates containing kanamycin, ampicillin, tetracycline, and chloramphenicol. Insertions were mapped by restriction analysis.

Construction of flagellin gene deletions. Deletions of the flagellin genes were constructed by removing selected restriction fragments from hybrid clone pSUP10.6 and replacing them with the omega cassette (25), which carries resistance markers for streptomycin and spectinomycin. For the first construct, pSUP10.6 was digested with *Eco*RI and the two large fragments were purified after electrophoresis in ultrapure agarose (International Biotechnologies, Inc., New Haven, Conn.). These two fragments were ligated with the omega cassette which had been also purified after *Eco*RI digestion of pHP45 (25). *E. coli* S17-1 was transformed with the ligation mixture, and recombinants were selected for spectinomycin resistance (Sp^r). Individual clones were analyzed for correct orientation by restriction mapping, and several isolates were shown to be missing the 1.1-kb *Eco*RI(a)-*Eco*RI(b) fragment (Fig. 1). One of these subclones was designated pSUP1.1 (Fig. 1C).

The second deletion was constructed by digesting pSUP1.1 with *Sal*I (note that pSUP1.1 does not contain *Sal*I(a), purifying the two arms minus the 2.2-kb *Sal*I(b)-*Sal*I(c) fragment, and subsequent ligation (Fig. 1C). An Sp^r transformant of *E. coli* S17-1 (31) was isolated, and the plasmid DNA was purified and digested with *Sal*I to confirm that the 2.2-kb *Sal*I(b)-*Sal*I(c) fragment had been deleted. This plasmid, designated pSUP3.3 (Fig. 1C), and pSUP1.1 were used for gene replacements in *C. crescentus* as described below.

Gene replacement protocol. Derivatives of plasmid pSUP10.6 that had been mutagenized by Tn*5* insertions or by in vitro insertion of the omega cassette were transferred from *E. coli* S17-1 to *C. crescentus* by filter matings. Matings were done by spotting 0.1 ml of the *E. coli* donor and 0.2 ml of the *C. crescentus* CB15 recipient on a nitrocellulose filter (0.22-μm pore size) and incubating at 30°C for 4 to 12 h on PYE plates. Transconjugants were selected by suspension of the cells in M2 medium and spreading dilutions on M2 minimal agar plates supplemented with kanamycin (150 μg/ml) or spectinomycin (50 μg/ml). Initially, we intended to differentiate single crossovers (plasmid cointegrates) from double crossovers (gene replacements) by screening for one of the plasmid-encoded antibiotic resistance markers.

To test the gene replacement protocol, we used a derivative of pCN200 (pCN301) which contains the insertion mutation *zcg-301*::Tn*5* just 3′ to the end of the 29-kDa flagellin gene (Fig. 1B). The *Eco*RI fragment containing this

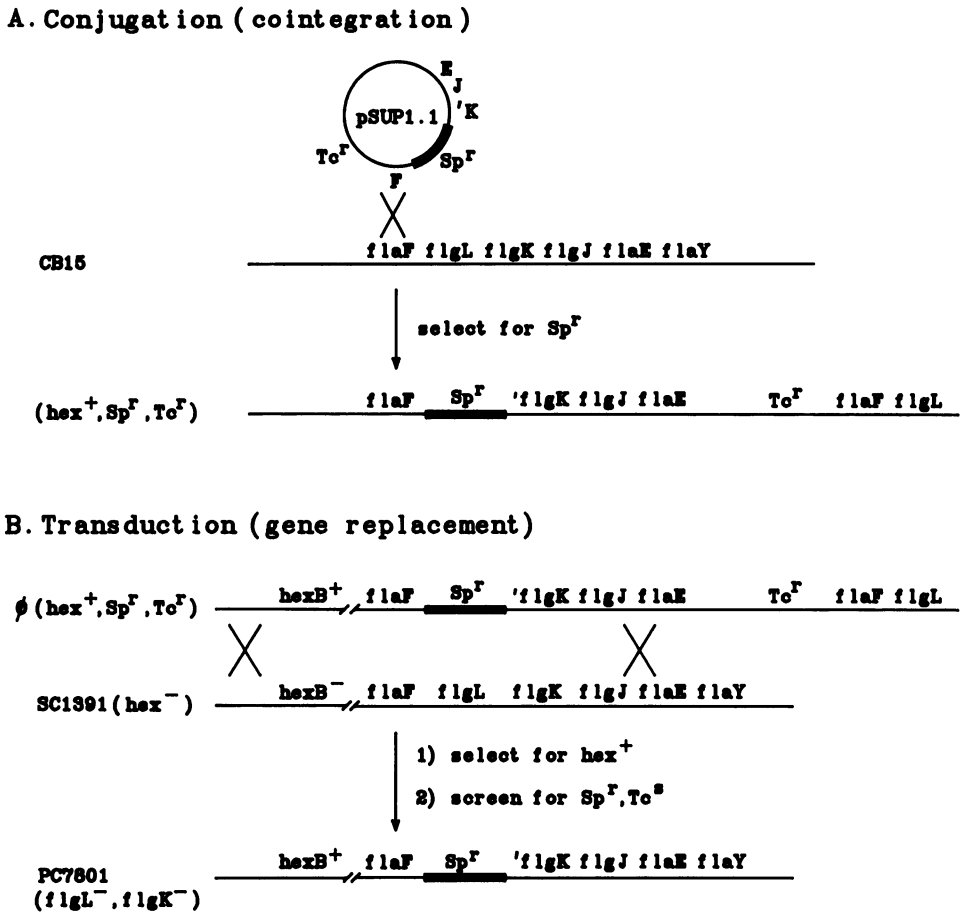

FIG. 2. *C. crescentus* gene replacement protocol used for construction of the *flgL flgK* deletion mutant PC7801 (see Materials and Methods).

Tn*5* was subcloned into pSUP202, a plasmid that does not replicate in *C. crescentus* cells, to give the hybrid plasmid pSPU301. The *E. coli* merodiploid S17-1(pSPU301) was then used to transfer plasmid pSPU301 directly to *C. crescentus* wild-type strain CB15. Km^r transconjugants were recovered on minimal medium at a frequency of 10^{-4} and scored for the tetracycline resistance (Tc^r) marker of the parent pSUP202 vector. Of 2,000 Km^r transconjugants scored, only 3 Tc^s isolates were recovered, indicating first that independent transposition of the Tn*5* element is relatively infrequent and second that there is a strong bias for single crossovers with cointegrate formation. To select for gene replacements, an "orgy" lysate was prepared on a pool of approximately 1,000 Km^r recombinants with transducing bacteriophage φCR30 and used to transduce the *hexB* mutant SC1391. *hexB* is 15% linked to *flaEY* (C. Gerardot and B. Ely, unpublished data), and of the 1,014 *hex*$^+$ recombinants isolated, 12% were Km^r Tc^r and 1% were Km^r Tc^s, or potential gene replacements. In experiments with insertions in other *C. crescentus* genes, we have observed that up to 50% of the transductants containing the Tn*5* insertion were Km^r Tc^s (unpublished data). Thus, transduction and selection for a linked marker to the region of interest apparently provide a strong selection for the double crossover and the gene replacement event (see below). The generalized replacement protocol is illustrated in Fig. 2 for the construction of the *flgL flgK* deletion mutant PC7801. In this experiment, the 1.1-kb *Eco*RI fragment of the *flaEY* cluster was replaced with the omega cassette, which confers spectinomycin resistance.

Motility assays. Swarm assays for motility were done by stabbing cells into 0.3% PYE agar and incubating the plates at 30°C for 24 to 36 h.

Radioimmunoassays. Radioimmunoassays were conducted as previously described (20), using monoclonal antibodies shown to react with both the 25- and 27-kDa flagellins (J. Sommer and A. Newton, unpublished data) or polyclonal rabbit antiflagellin immunoglobulin G. Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, enhanced by fluorography, dried, and subsequently exposed with preflashed X-ray film at −70°C.

RESULTS

Cloning and characterization of flagellin genes. A 40-kb DNA fragment with a Tn*5* insertion in *flaE* was cloned (pCN100) from *C. crescentus* SC1062 by using the cosmid vector pHC79 (10). Comparison of the DNA restriction patterns of pCN100 (data not shown) with the published restriction maps (9, 26) showed that the cloned insert originated from the *flaEY* gene cluster and contained the 29-kDa flagellin gene (*flgJ*). This conclusion was confirmed by DNA sequencing and comparison of the sequence obtained with the one published previously for the *flgJ* structural gene (9). We subsequently identified two additional flagellin genes on the 6.6-kb *Hin*dIII(a)-*Hin*dIII(c) fragment that contained *flgJ*

(Fig. 1A), one coding for the 25-kDa flagellin (*flgK*) and the other coding for the 27-kDa flagellin (*flgL*) (17). To obtain definitive proof for these flagellin gene assignments, to determine the role of the different flagellins in flagellum assembly and cell motility, and to study *fla* gene regulation, we constructed the mutants described below.

Tn*5* insertion mutations. Hybrid plasmids of pSUP202 (pSUP10.6 [31]) or pBR322 (pCN200) containing the 6.6-kb *Hin*dIII-*Hin*dIII fragment (Fig. 1B) were mutagenized with Tn*5* in *E. coli* HB101 (see Materials and Methods), and 20 insertions were isolated in the 6.6-kb insert. Restriction analysis showed that 14 insertions were within the 2,200-base-pair *Sal*I(b)-*Sal*I(c) fragment (301, 302 shown in Fig. 1B), which contains the 25- and 29-kDa flagellins; 3 insertions were mapped within the 1,100-bp *Sal*I(a)-*Sal*I(b) fragment (303, 312, 313; Fig. 1B), which contains the 27-kDa flagellin gene and the 5′ end of the 25-kDa flagellin gene; and 3 insertions were mapped in the 680-bp *Hin*dIII(a)-*Eco*RI(a) fragment (data not shown), which contains the 5′ end of the 27-kDa flagellin gene and part of *flaF*. The locations of the Tn*5* insertions used in these studies are shown in Fig. 1B and listed for each strain in Table 1.

Using the gene replacement protocol described in Materials and Methods, we isolated presumptive gene replacements for each of the Tn*5* insertions based on linkage of the kanamycin resistance marker to *hexB* and sensitivity to plasmid-encoded resistance markers. A Southern blot (32) was prepared with *Hin*dIII-digested chromosomal DNA isolated from one Km^r Tc^s isolate containing *zcg-301*::Tn*5* and probed with the ^{32}P-radiolabeled 6.6-kb *Hin*dIII-*Hin*dIII fragment of plasmid pSUP10.6. Comparison of this hybridization pattern with the genomic blot of the parent strain CB15 (Fig. 3, lane a) showed the generation of two new *Hin*dIII fragments in place of the 8-kb wild-type fragment (Fig. 3, lane b) and confirmed that the recombinant is a genomic replacement. A Tn*5* insertion mutant of the 27-kDa flagellin gene was constructed by the same procedure, and the location of the *flgL312*::Tn*5* replacement in the genome was also confirmed by Southern analysis; since the site of Tn*5* insertion is approximately 1 kb from the *Hin*dIII-a site, digestion at *Hin*dIII sites a and b (Fig. 1A) and at the two *Hin*dIII sites within the IS*50* sequences of the Tn*5* (12) generated fragments of 2.2 kb and ca. 8 kb (Fig. 3, lane c). The other *fla* gene replacements discussed below were constructed and analyzed in the same way (Table 1).

Construction of deletion mutants. We were also able to use the gene replacement method just described to construct deletion mutants of the *flaEY* cluster. One of the deletions was prepared by removing the 1.1-kb *Eco*RI(a)-*Eco*RI(b) fragment from the insert of plasmid pSUP10.6 and replacing it with the streptomycin-spectinomycin resistance omega cassette (25). This modified plasmid, designated pSUP1.1, lacked most of the 27-kDa flagellin gene and the promoter region of the 25-kDa flagellin gene (Fig. 1C). A second deletion was constructed by digesting pSUP1.1 DNA with *Sal*I to remove the 2.2-kb *Sal*I(b)-*Sal*I(c) fragment (Materials and Methods). This construct, pSUP3.3, lacked all three flagellin genes (Fig. 1C). The presence of the spectinomycin omega cassette in plasmids pSUP1.1 and pSUP3.3 allowed the isolation of gene replacements in *C. crescentus* by selection of spectinomycin-resistant recombinants by the same procedure used for the Tn*5* insertions (see Fig. 2 and previous section).

DNA from strain PC7801 containing the omega cassette was analyzed by Southern hybridization with the 1.1-kb *Eco*RI-*Eco*RI fragment as the probe and was found to be missing the 1.1-kb *Eco*RI-*Eco*RI fragment (Fig. 3, lane e). A *Hin*dIII digest of DNA from strain PC7810 generated a 0.7-kb fragment [*Hin*dIII(a)-*Hin*dIII(d) fragment] and a 4-kb fragment [*Hin*dIII(e)-*Hin*dIII(b) fragment]; this genomic digestion of DNA from the strain PC7810 pattern is consistent with the deletion of the 1.1- and 2.2-kb fragments (Fig. 1A and C).

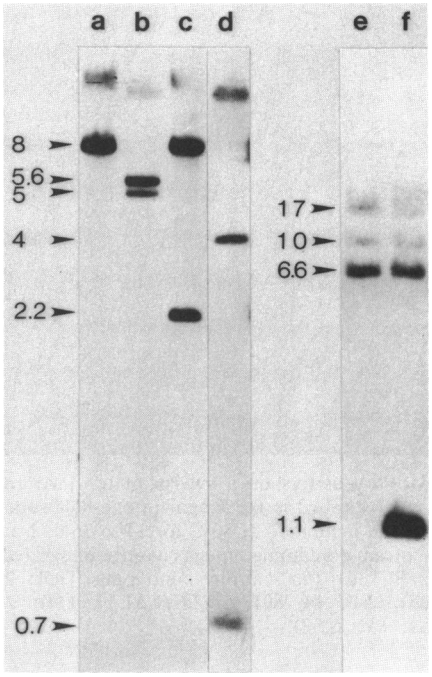

FIG. 3. Southern hybridization analysis of DNA from replacement mutants. Genomic DNAs from strains indicated below were restricted with *Hin*dIII (lanes a through d) or *Eco*RI (lanes e and f), electrophoresed, transferred to nitrocellulose filters, and hybridized to nick-translated DNA probes: 6.6-kb *Hin*dIII(a)-*Hin*dIII(c) fragment for lanes a through d and 1.1-kb *Eco*RI a-*Eco*RI b fragment for lanes e and f. Lanes: a, CB15; b, PC7301; c, PC7312; d, PC7810; e, PC7801; and f, CB15. Fragment sizes are given in kilobases. The length of the *Hin*dIII(a)-*Hin*dIII(b) fragment in the wild-type CB15 genome (Fig. 1) is approximately 8 kb. In Tn*5* mutants, two fragments of various sizes, totaling ca. 8 kb plus 2.4 kb, are obtained by *Hin*dIII digestion depending on the location of the insertions owing to two symmetrically located *Hin*dIII sites in the IS*50* sequences of Tn*5* (1.2 kb from the ends [12]). In PC7810 (lane d, and also Fig. 1C), the 0.7-kb fragment corresponds to *Hin*dIII-a-*Hin*dIII-d and the 4-kb fragment corresponds to *Hin*dIII(e)-*Hin*dIII(b) minus *Sal*I(b)-*Sal*I(c). *Eco*RI digestion generates four fragments in CB15, of which 1.1-kb (Ra to Rb) and 10-kb (Rb to Rc) fragments are located in the *flaEY* region (see the legend to Fig. 1A).

Effect of flagellin gene mutations on flagellin synthesis and motility. The Tn*5* insertion and deletion mutations described above were examined for their effect on flagellin synthesis by radioimmunoassay and on motility by light microscopy and swarm plate assay. Since we found that the *hexB* auxotroph SC1391 used to construct all the replacements contains an allele for impaired motility, isogenic strains were constructed by transducing replacement mutations back into wild-type *C. crescentus* CB15 (Table 1). These strains were used for both the radioimmunoassays and the motility assays.

The swarm plate assay of strain PC7321, which contains the Tn*5* downstream from the 29-kDa flagellin gene, showed that this insertion has no effect on motility (Fig. 4, 1-c);

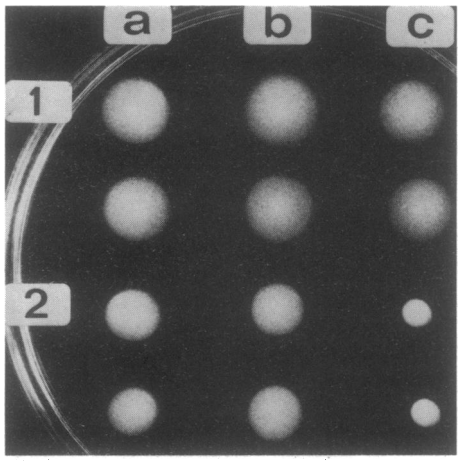

FIG. 4. Motility assays on a swarm plate. Two colonies from each strain were stabbed in 0.3% agar in one-half concentration of PYE medium and incubated at 30°C for 24 to 36 h. 1-a, CB15; 1-b, PC7811 (*fla*$^+$ strain containing omega cassette integrated in chromosome); 1-c, PC7301 (*fla*$^+$ strain containing Tn*5*); 2-a, PC7312 (*flgL312*::Tn*5*); 2-b, PC7801 (*flgLK*, Δ1.1, +Ω); 2-c, PC7810 (*flgLKJ*, Δ1.1, +Ω, Δ2.2).

radioimmunoassay also showed that this Tn*5* insertion strain synthesizes 25-, 27-, and 29-kDa flagellins (Fig. 5, lane f). We confirmed the latter result by constructing a strain which contains the *zcg-301*::Tn*5* insertion in a background with a mutation in *flaW*. Since *flaW* mutants do not express the 25- and 27-kDa flagellin genes (22) the presence of the 29-kDa flagellin is readily apparent, as seen for the Tn*5* insertion mutant (Fig. 5, lane e) and the parent *flaW* mutant SC259 (Fig. 5, lane d).

Tn*5* insertion mutants PC7302 and PC7303 of the 25-kDa flagellin gene, which contain *flgK302*::Tn*5* and *flgK303*::Tn*5*, respectively, were assayed in a separate experiment and shown to synthesize 25-kDa flagellin, as well as the 27-kDa flagellin (Fig. 5, lanes l and m); for this set of assays, a monoclonal antiflagellin mouse serum was used which does not cross-react with 29-kDa flagellin (see the legend to Fig. 4). These results show that the 25-kDa flagellin gene in *flaEY* is not required for flagellum function and that the beta-flagellin cluster which maps outside of the *flaEY* gene cluster contains active copies of the 25-kDa flagellin gene. The presence of multiple 25-kDa flagellin genes has been suggested previously (16), and support for this conclusion is seen in the genomic Southern analysis of flagellin deletion mutant PC7801 in which only the 1.1- and 10-kb *Eco*RI fragments originate from the *flaEY* gene cluster (Fig. 3, lane e; see legend to Fig. 1A).

The failure of insertion mutants which contain either *flgL312*::Tn*5* or *flgL313*::Tn*5* to synthesize the 27-kDa flagellin (Fig. 5, lanes h and J) is consistent with our earlier assignment of this gene (17). As a control in these experiments, we also examined recombinants of the *flgL* insertions derived from single crossovers that resulted in a recombinant with the disrupted *flgL* copy plus the intact *flgL* gene. Both of these Km^r Tc^r recombinants, PC7314 and PC7315, made the 27-kDa flagellin (Fig. 5, lanes i and k). Further support for the assignment of the 27-kDa flagellin to *flgL* is the presence of a DNA sequence within the open reading frame of this gene encoding the B13 peptide (15 amino acids in length) identified by Weissborn et al. (33) as unique to the 27-kDa flagellin (C. R. Wheeless, S. A. Minnich, and A. Newton, unpublished data).

The two 27-kDa flagellin gene (*flgL*) mutants examined synthesize 25-kDa flagellin, they are motile by light microscopy, and they display almost normal motility, as assayed on swarm plates (Fig. 4, 2-a). Consequently, we conclude that the 25-kDa flagellin can assemble to form an active filament in the absence of 27-kDa flagellin. These conclusions were confirmed by the behavior of deletion mutant PC7801, which lacks most of the 27-kDa structural gene and part of the 25-kDa flagellin gene (Fig. 1C). This strain also makes no 27-kDa flagellin (Fig. 5, lane b), and like Tn*5* insertion mutants of *flgK*, the 25-kDa flagellin synthesized is apparently encoded by the 25-kDa flagellin gene in the beta-flagellin cluster. We have shown previously that a transcript from *flgK* cannot be detected in strain PC7801 (17). The isogenic derivative of strain PC7801 (PC7802) is slightly less motile than either wild-type strain CB15 or strain PC7301 (Fig. 4, 2-b) and identical in motility on swarm plates to *flgL*::Tn*5*, PC7330 (Fig. 4, 2-a). These results suggest that the slight motility defect observed in strains PC7802 and PC7330 is significant and results from the failure of these strains to synthesize 27-kDa flagellin (Fig. 5, lanes b and h).

Since Tn*5* insertions were not initially isolated in the 29-kDa flagellin gene, we examined mutant PC7810 and its isogenic derivative strain PC7809 in which all or part of the 27-, 25-, and 29-kDa flagellin genes are deleted (Fig. 1C). This strain does not synthesize detectable 29- or 27-kDa flagellin (Fig. 5, lane c). Mutant PC7809 is much more severely impaired in motility than strain PC7802, which is deleted for only the 27- and 25-kDa flagellins (Fig. 4, 2-c). This result indicates that the 29-kDa flagellin is also required

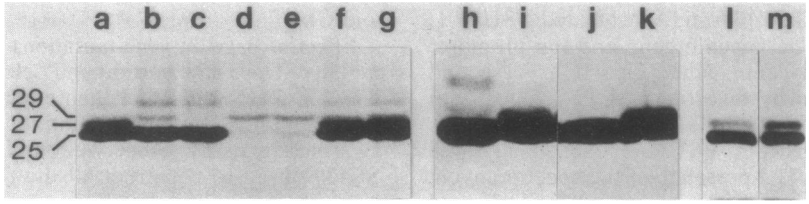

FIG. 5. Radioimmunoassays of replacement mutants. Lysates from cultures labeled with [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) were reacted with antiflagellin antibody, and immunoprecipitates were fractionated on 12.5% (lanes a to g) or 10% (lanes h to m) polyacrylamide-sodium dodecyl sulfate gels. Gels were fluorographed and exposed to X-ray films. Lanes: a, CB15; b, PC7801 (*fla*$^+$); c, PC7809 (*flgLKJ* deletion); d, SC259; e, SC259 with *zcg-301*::Tn*5*; f, CB15 with *zcg-301*::Tn*5*; g, CB15; h, PC7312 (*flgL312*::Tn*5*); i, PC7314 (*flgL*$^+$ *flgL312*::Tn*5*); j, PC7313 (*flgL313*::Tn*5*); k, PC7315 (*flgL*$^+$ *flgL313*::Tn*5*); l, PC7302 (*flgK302*::Tn*5*); m, PC7303 (*flgK303*::Tn*5*). Strains PC7314 and PC7315 are Tc^r Km^r single-crossover recombinants. Molecular weights are given in kilodaltons. Experiments shown in lanes a through g were done with a polyclonal antiflagellin immunoglobulin G from rabbits, and those in lanes h through m were done with a monoclonal mouse serum. The 29-kDa flagellins often did not show up with the latter even in CB15 extract.

for normal flagellum function in *C. crescentus* (see Discussion).

DISCUSSION

The flagellum of *C. crescentus* is assembled from the 27- and 25-kDa flagellins (14, 29, 33), and there is now evidence that the sequence-related 29-kDa flagellin is also assembled into the flagellar filament (L. Shapiro, personal communication). The function of the flagellins has remained a matter of speculation, since the only flagellin mutations identified in extensive screens of *fla* mutants were large deletions that extend into flanking *fla* genes of the *flaEY* cluster (3, 26). The method of gene replacement described in this report was developed to assess the role of the individual flagellins in flagellum function and flagellin gene regulation. As discussed below, our results indicate that the 27- and 29-kDa flagellins are not essential for motility but that both these proteins are required for the assembly of a fully active flagellum in *C. crescentus*.

From the analysis of mutants with Tn*5* insertions in the 27- and 25-kDa flagellin genes and deletions of the 29-, 27-, and 25-kDa flagellin genes of the *flaEY* cluster, we draw the following conclusions. (i) The 29-, 27-, and 25-kDa flagellin genes of the alpha-flagellin cluster are not absolutely essential for motility as assayed on swarm plates; all the flagellin mutants in the *flaEY* cluster were at least partially motile, which may explain the previous failure to isolate specific flagellin mutations. (ii) Copies of the 25-kDa flagellin gene in the beta-flagellin gene cluster, which maps outside the *flaEY* cluster by Southern anlaysis (16) (Fig. 3), are expressed; mutants deleted for the three flagellin genes in the alpha cluster synthesize levels of 25-kDa flagellin that cannot be distinguished from wild-type levels. (iii) The 25-kDa flagellin alone is sufficient for the assembly of a partially active flagellum (see i above). (iv) Wild-type cell motility assayed on swarm plates requires the 27- and 29-kDa flagellin genes.

The conclusion that the 27- and 29-kDa flagellin genes are required for fully motile cells is based on the observation that deletions or insertion mutations of the 27-kDa flagellin gene cause slight defects in motility. More recently, we have shown that Tn*5* insertion mutations of the 29-kDa flagellin gene also cause slight defects in motility (unpublished data). A mutant with a deletion removing both the 27- and 29-kDa flagellin genes (PC7810) displays a more severe motility defect than a mutant with a Tn*5* insertion in either the 27- or 29-kDa flagellin gene alone. The defects observed in 27- and 29-kDa flagellin mutants could result from their failure to assemble fully active flagella and/or a defect in chemotaxis, which would also result in smaller swarms as assayed on motility agar (Fig. 4). An impaired motility phenotype has also been observed in mutants with spontaneous deletion mutations of the 29-kDa flagellin gene (L. Shapiro, personal communication) and the 27-kDa flagellin gene (P. V. Schoenlein and B. Ely, personal communication) when they were complemented for adjacent *fla* genes that had been removed by the deletions.

The availability of flagellin gene mutations in *C. crescentus* also allowed us to address directly several questions about *fla* gene regulation for the first time. Results from several laboratories have shown that *fla* genes are arranged in a regulatory hierarchy and that the expression of these genes is mediated at least in part by a cascade of *trans*-acting gene products (17, 21, 28, 30). We have shown previously that genes in transcription unit III of the hook operon are required in *trans* for expression of transcription unit II and that the hook protein structural gene (*flaK*), which is located in transcription unit II, is required for synthesis of the 27- and 25-kDa flagellins (17, 21, 22). This gene organization does not account, however, for sequential flagellin gene expression in which the 27-kDa flagellin and flagellin gene transcript appear before those from the 25-kDa flagellin gene in the *C. crescentus* cell cycle (15, 17). A plausible mechanism for the sequential staging of flagellin gene expression would be the requirement of the 27-kDa flagellin for activation of the 25-kDa flagellin gene. The observation reported here that 27-kDa flagellin gene mutants synthesize the 25-kDa flagellin at essentially wild-type rates (Fig. 5, lanes h and j) eliminates this simple model. The synthesis of normal levels of the 5′ transcript from the *flgL* gene in these insertion mutants also indicates that transcription of the 27-kDa flagellin gene is not autoregulated.

A more complex mechanism for the staging of flagellin gene expression in *C. crescentus* has been suggested by the observation that the *flaK*, or hook protein, gene is required directly or indirectly for transcription and translation of the 25-kDa flagellin gene, but is required only for translation of the 27-kDa flagellin genes (S. A. Minnich and A. Newton, unpublished data). If the transcriptional activator of the 25-kDa flagellin gene was the intracellular 70-kDa *flaK* gene product that accumulates after the completion of hook assembly, then the time required for hook assembly might account in part for the delayed appearance of the 25-kDa flagellin transcript.

The method of gene replacement described in this report has also been used successfully for the construction of insertions in the hook gene cluster and in the rRNA genes of *C. crescentus* (N. Ohta and A. Newton, unpublished data), which indicates that the procedure may be generally useful in the genetic analysis of this bacterium. It may also have wider application in the genetic studies of gram-negative, nonenteric bacteria. In this context, we should emphasize several observations. The Km^r recombinants isolated initially after mating the hybrid pSUP202 plasmids from *E. coli* to *C. crescentus* were predominantly, if not exclusively, plasmid-genome cointegrates generated by single crossovers (Fig. 2A). The transduction of these Km^r constructs to the target region (*flaEY*) by selection of the linked *hexB* marker selects for the double crossover and gene replacement in the desired region of the chromosome (Fig. 2B). Transduction of the marker thus seems to be required for recovery of the gene replacement; it may be that linear DNA is more efficient than circular DNA in recombining by double crossovers. Although we do not understand the operative mechanism, we have been able to use this observation to translocate genes to different regions of the *C. crescentus* chromosome. This has been accomplished by selecting for cointegrate formation among transconjugants that result from recombination between two Tn*5* elements, one carried by the hybrid pSUP202 plasmid and the other at a selected position on the chromosome (N. Ohta and A. Newton, unpublished data).

In summary, we made the following observations. The gene replacement protocol described here shows general utility for mutant constructions in *C. crescentus* with cloned DNA fragments mutagenized in vivo by Tn*5* insertions or by insertion of the omega cassette in vitro. Our results indicate that mutations in any of the three flagellin genes of the *flaEY* cluster do not result in a Mot^- phenotype. Loss of the 29-kDa flagellin and the 27-kDa flagellin does produce a partial defect in motility or chemotaxis, however. We are

examining the requirement of these latter two flagellins for motility in more detail.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants GM22299 and GM25644 from the National Institutes of Health and grant MV-386 from the American Cancer Society.

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