Control of synthesis and positioning of a *Caulobacter crescentus* flagellar protein

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The *Caulobacter crescentus* flagellum is assembled during a defined time period in the cell cycle. Two genes encoding the major components of the flagellar filament, the 25K and the 27.5K flagellins, are expressed coincident with flagellar assembly. A third gene, *flgJ*, is also temporally regulated. The synthesis of the product of *flgJ*, the 29K flagellin, occurs prior to the synthesis of the other flagellin proteins. We demonstrate here that the time of initiation of *flgJ* expression is independent of chromosomal location but is dependent upon *cis*-acting sequences present upstream of the *flgJ* structural gene. Evidence that there is transcriptional control of *flgJ* expression includes the following: (1) The initial appearance of *flgJ* message was coincident with the onset of 29K flagellin protein synthesis, and (2) expression of an NPT II reporter gene driven by the *flgJ* promoter was temporally correct. Post-transcriptional regulation might contribute to the control of expression, because the *flgJ* mRNA persisted for a longer period of time than did the synthesis of the 29K protein. The 29K flagellin was found only in the progeny swarmer cell after cell division. In a mutant strain that failed to assemble a flagellum, the 29K flagellin still segregated to the presumptive swarmer cell, demonstrating that positioning of the protein is independent of filament assembly. Analysis of a chimeric *flgJ*-NPT II transcriptional fusion showed that the *flgJ* regulatory sequences do not control the segregation of the 29K flagellin to the swarmer cell progeny, suggesting that correct segregation depends on the protein product.

[Key Words: *Caulobacter crescentus*; flagellar protein; *flgJ* expression]

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The *Caulobacter crescentus* flagellum and chemotaxis apparatus are only present transiently during the cell cycle. A single polar flagellum is synthesized just prior to cell division; consequently, only one daughter cell carries a flagellum. The flagellum is a complex structure containing three subassemblies: a basal body, a hook, and a filament (Johnson et al. 1979; Wagenknecht et al. 1981; Stallmeyer et al. 1985). Over 30 flagellar (*fla*) genes involved in the biogenesis of this structure have been identified, and an additional 11 genes are required for motility and chemotaxis (Ely et al. 1984, 1986). The genes for the filament include a cluster containing *flgJ* (29K flagellin), *flgK* (25 flagellin), and *flgL* (27.5K flagellin). An additional three genes encoding 25K flagellins are located elsewhere on the chromosome (N. Agabian, unpubl.). The gene encoding the hook protein *flaK* resides in a separate cluster of *fla* genes (Chen et al. 1986).

All flagellar and chemotaxis proteins that have been identified have been found to be synthesized at a specific time in the cell cycle, with the peak of synthesis occurring around the time of flagellar assembly. Evidence has been presented suggesting that the time of expression of

the *Caulobacter* flagellar genes is regulated at the transcriptional level and that the amount of flagellar and chemotaxis gene expression is modulated by a *trans*-acting cascade of *fla* gene products (Champer et al. 1985, 1987; Chen et al. 1986). The mechanisms that control the time of *fla* gene expression appear to function independently of the regulatory cascade that controls the amount of gene expression (Bryan et al. 1987).

The assembly of the flagellum at one pole of the predivisional cell requires that the newly synthesized flagellar proteins be sequestered to a specific cellular location. The proteins involved in chemotaxis are also positionally biased to the flagellar pole of the predivisional cell and are segregated to the swarmer cell progeny upon division (Gomes and Shapiro 1984; Nathan et al. 1986). Thus, the biogenesis of the flagellum and chemotaxis apparatus depends on complex regulatory signals that control the time of synthesis, the amount of synthesis, and the positioning of a large number of flagellar and chemotaxis proteins within the cell.

To identify the regulatory signals that control the temporal and spatial expression of a specific *fla* gene, we have studied the expression of the *flgJ* gene. *flgJ* encodes a 29K flagellin (Gill and Agabian 1983) that is part of a family of differentially expressed flagellins (Lagenaur

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and Agabian 1978). The two other flagellins, 27.5K and 25K, comprise the major portion of the flagellar filament, with the 27.5K protein occupying the hook-proximal position (Koyasu et al. 1981; Weissborn et al. 1982). The *flgJ* gene has been isolated (Milhausen et al. 1982; Purucker et al. 1982) and sequenced (Gill and Agabian 1983). Lagenaur and Agabian (1978) have shown that the 29K flagellin is synthesized prior to the synthesis of the 27.5K and 25K flagellins. We show here that the timing of transcription of *flgJ* is dependent on nucleotide sequences in the 5'-regulatory region of the gene and that this temporal control is independent of whether the gene is located on the chromosome or on a low-copy-number plasmid. We also provide evidence that the protein-coding portion of the *flgJ* gene is necessary for the segregation of the 29K product to only one of the progeny cells (the swarmer cell). The correct segregation of this protein to the swarmer cell occurs in mutants unable to assemble a flagellum. Therefore, the signals that contribute to the positioning of the protein appear to be independent of the flagellar assembly process.

Results

Temporal expression of the flgJ gene from chromosomal or plasmid locations

The time of synthesis of the three flagellins in synchronized cells was determined by immunoprecipitation of [³⁵S]methionine pulse-labeled cultures with anti-flagellin antibody (Fig. 1). The polyclonal anti-flagellin antibody cross-reacts with all three flagellin species because the amino acid sequence of these proteins has been highly conserved (Gill and Agabian 1982; Weissborn et al. 1982).

Analysis of wild-type cultures showed that the 25K flagellin is synthesized in the swarmer cell after cell division (Fig. 1A). Following differentiation of the swarmer cell into a stalked cell, the 29K flagellin is the first flagellin made, followed by the 27.5K flagellin and the 25K flagellin. This temporal order of synthesis reflects the position of these flagellins in the filament. Recently, we have been able to detect the 29K flagellin in a very short hook-proximal region of the filament using immunomicroscopy (A. Driks and R. Bryan, unpubl.). The rest of the hook-proximal portion of the filament is composed of the 27.5K flagellin, and the remainder of the filament, by the 25K flagellin (Koyasu et al. 1981; Weissborn et al. 1982). The onset of synthesis of the 29K flagellin occurred at 0.5 division units, and its synthesis was detected for only a brief period, prior to the onset of flagellar assembly (Fig. 1A).

To determine whether the temporal expression of the *flgJ* gene was dependent on its chromosomal location, a strain, AE9002 (Table 1), was constructed in which the *flgJ* gene was deleted from the chromosome. A plasmid, pRB3, containing *flgJ* (Fig. 2A) was then mated into the deletion strain. Details of the strain construction and mapping of the deletion are described in Materials and methods. As shown in Figure 2A, AE9002 contains a deletion of *flgJ*, *flaE*, and part of *flaY*, as well as a portion of

Figure 1. The time of synthesis of the three flagellins during the *C. crescentus* cell cycle. Aliquots of synchronized cultures of wild-type CB15N (A) and AE9002/pRB3 (B) were pulse-labeled for 10 min with [³⁵S]methionine (15 μ Ci) at specific stages in the cell cycle, indicated by the schematic shown below B. Proteins were immunoprecipitated with anti-flagellin antibody, as described in Materials and methods, and then separated by electrophoresis through a 10% polyacrylamide-SDS gel. Autoradiograms of the separated proteins are shown, and the 29K, 27.5K, and 25K flagellins are indicated. Aliquots of cells were pulse-labeled at 0, 0.2, 0.5, 0.6, 0.75, and 1.0 division units, and the immunoprecipitated proteins are shown in lanes 1–6, respectively. Division units represent fractions of the cell-division cycle, with 1 unit equivalent to the generation time. The generation time of these cultures, grown in minimal glucose M2 media, was 160 min.

flgK, one of the structural genes for the 25K flagellin (Minnich and Newton 1987). The plasmid pRB3, containing intact *flgJ*, *flaE*, and *flaY* genes, as well as a portion of the *flgK* gene, was mobilized from *Escherichia coli* HB101 into AE9002. The presence of pRB3 enabled AE9002 to swim and allowed the synthesis of all three flagellins (Fig. 2B, lane 3). AE9002 carrying the plasmid pRB7 (Fig. 2A), with just the *flaE* and *flaY* genes, was able to swim but failed to synthesize the 29K flagellin (Fig. 2B, lane 2). Thus, the 29K flagellin does not appear to be required for motility, nor does motility appear to depend on the synthesis of the 25K flagellin encoded by *flgK*. Although *flgJ* is the only gene encoding the 29K flagellin, there is another group of 25K flagellin genes elsewhere on the chromosome (Gill and Agabian 1983).

To ascertain whether the *flgJ* gene on pRB3 had maintained its plasmid location, motile cells were selected by streaking AE9002/pRB3 twice onto a swarmer agar plate.

Table 1. Bacterial strains

Strain	Genotype	Source/derivation/reference
(a) <i>C. crescentus</i>		
AE5000	wild-type	Poindexter (1964)
CB15N	wild-type, synchronizable	Evinger and Agabian (1977)
AE5478	<i>cysD</i> :: Tn5, <i>ts104</i> /pVS1	David Hodgson
AE9001	<i>cysD</i> :: Tn5 in CB15N/pVS1	AE5478 × CB15N
SC512	<i>flaE156</i>	Johnson and Ely (1979); Purucker et al. (1982)
AE9002	<i>flaE156</i> in CB15N	0CR30-mediated transduction of AE9001 × SC512; cured of pVS1
AE8006	<i>flaK</i> :: Tn5-VB32	Champer et al. (1987)
SC1997	<i>flaN</i> :: Tn5	Bryan et al. (1987)
(b) <i>E. coli</i>		
HB101	<i>rB⁻</i> , <i>mB⁻</i> , <i>recA13</i> , <i>suII</i> , <i>leuB6</i> , <i>B1⁻</i> , <i>proA2</i> , <i>lacZ4</i> , <i>Sm^R</i>	Cold Spring Harbor Laboratory

Cells from the border of the swarm were propagated, and DNA was isolated for Southern blot analysis. The DNA was digested with the restriction enzymes *Hind*III and *Bam*HI and analyzed by Southern blot hybridization using as probe the nick-translated *Eco*RI–*Bam*HI (probe 1) fragment shown in Figure 2A. If the plasmid pRB3 recombined into the chromosome between the left *Eco*RI site and the left end of the deletion, the *flgK* gene would be restored, and *flgJ* would be restored to its chromosomal location between *flgK* and *flaE*. Evidence of this event would be the restoration of the *Hind*III and *Bam*HI fragments found in wild-type DNA. There was no evidence that this recombination event had occurred (data not shown). The only bands visible following Southern blot hybridization using the probes shown in Figure 2A demonstrated that the chromosomal deletion was still present and that this recombination event had not occurred. If the plasmid recombined into the chromosome between the right end of the deletion and the right *Eco*RI site, a pattern of hybridization indistinguishable from that obtained without recombination would be obtained, but the chromosomal order of *flgK*, *flgJ*, and *flaE* genes would not be restored.

Having determined that *flgJ* in AE9002/pRB3 had not regained its wild-type chromosomal location, we synchronized cultures of this strain. At various times in

the cell cycle, aliquots were pulse-labeled with [³⁵S]methionine, and the cell extracts were immunoprecipitated with flagellin antisera (Fig. 1B). As expected from the partial deletion in one of the 25K flagellin genes, the level of the 25K flagellin was lower in these cells. The amount of the 29K flagellin was elevated relative to the level found in wild type (Fig. 1A), probably due to plasmid copy number (approximately five copies per cell). The time of initiation of synthesis of the 29K protein, however, was the same as in wild-type cells, demonstrating that the signals that control timing are present in pRB3. These results also suggest that the gene

Figure 2. (A) Restriction map of a region of the *C. crescentus* CB15 chromosome carrying the *flgJ*, *flaE*, and *flaY* genes. The boxed portion shows the location and extent of the chromosomal deletion in AE9002. The extent of the pRB3 insert containing part of *flgK* (25K flagellin), as well as the *flgJ* (29K flagellin), *flaE*, and *flaY* genes, and the pRB7 insert containing the *flaE* and *flaY* genes are shown, as are the probes used for Southern hybridization. The restriction enzymes used were *Bam*HI (B), *Hind*III (H), *Eco*RI (E), *Pst*I (P), and *Sal*I (S). (B) Flagellin composition of the deletion strain AE9002 containing pRK290 vector (1), pRB7 (2), or pRB3 (3). The flagellins were detected by immunoprecipitation of [³⁵S]methionine pulse-labeled cultures with anti-flagellin antibody. The immunoprecipitated proteins were separated by electrophoresis through 10% polyacrylamide–SDS gels.

Synthesis of NPT II in CB15N/pZL1451 was measured in synchronized cultures to determine whether the 5'-regulatory sequences of *flgI* are sufficient to control the

Figure 3. Nuclease S1 protection assays of *flgI* transcripts as a function of the cell cycle. (A) Schematic representation of nuclease S1 analysis. (Top) A partial restriction map of the 5' region and the protein-coding sequence of the *flgI* gene. The restriction fragments used as probes are indicated by a solid line below the restriction map. The line ends in a dot, which indicates the 5' end label. The *PvuII*-*ClaI* probe is 810 bp in length, and the *NaeI*-*TaqI* probe is 280 bp in length. The wavy lines indicate the protected RNA fragment, 280 bp in the first case and 180 bp in the latter case. (B) Cells of strain AE9002/pRB3 were grown in rich PYE media and synchronized as described in Materials and methods. The generation time was 90 min. Aliquots were taken at 0, 0.5, 0.8, and 1.0 division unit, shown in lanes 1-4, respectively. Lane 5 is a control of the probe and S1 without any RNA. RNA was purified as described previously (Amemiya et al. 1980). The level of *flgI* mRNA in each sample was determined by S1 nuclease assays with the *NaeI*-*TaqI* probe, as described in Materials and methods. Samples were separated by electrophoresis in a 6% polyacrylamide/urea gel.

temporal expression of the NPT II-coding region (Fig. 5B). Immunoprecipitation of pulse-labeled synchronized cells (Fig. 5B) showed that NPT II first appeared at 0.5 division units, reached a maximum at 0.75 division units, and then diminished as the culture approached cell division. This onset of synthesis of NPT II occurred

Figure 4. Schematic diagram showing the construction of a chimeric plasmid containing the promoter and amino-terminal sequences of *flgI* in a transcription fusion with the gene encoding NPT II. A 1.7-kb *Bam*HI–*Bcl*II fragment, derived from a fusion of the small *Bam*HI–*Sal*I fragment from pBR322, and a 1.4-kb *Sal*I–*Bcl*II fragment of *C. crescentus* sequences, containing 340 nucleotides of the *flgI*-coding region and the entire 5' regulatory region, was inserted in both orientations into the unique *Bgl*III site that is immediately upstream to the promoterless *neo* gene present in pZL250. pZL250 was constructed as described in Materials and methods. pZL451 and pZL452 were inserted into pRK291. The pZL1451 plasmid contains an in-frame translation stop signal between the 5' fragment of the *flgI* gene and the ATG of the *neo* gene, ensuring that an intact protein product, NPT II, could be synthesized. pZL1452 contains the *flgI* promoter region in opposite orientation with respect to the *neo* gene. Restriction enzymes used were *Bam*HI (B), *Sal*I (S), *Bcl*II (Bc), *Eco*RI (E), and *Bgl*III (Bg).

at the same time point in the cell cycle as the onset of synthesis of the 29K flagellin (Fig. 1). Because NPT II protein synthesis measures the presence of the chimeric mRNA originating at the *flgI* promoter, these results are in agreement with the S1 nuclease protection assays of *flgI* mRNA (Fig. 3B). When the *neo* gene is driven by its own promoter in a Tn5 insertion mutant, it is constitutively expressed throughout the cell cycle (Champer et al. 1987). These results provide direct evidence that the temporal turn-on of the *flgI* gene in the *C. crescentus* cell cycle is controlled by sequences in the 5' regulatory region of the gene.

Segregation of the 29K flagellin to the swarmer cell progeny

The assembly of the flagellum occurs at one pole of the predivisional cell prior to cell division. Flagellins that were pulse-labeled with [³⁵S]methionine during their synthesis in the predivisional cell were recovered only in the swarmer cell progeny following cell division (Fig. 6A). The autoradiograph shown was overexposed to demonstrate that almost all of the newly synthesized flagellins can be chased into the flagellum-bearing

swarmer cell. These results suggest that either all the flagellins are assembled into the filament or both assembled and nonassembled flagellins are segregated to the pole of the predivisional cell prior to division. In strain AE9002/pRB3, in which one of the flagellins, the 29K subunit, is somewhat overproduced because the gene resides on a plasmid with a copy number of approximately five, we found that the excess 29K flagellin also segregated to the swarmer cell (Fig. 6B). Huguenel and Newton (1984) presented evidence that a pool of free flagellins can be detected in a polar compartment in the predivisional cell. In addition, we have recently shown that the newly synthesized MCP chemotaxis receptor is preferentially inserted into the membrane of the swarmer portion of the predivisional cell (Nathan et al. 1986). These results suggest that some mechanism other than assembly into a complex structure contributes to the positioning of the flagellar and chemotaxis proteins in the predivisional cell and their subsequent segregation to the swarmer cell progeny. This argument is supported by the results of the experiment that shows the segregation pattern of the 29K flagellin in a mutant unable to assemble a flagellum (Fig. 6E). The mutant strain AE8006 has a transposon (Tn5–VB32) insertion in the *flaK* gene that normally encodes the hook protein. Sequence analysis showed that the insertion is at codon 8 of the hook protein structural gene (H. Xu and J. Kaplan, unpubl.). In the absence of a hook protein, neither the hook nor the filament can be assembled. Hook protein null mutations result in a down-regulation of both the 25K and the 27.5K flagellins and the up-regulation of the 29K flagellin. Pulse-chase experiments with AE8006 show clearly that the 29K flagellin, synthesized prior to cell division, segregated to the swarmer progeny cell in this strain, even though it could not be assembled into a filament (Fig. 6E).

Having observed that a given flagellar protein does not have to be assembled into a flagellum to be segregated to the swarmer cell, we can now ask whether the 5' regulatory region of a gene encoding a "segregated" protein contributes to its correct positioning in the predivisional cell. This question was approached by analyzing the segregation pattern of the foreign protein, NPT II, when its expression is driven by the 5' regulatory region of the *flgI* gene (see Fig. 5A). Cultures of CB15N/pZL1451 were synchronized, and the predivisional cells were pulse-labeled with [³⁵S]methionine. Cell division was allowed to proceed in the absence of label, the progeny cells were separated, and the presence of labeled NPT II was as-

Table 2. Plasmids and kanamycin concentrations that allow growth

Strain	Kanamycin (μg/ml)
CB15N/pZL1451	150
AE9002/pZL1451	150
CB15N/pZL1452	<20
AE9002/pZL1452	<20

Figure 5. The expression of NPT II from the chimeric *flgJ-neo* gene in the plasmid pZL1451. Cultures of *C. crescentus* CB15N/pZL1451 were synchronized, and the synthesis of NPT II was measured in aliquots of cells pulse-labeled with [^{35} S]methionine. Cell extracts were immunoprecipitated with anti-NPT II antibody, as described in Materials and methods. (A) A restriction map of the junction between the *flgJ* promoter, a portion of the 29K 5'-coding region, and the promoterless *neo* gene. Due to the presence of an in-frame translation stop signal between the *flgJ* fragment and the *neo* gene, the chimeric mRNA yields free NPT II. (B) An autoradiogram of a 10% polyacrylamide-SDS gel, showing the level of synthesis of NPT II at 0, 0.25, 0.5, 0.6, 0.75, and 1.0 cell-cycle division units. The culture, grown in minimal glucose M2 media, had a generation time of 140 min. Lanes 1–6 correspond to the specific stages of the cell cycle shown diagrammatically below the gel.

sayed by immunoprecipitation (Fig. 6C). The NPT was not segregated to the swarmer cell but was found predominantly in the stalked cell progeny. In a control experiment, the segregation pattern of NPT II expressed from its own promoter in an intact Tn5, inserted in a *flaE* gene, was found to be the same as that found with the chimeric gene encoding NPT II in CB15N/pZL1451 (Fig. 6D). These results imply that the nucleotide sequences responsible for differential expression of the 29K flagellin do not contribute to the positioning of the protein within the cell but that the protein product is responsible for its localization.

Discussion

Temporal regulation of flagellin gene expression

The temporal expression of one of the *Caulobacter* flagellin genes, *flgJ*, as well as the control of the placement of its gene product, the 29K flagellin, have been characterized in this study. Our initial experiments confirmed earlier work (Lagenaur and Agabian 1978) that established the time of synthesis of the 29K flagellin protein at an early time in the stalked cell cycle relative to the other flagellin proteins. Nuclease S1 protection experiments showed that the initial appearance of the *flgJ* message coincided with the onset of 29K flagellin synthesis. When the 29K flagellin was expressed from a plasmid in a *Caulobacter* strain deleted for *flgJ* on the chromosome, the time of initiation of *flgJ* mRNA and 29K flagellin synthesis remained intact. Thus, the temporal regulation of *flgJ* expression is independent of its chromosomal location, and all the sequences necessary for temporal control are contained on the cloned DNA fragment with an intact *flgJ* gene.

A transcription fusion was constructed in which the *flgJ* promoter region drives the reporter gene encoding NPT II. Analysis of strains harboring this chimeric construct showed that NPT II synthesis was initiated at the same time in the cell cycle as the synthesis of the 29K flagellin from the intact gene. These results clearly demonstrate that the sequences 5' to the structural gene determine the time of initiation of expression of *flgJ*.

The contribution of 5' regulatory sequences to cell-cycle control has been characterized in a variety of systems. Artishevsky et al. (1985) showed that 5'-flanking sequences from a hamster histone H3 gene were sufficient to regulate bacterial coding sequences in a cell-cycle manner. Recently, Osley et al. (1986) demonstrated that the periodic expression of yeast histone genes was dependent on sequences localized to the 5'-promoter region. Studies from our laboratory (Champer et al. 1985, 1987) showed that a reporter gene encoding NPT II was expressed at the correct time in the cell cycle when placed under the control of promoters for several different temporally regulated flagellar genes.

Although the time of initiation of *flgJ* mRNA and protein synthesis was coincident at 0.5 division units, the *flgJ* mRNA persisted after 29K flagellin synthesis had ceased. In addition, the synthesis of NPT II from the transcription fusion driven by the *flgJ* promoter continued after the period of 29K flagellin synthesis. These observations suggest that post-transcriptional control might contribute to the temporal regulation of *fla*-gene expression.

The initiation of synthesis of the hook protein and the 25K and 27.5K flagellins occurs close to their time of assembly into the flagellar structure. However, the time of synthesis of the 29K flagellin occurs relatively early in the cell cycle, prior to the synthesis of other known flagellar components. The early time of synthesis of the 29K flagellin suggests that it functions at a relatively early stage in the assembly process. The role of the 29K flagellin in the flagellar assembly process is not known. Analysis of the filament structure by immunomicro-

Figure 6. Autoradiograms of pulse-chase experiments in which predivisional cells (PD), shown schematically (*top left*), were pulse-labeled with [^{35} S]methionine (15 μ Ci) for 20 min and then chased into progeny stalked (ST) and swarmer (SW) cells. The progeny cells were separated and lysed, and the proteins were immunoprecipitated with anti-flagellin antibody (A, B, and E) or anti-NPT II antibody (C and D). The immunoprecipitated proteins were separated by electrophoresis in 10% polyacrylamide–SDS gels. The *C. crescentus* strains used in each panel were CB15N (A); AE9002/pRB3 (B); CB15N/pZL1451 (C); SC1997 (D); and AE8006 (E).

scopy of wild-type and *flgJ* deletion strains suggests that in wild-type strains, the 29K flagellin resides at the hook–filament junction (R. Bryan and A. Driks, unpubl.). The observation that strains carrying a deletion in the *flgJ* gene are able to assemble a flagellum and are motile clearly demonstrates that the 29K flagellin is not essential for motility. We have recently determined, however, that *flgJ* deletion strains form swarm colonies at a slower rate than those formed by isogenic wild-type strains. It remains to be determined whether *flgJ* deletion strains have fewer flagellum-bearing cells or whether the number of swarmer cells is comparable to wild type, but they swim more slowly.

Asymmetric distribution of flagellar proteins

The generation of different progeny cells from a binary fission requires the asymmetric distribution of proteins and structures in the predivisional cell and their ultimate segregation with the appropriate progeny cell. Asymmetric segregation of *Caulobacter* proteins has been described previously for the chemotaxis methylation machinery, which is positionally biased toward the portion of the predivisional cell that bears the flagellum (Gomes and Shapiro 1984), and several heat shock proteins, which segregate to the nonmotile stalked cell (Reuter and Shapiro 1987). In this paper we have addressed the possible mechanisms that contribute to the segregation of a flagellar protein to the progeny swarmer cell. We have shown that the newly synthesized 29K flagellin segregates to the progeny swarmer cell upon division. Because the flagellins are the components of the flagellar filament, a possible mechanism for their segregation could rely on their assembly into the flagellum that is formed in the predivisional cell. Consequently, the flagellar components would then segregate to the swarmer cell upon division. This mechanism

would require that most, if not all, flagellin monomers be assembled into the filament. However, even in mutant cells unable to assemble either a hook or a filament, the 29K flagellin still segregated to the progeny cell that would have carried a flagellum. Because incorporation of the flagellar protein into the assembled structure did not appear to be required for segregation to the correct progeny cell, we asked if the reporter protein NPT II, whose transcription is driven by the *flgJ* promoter region, was correctly positioned. NPT II that was synthesized in the predivisional cell was not segregated to the progeny cell in a specific manner. Therefore, the protein must carry signals for its proper segregation.

Positioning of a protein within the cell could be accomplished in a number of different ways: (1) The location of a gene on a chromosome could be important. It is possible that a gene encoding a protein that has to be positioned could be oriented and transcribed in such a way that upon its synthesis, the protein is near or at a specific membrane-binding site. This does not appear to be the case for the positioning of the 29K flagellin. The gene for this protein can be located on a plasmid without altering its pattern of segregation. (2) Transcription could specifically come from that portion of the replicating chromosome that will ultimately reside in the swarmer portion of the predivisional cell. Because transcription and translation are coupled, the gene would be expressed at the site of residence of the protein. A possible way to initiate transcription from only one of the newly replicated regions of the chromosome could be to utilize the 5' regulatory sequences as a binding site for a soluble factor present only in one part of the predivisional cell. Clearly, in the case of 29K flagellin, the 5' regulatory region is not the sole determining factor for its segregation to the swarmer cell because the NPT II gene product from the chimeric fusion gene was not preferentially segregated to the swarmer cell. (3) The

mRNA could migrate to the correct portion of the predivisional cell, either because of a nascent polypeptide chain or because of properties inherent to the RNA itself. If the mRNA were localized in the swarmer cell portion of the predivisional cell, it is likely that the NPT II synthesized from the chimeric *flgJ*–NPT II mRNA in the predivisional cell would be segregated to the progeny swarmer cell. We did not find NPT II protein segregating to the swarmer cell. This could mean that the chimeric mRNA is not positioned in the predivisional cell. However, it is also possible that the *flgJ*–NPT II chimeric mRNA is confined to the swarmer portion of the predivisional cell but that the newly synthesized NPT II diffuses to the rest of the predivisional cell before division occurs. Milhausen and Agabian (1983) found that mRNA for the 25K flagellin segregated to the daughter swarmer cell upon cell division, suggesting that mRNA segregation does occur in some cases. (4) The free flagellin protein could segregate by itself. We have shown that the 5' regulatory sequences are not solely responsible for protein segregation. The possibility must therefore be considered that an amino acid sequence in the positioned protein is responsible for correct segregation. Furthermore, the site on the cell that "sees" this amino acid sequence cannot be the hook and filament but must be a receptor at or near the position of flagellar assembly. This putative receptor might be laid down at the site of cell division, as it is this site in both the swarmer and stalked cell that ultimately bears the flagellum. Experiments are in progress to define the portion of the protein-coding region that is essential for the proper segregation of the 29K flagellin.

Materials and methods

Restriction enzymes were obtained from New England Biolabs and Boehringer-Mannheim. Calf intestine alkaline phosphatase, T4 polynucleotide kinase, and nuclease S1 were purchased from Boehringer-Mannheim. [γ - ^{32} P]ATP (10 Ci/mmol) and [^{35} S]methionine (1150 Ci/mmol) were obtained from Amer-

sham Corporation. Staph A cells were purchased from Bethesda Research Laboratory. RNA used for nuclease S1 assays was extracted with phenol at 65°C, as described previously (Amemiya et al. 1980).

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Tables 1 and 3, respectively. *C. crescentus* was grown at 30°C in minimal glucose M2 or in rich PYE media (Contreras et al. 1978). *C. crescentus* wild-type CB15N and derivative strains were synchronized following the separation of swarmer cells in a Ludox density gradient, as reported by Evinger and Agabian (1977) and as described previously (Mansour et al. 1980). Motile colonies were detected on semisolid swarm plates containing 0.3% agar in PYE, as described previously (Bryan et al. 1984).

Construction of AE9002 from SC512

C. crescentus strain SC512 carries the spontaneous *flaE156* deletion (Johnson et al. 1979; Purucker et al. 1982). AE9002 was constructed by genetically moving a region of SC512 DNA containing this deletion into the synchronizable, wild-type background of CB15N (Evinger and Agabian 1977). The first step in this construction was to mate AE5478 (*cysD*::Tn5, *ts104*, pVS1) with CB15N. CB15N colonies that had obtained *cysD*::Tn5 formed kanamycin-resistant (Kan^r) progeny at 30°C, the restrictive temperature for AE5478. The Kan^r progeny were screened for the presence of a Cys⁻ phenotype. Because *cysD* and *flaE* can be cotransduced, one *cysD*::Tn5 CB15N derivative AE9001 was transduced to Cys⁺ prototrophy, using the transducing phage 0Cr30 grown on strain SC512 (*flaE156*). Of 27 transductants tested, 10 had a Fla⁻ phenotype. The plasmid pVS1, resident subsequent to the original mating, was cured from one Fla⁻ transductant, AE9002, by growth of the strain in PYE liquid culture. To show that the deletion in SC512 had been transferred correctly to AE9002, DNA was isolated from SC512 and AE9002, digested with the restriction enzymes *Hind*III and *Bam*HI, and analyzed by Southern blot hybridization using probe 1, shown in Figure 2. The same size restriction fragments were detected in the two strains (data not shown). Furthermore, the pattern of plasmid complementation was identical in the two strains.

The deletion in SC512 was shown previously to be approximately 3 kb in length (Purucker et al. 1982). Digestions of

Table 3. List of plasmids

Plasmid	Length (kbp)	Relevant properties	Reference/source
pBR322	4.4	Ap ^R , Tc ^R	Bolivar et al. (1977)
pBR325	5.9	Ap ^R , Tc ^R , Cm ^R	Bolivar (1978)
pFB1	8.1	Ap ^R , Cm ^R , <i>flgJ</i> insert, pBR325 vector	Champer et al. (1985)
pRB3	30	Tc ^R , <i>flgJ</i> , <i>flaE</i> , <i>flaY</i> insert, pRK290 vector	Bryan et al. (1987)
pRB7	23	Tc ^R , <i>flaE</i> , <i>flaY</i> insert, pRK290 vector	Bryan et al. (1987)
pRK2013	49	Kn ^R , <i>ColE1</i> replicon, <i>Tra</i> ⁺ , RK2 derivative	Figurski and Helinski (1979)
pRK290	20	Tc ^R , RK2 replicon, <i>Tra</i> ⁻ , <i>mob</i> ⁻ , incompatibility group P-1	Ditta et al. (1980)
pRK291	20	Tc ^R , RK2 replicon, <i>Tra</i> ⁻ , <i>mob</i> ⁻ , incompatibility group P-1	Ditta et al. (1985)
pVS1	66	Tc ^R , Kn ^S derivative of RP4	Barrett et al. (1982)
pKM2	5.1	Ap ^R , Kn ^R	Beck et al. (1982)
pZL250	4.9	Ap ^R	this study; derived from pKM2
pZL451	6.6	Ap ^R , <i>flgJ</i> promoter insert, correct orientation	this study
pZL452	6.6	Ap ^R , <i>flgJ</i> promoter insert, incorrect orientation	this study
pZL1451	26.6	Ap ^R , Tc ^R	derived from pZL451
pZL1452	26.6	Ap ^R , Tc ^R	derived from pZL452

SC512 DNA with *EcoRI* and *BamHI*, as well as with *EcoRI* and *HindIII*, followed by Southern blot hybridization to probe 2 (Fig. 2A), indicated that the size of the deletion was approximately 3.4 kb (data not shown). The end points of this deletion were mapped by digesting SC512 DNA with *PstI* and *SalI* and analyzing the fragments by Southern blot hybridization. Following *PstI* digestion, both probe 1 and probe 2 (Fig. 2A) hybridized to a fusion fragment of 4.6 kb. This is consistent with either a 3.8-kb deletion spanning all of the internal *PstI* sites, as shown in Fig. 2A, or with a 2.9-kb deletion extending from the *PstI* site in *flgJ* to the *PstI* site in *flaY*. When Southern hybridization of *SalI*-digested SC512 DNA was done, probe 2 hybridized to a 2.1-kb fragment, slightly smaller than the 2.2-kb *SalI* fragment found in wild-type DNA. This size is consistent with what would be found if the deletion extended from *flgK* to *flaY*, as shown in Figure 2A. If the deletion extended only from the *PstI* site in *flgJ* to the *PstI* site in *flaY*, the *SalI* fragment in SC512 hybridizing to probe 2 would have been larger, not smaller, than the corresponding *SalI* fragment from the wild-type DNA.

Immunoprecipitation

The synthesis of the flagellins and NPT II was measured in cultures pulse-labeled with [³⁵S]methionine by the immunoprecipitation procedure described by Gomes and Shapiro (1984). [³⁵S]Methionine-labeled *C. crescentus* cells were lysed, preadsorbed with Staph A cells, and immunoprecipitated with antisera raised against purified flagellins (Shapiro et al. 1982) or NPT II (Champer et al. 1985). The immunoprecipitated proteins were separated by electrophoresis through 10% or 12% polyacrylamide–SDS gels. The gels were fixed, stained, enhanced, and subjected to autoradiography.

S1 nuclease assay

S1 nuclease assays were performed by a modification (Amemiya et al. 1986) of the procedure of Berk and Sharp (1977). The probes used were derived from the plasmid pRB1 (Champer et al. 1985). This plasmid contains a 2.2-kb *SalI* fragment encoding the entire *flgJ* gene. The *PvuII*–*ClaI* probe was obtained by phosphorylating the *ClaI* end with phage T4 polynucleotide kinase, redigesting the DNA with *PvuII*, and isolating the 0.8-kb fragment by electrophoresis through a 5% (wt/vol) polyacrylamide gel. The *NaeI*–*TaqI* probe was prepared by 5' end-labeling the *TaqI* site, redigesting the DNA with *NaeI*, and isolating the 0.28-kb fragment by electrophoresis through a 5% (wt/vol) polyacrylamide gel. The labeled fragment was then mixed with 100 µg of RNA, precipitated with ethanol, and dried briefly under vacuum. Following hybridization and S1 digestion, the samples were analyzed on a 6% (wt/vol) polyacrylamide gel with 7 M urea, using 0.5 × TBE buffer (Maniatis et al. 1982). RNA was isolated from synchronized cultures, as described by Amemiya et al. (1980).

Construction of NPT II transcription fusion

A 1.7-kb *BamHI*–*BclI* fragment carrying the *flgJ* promoter was ligated into the unique *BglII* site of pZL250 (Fig. 4). pZL250 was made as follows: The small *EcoRI*–*BamHI* fragment of pBR322 was deleted, and a *BglII* site was manufactured in its place. The *BglII*–*SalI* fragment, containing the *neo* gene of Tn5, was then inserted in place of the *BglII*–*SalI* fragment of the pBR322 derivative. The resultant two plasmids, pZL451, where the direction of transcription is toward the *neo* gene, and pZL452, where direction of transcription is away from the *neo* gene, were cut at their respective unique *EcoRI* sites and ligated into the *EcoRI*

site of pRK291. These recombinants, pZL451 and pZL452, were then mated into *Caulobacter* strains CB15N and AE9002.

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Control of synthesis and positioning of a *Caulobacter crescentus* flagellar protein.

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