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Structural and Genetic Analysis of a Mutant of *Rhodobacter sphaeroides* WS8 Deficient in Hook Length Control

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Motility in the photosynthetic bacterium *Rhodobacter sphaeroides* is achieved by the unidirectional rotation of a single subpolar flagellum. In this study, transposon mutagenesis was used to obtain nonmotile flagellar mutants from this bacterium. We report here the isolation and characterization of a mutant that shows a polyhook phenotype. Morphological characterization of the mutant was done by electron microscopy. Polyhooks were obtained by shearing and were used to purify the hook protein monomer (FlgE). The apparent molecular mass of the hook protein was 50 kDa. N-terminal amino acid sequencing and comparisons with the hook proteins of other flagellated bacteria indicated that the *Rhodobacter* hook protein has consensus sequences common to axial flagellar components. A 25-kb fragment from an *R. sphaeroides* WS8 cosmid library restored wild-type flagellation and motility to the mutant. Using DNA adjacent to the inserted transposon as a probe, we identified a 4.6-kb *Sal*I restriction fragment that contained the gene responsible for the polyhook phenotype. Nucleotide sequence analysis of this region revealed an open reading frame with a deduced amino acid sequence that was 23.4% identical to that of FliK of *Salmonella typhimurium*, the polypeptide responsible for hook length control in that enteric bacterium. The relevance of a gene homologous to *fliK* in the uniflagellated bacterium *R. sphaeroides* is discussed.

Many bacterial species have the ability to move, and they alter the direction of their movement in response to different external stimuli, which enables them to migrate to more favorable environments (33). Swimming motility (as opposed to gliding and other types of motility) involves the function of a complex structure called the flagellum. The flagellum is composed of three main parts, the basal body, the hook, and the filament (for recent reviews, see references 9, 33, and 46). Much of the information generated to date regarding flagellar structure, assembly, function, and genetics has been obtained from the gram-negative organisms *Escherichia coli* and *Salmonella typhimurium* (reviewed in references 20 and 32). These enteric bacteria possess between 5 and 10 peritrichous flagella per cell. The flagellum can rotate either counterclockwise, causing the filaments to form a bundle that produces translational motion, or clockwise (CW), causing the cell to tumble and reorient (31, 49).

The filament is connected to the basal body through the hook, which works as a flexible coupling that allows torque generated by the motor to be transmitted to the filament (10, 11). For this purpose, the hook of the bacterial flagellum requires a rather well-defined length (23). A shorter hook would not generate a sufficient bend angle (23), and presumably a long flexible coupling would not permit effectively directed transmission of torque (57). The mean length that has been established for different bacterial species varies from 55 ± 6 nm for the wild-type hook of *S. typhimurium* (15) to 69 ± 8 nm for *Treponema phagedenis* (29) and up to 105 nm for *Campylobacter coli*, the longest hook yet described (43).

Nonmotile mutants with abnormally long flagellar hooks and without a flagellar filament have previously been isolated and termed polyhook mutants (41, 48). The flagellar gene *fliK* has previously been cloned (16), and its protein product has been identified as being responsible for controlling hook length (41, 55). FliK has not been detected within the final flagellar structure, and the means by which it regulates hook length is not known (15). Nevertheless, different mechanisms for hook length control have been proposed, based on the identification of pseudorevertants of *fliK* mutants that recovered the ability to assemble filaments on the tips of polyhooks. These pseudorevertants have been called polyhook filament mutants. The reverting mutations have previously been isolated in the *fliK* or *flhB* gene (15, 25, 55). FlhB is an integral membrane protein with a C-terminal cytoplasmic domain (35) that has been proposed to form part of the export apparatus (25) and to participate in the determination of hook length (15, 25). Recently, it has been shown that the negative regulator FlgM (25) and the newly identified protein RflH (26) are also involved in this process of hook length control. In *S. typhimurium*, the assembly of the hook is assisted by a scaffolding protein, FlgD. When the hook reaches its final length, FlgD is replaced by FlgK, the first hook-associated protein (HAP1). In polyhook mutants, this replacement does not occur and FlgD remains at the tip of the polyhook (39).

Rhodobacter sphaeroides is a purple nonsulfur photosynthetic bacterium that swims by using a single medially located flagellum that rotates unidirectionally CW. In contrast to the CW-counter clockwise switching swimming behavior of enteric bacteria, a change in the swimming direction in *R. sphaeroides*

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
R. sphaeroides		
WS8	Wild type; spontaneous Nal^r	52
PG	WS8 Tn*phoA* derivative; nonmotile; Km^r Nal^r	This work
PG2	WS8 Tn*phoA* derivative; nonmotile; Km^r Nal^r	This work
E. coli		
S17-1	Donor for conjugations; Pro$^-$ Res$^-$ Mod$^+$ *recA*-integrated plasmid (RP4-Tc::Mu) (Km::Tn*7*) Tra$^+$	50
HB101	*supE44 hsdS20*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	44
Plasmids		
pU1800	pSUP203 derivative carrying Tn*phoA*; Cm^r Tc^r Km^r	36
c140	pLA2917 derivative carrying 25 kb of *R. sphaeroides* WS8 DNA; Tc^r	52
pTnPG	5.7-kb *Sal*I fragment cloned into pUC19 containing 4.6 kb of Tn*phoA* plus 1.1 kb of PG DNA flanking the site of transposon insertion; Km^r Amp^r	This work
pBG0404	4.6-kb *Sal*I fragment from c140 cloned into pTZ19R; Amp^r	This work
pBG0206	2.4-kb *Sal*I-*Eco*RI subclone from pBG0404 cloned into pTZ18R; Amp^r	This work
pBG0007	0.7-kb *Pst*I fragment derived from pBG0206 cloned into pTZ18R; Amp^r	This work
pBG0108	pBG0206 with a 0.5-kb *Sal*I-*Not*I deletion; Amp^r	This work

involves a rotational stop during which Brownian motion reorients the cell prior to renewed swimming (4).

In this work, we report the isolation of a polyhook mutant (PG) of *R. sphaeroides*, a bacterium that, unlike enteric bacteria, possesses a straight hook (53). This mutant was particularly useful for the purification of the hook monomer FlgE. The finding of a polyhook mutant in a uniflagellated bacterium with start-stop swimming behavior indicates that hook length may be regulated by a mechanism shared by many bacterial species.

MATERIALS AND METHODS

Bacterial strains and growth. The bacterial strains and plasmids used in this work are described in Table 1. *R. sphaeroides* cell cultures were grown photoheterotrophically in Sistrom's medium (51) under continuous illumination at 30°C in liquid or solid medium (1.5% agar). *E. coli* strains were grown in Luria broth at 37°C with appropriate antibiotic selection (44). The antibiotics used for the selection of *R. sphaeroides* recombinant strains were as follows: kanamycin, 25 μg ml^{-1}; tetracycline, 1 μg ml^{-1}; and nalidixic acid, 20 μg ml^{-1}.

Isolation of the polyhook mutant. Transposon mutagenesis was performed by introducing the transposable element Tn*phoA* on suicide plasmid pU1800 from the donor *E. coli* strain, S17-1, into wild-type *R. sphaeroides* WS8-N by diparental conjugation (36). The mutagenized population was screened for nonmotile mutants by tryptone-yeast extract swarm assays as described previously (53). Cells were also analyzed by high-intensity dark-field microscopy (30) and by electron microscopy.

Purification of FlgE. A polyhook mutant (PG) was used for the purification of hook protein FlgE. Early-exponential-growth phase cells were sheared by passaging them through a 18- by 38-gauge cannula several times. Whole cells and cell debris were removed by two low-speed centrifugations (10,000 × *g* for 15 min), and the resulting supernatant was subjected to ultracentrifugation (100,000 × *g* for 60 min). The resulting pellet was suspended in CsCl (0.4 g ml^{-1}) and subjected to density centrifugation (2). The purity of the isolated hook protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15.0% polyacrylamide gels (27) and silver stained (38). Flagellar filaments were also isolated from wild-type cells by shearing, followed by differential centrifugation and cesium chloride gradient centrifugation. Few of the resulting filaments contained a hook attached to the rest of the structure, as observed by negative staining.

For N-terminal sequence analysis of FlgE, protein was transferred to a ProBlott membrane (Applied Biosystems, Foster City, Calif.) with 100 mM 3-cyclohexilamino-1-propane sulfonic acid buffer (pH 11) by the method of LeGendre and Matsudaira (28). Blotted protein was stained with Coomassie blue and cut from the ProBlott membrane. Amino-terminal sequence analysis was carried out at the W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University, New Haven, Conn.

Electron microscopy. Purified polyhooks, sheared flagella, or bacterial cell suspensions (10 μl) were applied to Formvar-coated grids. Samples were negatively stained with 1% uranyl acetate and observed with a JEM-1200EXII electron microscope (Jeol, Tokyo, Japan). Micrographs were taken at an accelerating voltage of 80 kV.

Recombinant DNA techniques. The isolation of chromosomal DNA was performed essentially as described elsewhere (44). Plasmid DNA was obtained by standard procedures (44) or with plasmid midiprep columns from Qiagen (Chatsworth, Calif.). Restriction enzymes and T4 DNA ligase were used according to the manufacturer's recommendations. Standard methods were used for *E. coli* transformation, restriction endonuclease analysis, ligation, and other related techniques (44).

The dideoxy chain termination method was used for sequencing both single- and double-stranded DNAs (45). The sequencing reactions were carried out with Taquenase as instructed by the manufacturer of the delta*Taq* DNA polymerase system (version 2.0; U.S. Biochemical Corp., Cleveland, Ohio). Sequencing of cloned DNA was accomplished with ^{35}S-dCTP and the analog 7-deaza-dGTP to avoid problems associated with sequencing GC-rich DNA. Subclones for nucleotide sequencing were constructed by using the indicated restriction sites in plasmid pBG0206 (see Fig. 6A) and are described in Table 1.

Southern hybridization was done by the procedure described for the nonradioactive Photogene detection kit (Gibco, Life Technologies). Probes were labelled with biotin-14-dATP by the nick translation technique according to the protocol provided by the manufacturer (Bionick labelling system; Gibco BRL). Detection of the hybridization signal was done according to the manufacturer's (PhotoGene; Gibco BRL) protocol.

Complementation analysis. Genetic complementation of the mutant was carried out with a cosmid library of *R. sphaeroides* WS8 total DNA (53). Cosmids were introduced into *R. sphaeroides* mutant strains by diparental conjugation from *E. coli* S17-1. The complemented mutant was selected for its resistance to kanamycin (coded by Tn*phoA*) and tetracycline (cosmid encoded) and tested for motility as described above.

Nucleotide sequence accession number. The GenBank nucleotide sequence accession number for *R. sphaeroides fliK* is U86454.

RESULTS

Isolation and morphological characterization of a polyhook mutant. Transposon mutagenesis with subsequent motility screening was used to obtain flagellar mutants. A nonmotile mutant was identified in swarm tests on soft agar plates. The mutant was unable to form the diffuse ring that is characteristic of the wild-type strain when it moves away from the inoculation point (Fig. 1). The mutant was also incapable of swimming when a liquid culture was observed by either phase-contrast or dark-field high-intensity microscopy.

Further characterization was carried out by electron microscopy, which revealed an unusually short flagellar structure attached to the cell (Fig. 2a). This mutant structure was compared to the wild-type flagellum in order to establish its composition. Material sheared from wild-type *Rhodobacter* cells was shown to be rich in filaments, many of which possessed hooks. The wild-type flagellum shows a clearly different pattern in the arrangement of subunits between the hook and the filament (Fig. 2b). The filament has a vertical striation along its length, whereas the hook shows a cross-hatched helical pattern. Furthermore, these two structures are separated by a clearly distinguishable region at the interface that probably consists of HAPs (Fig. 2b). The wild-type hook structure had an average length of ca. 100 nm. When mutant cells were observed at a higher magnification, the pattern of their external structures was similar to that of the hook (compare Fig. 2c with b). This structure was longer (ca. 300 nm) than were hooks from wild-type cells, and no HAP proteins or filaments

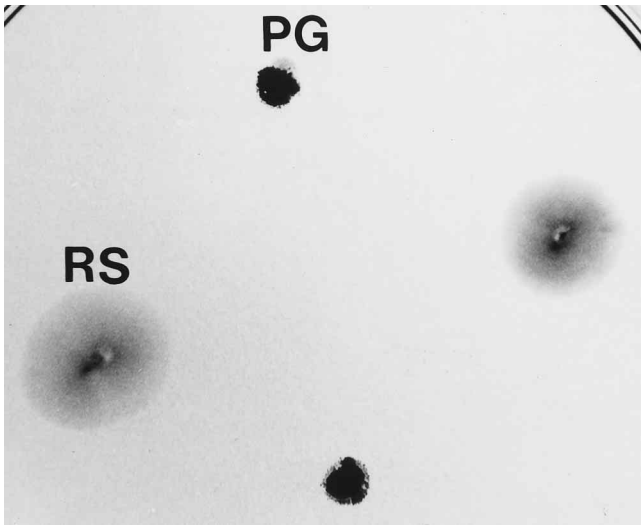

FIG. 1. Swarming assay of mutant and wild-type *R. sphaeroides* WS8. Wild-type *R. sphaeroides* (RS; left and right) and PG mutant (top and bottom) colonies were stabbed into the swarm agar, and the plates were incubated aerobically at 30°C for 48 h. Tryptone-yeast extract agar was composed of 0.03% (wt/vol) Bacto Peptone, 0.03% (wt/vol) yeast extract, and 0.3% (wt/vol) Bacto Agar (Difco Laboratories).

were detected at the tips of these structures. Therefore, based on microscopic analysis, the mutant seems to produce a hook of anomalous length, similar to the polyhooks described previously for enteric bacteria (41, 48).

The structures sheared from mutant cells were also analyzed by electron microscopy. They showed the same subunit pattern as that of the wild-type hook (Fig. 3; compare with Fig. 2b). Mutant polyhooks varied in length (from ca. 200 to 1,500 nm), a range comparable to what has previously been observed for polyhooks in enteric bacteria (15).

Purification of FlgE. In order to ascertain the protein composition of the flagellar structures of wild-type and mutant strains, we sheared and purified the external flagellar structures of both strains and analyzed them by SDS-PAGE (Fig. 4). Figure 4, lane 2, shows that wild-type filaments are composed mainly of two proteins, one with an apparent molecular mass of 55,000 Da, which has previously been identified as flagellin (FliC) (52), and a minor component with a molecular mass of 50,000 Da. This result suggests that the minor component is the hook protein. Figure 4, lane 3, shows the electrophoretic profile of the sheared external structure from the polyhook mutant. The only protein present has a molecular mass of ca. 50,000 Da. From this analysis, it is clear that the mutant strain lacks flagellin (Fig. 4; compare lanes 2 and 3).

In order to confirm the identity of the protein sheared from the mutant, we performed N-terminal sequence analysis of the 50,000-Da band. Figure 5 shows the sequence alignment of the N termini of hook proteins from various flagellated bacteria. The *R. sphaeroides* sequence contains the N-terminal consensus sequence SGL as well as the three conserved heptad repeats that have previously been argued to be important for quaternary interactions that take place between subunits of axial proteins (17).

Cloning of the wild-type *fliK* gene. The Tn*phoA* insertion that caused the polyhook phenotype was localized by Southern blot analysis with a 3.3-kb internal *Hin*dIII fragment from plasmid pU1800 as a probe (Fig. 6A). Chromosomal DNA isolated from the mutant was digested with the restriction enzyme *Sac*I. Since there is no cleavage site for *Sac*I in Tn*phoA*, the single band observed in Southern blot analysis confirmed that there was a single insertion of Tn*phoA* in the chromosome (data not shown).

The DNA flanking the site of the Tn*phoA* insertion was cloned into pUC19 by selection for the kanamycin resistance conferred by Tn*phoA*. One such clone (pTnPG) was found to contain a 5.7-kb DNA fragment that included a 4.6-kb fragment of Tn*phoA* DNA plus a 1.1-kb fragment of *R. sphaeroides* DNA fused to the *phoA* end of the transposon (Fig. 6A).

Using an *R. sphaeroides* WS8 genomic cosmid library, we

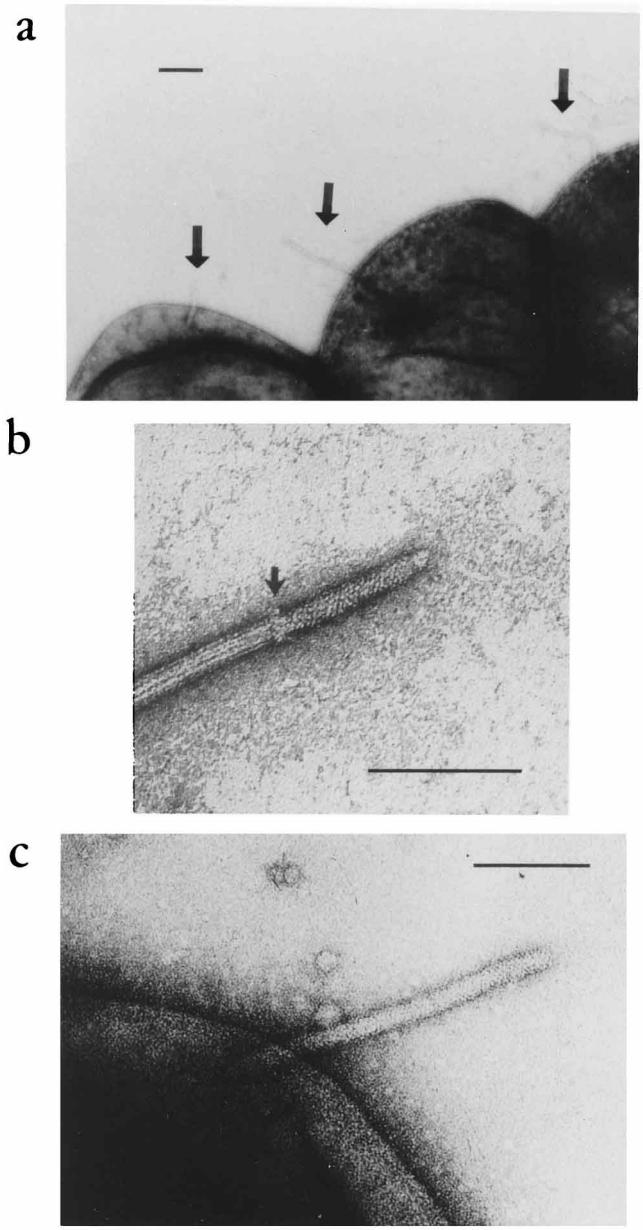

FIG. 2. Negatively stained electron micrographs of hook and polyhook structures. (a) PG mutant. Arrows point to short flagellar structures produced by the mutant. Bar, 200 nm. (b) A sheared flagellar filament from wild-type *R. sphaeroides* WS8 with an associated hook. The arrow points to a region that may be occupied by HAPs. Bar, 100 nm. (c) Higher magnification of the PG mutant. Bar, 100 nm.

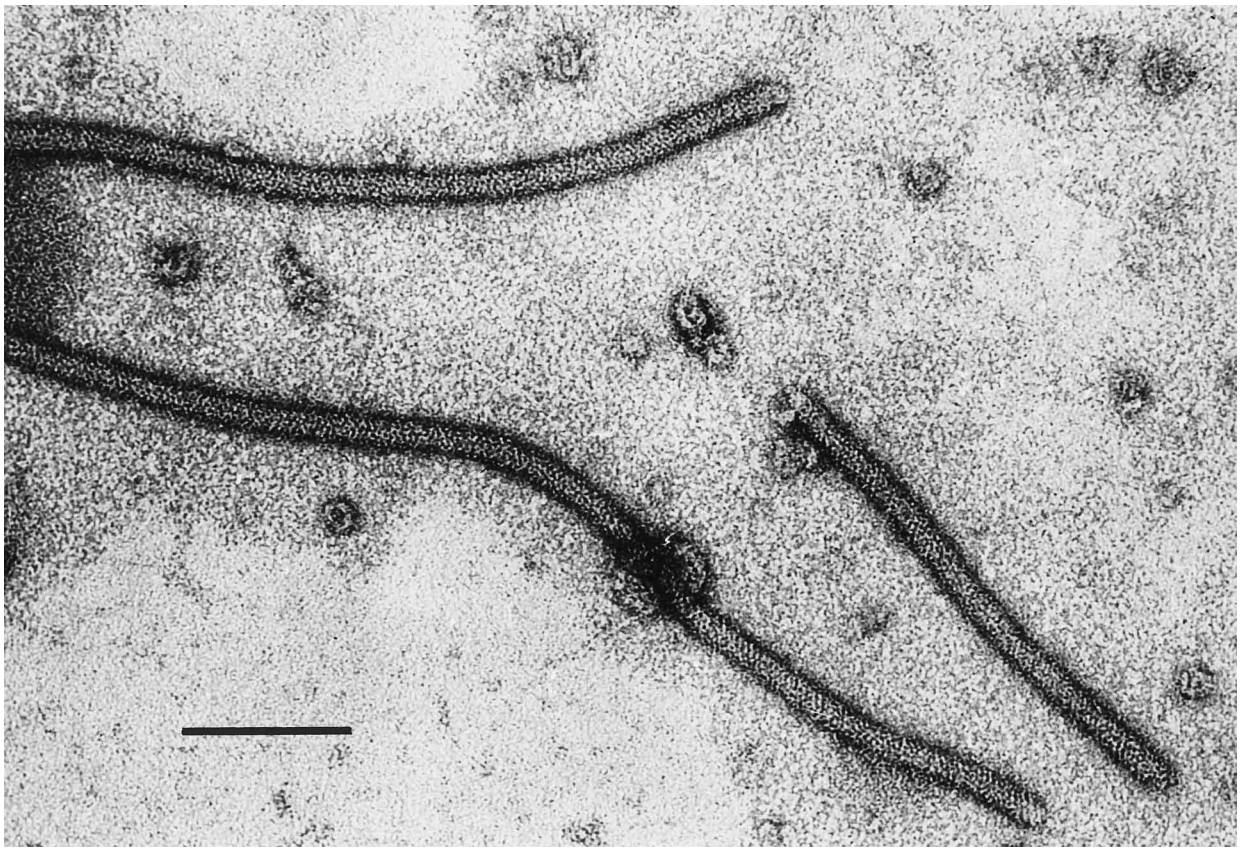

FIG. 3. Electron micrograph of negatively stained, sheared flagellar structures from a culture of the polyhook mutant. Bar, 100 nm.

identified a cosmid clone (c140) that restored motility and the ability to form normal hooks and filaments to the polyhook mutant (data not shown). A 1.4-kb *Sal*I-*Dra*I fragment from pTnPG was used to probe Southern blots made with different digests from the cosmid (Fig. 6A). A 4.6-kb *Sal*I restriction fragment which hybridized to the probe was identified (Fig. 6B) and cloned into plasmid pTZ19R to generate plasmid pBG0404 (Fig. 6A), which was used for nucleotide sequence analysis.

Sequencing reactions were initially run with pTnPG and both the universal sequencing primer M13 −40 (forward) and a primer complementary to the *phoA* end of the Tn*phoA* sequence. In addition, this fragment was sequenced with two internal primers. Sequencing was extended to the 3′ end of the cloned gene with plasmid pBG0404 or the subclones listed in Table 1 (pBG0206, pBG0007, and pBG0108).

One complete open reading frame (ORF) of 1,425 bp was identified on the 2.4-kb *Sal*I-*Eco*RI DNA segment from pBG0404. The nucleotide sequence has a GC content of 77%, which is in accord with the high GC bias of this bacterium (42). There is a putative ribosome binding site (GAGG) 11 bp upstream of the initiation codon. Tn*phoA* was inserted 1,132 bp downstream from the start codon. We isolated a second mutant whose Tn*phoA* insertion was found to be on the same ORF. The Tn*phoA* insertion in this mutant (polyhook mutant 2) also generated a polyhook phenotype (data not shown) and was localized 550 bp downstream from the initiation codon. The ORF whose disruption was responsible for the polyhook phenotype of both mutants codes for a polypeptide of 474 residues with a predicted mass of 45,745 Da. A comparison of the deduced amino acid sequence with the polypeptide sequence of *S. typhimurium* FliK (Fig. 7) showed 23.4% identity and 44% similarity. As noted previously for the FliK sequences from *S. typhimurium* and *E. coli* (23), there is a high proline

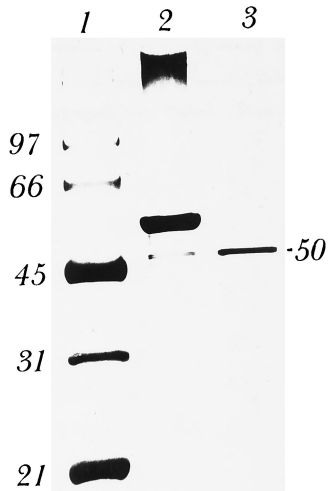

FIG. 4. SDS-PAGE of wild-type filaments and sheared polyhooks. Lane 1, molecular mass markers: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21 kDa); lane 2, sheared wild-type filaments from *R. sphaeroides* WS8 (10 μg); lane 3, sheared polyhooks (10 μg).

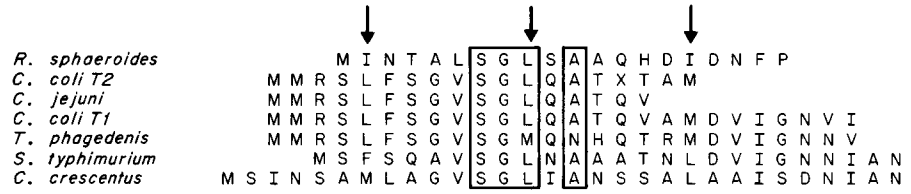

FIG. 5. Comparison of the N-terminal amino acid sequences of hook proteins from *R. sphaeroides* WS8, *C. coli* VC167-T2 (T2), *C. jejuni* VC74, *C. coli* VC167-T1 (T1) (43), *T. phagedenis* (29), *S. typhimurium* (17), and *Caulobacter crescentus* (40). Arrows point to the hydrophobic residues present at 7-residue intervals. The SGL consensus sequence and a highly conserved alanine (missing in *T. phagedenis*) are boxed.

content. Proline residues were located mainly in the central region, which most resembles FliK of enteric bacteria (23). An amino acid sequence comparison of the C-terminal regions of FliK proteins from various bacteria (Fig. 8) revealed five highly conserved leucine residues. On the basis of the sequence similarity and other properties of this protein, we have designated this ORF *fliK*, the gene that encodes the hook length control protein in *R. sphaeroides.*

DISCUSSION

The motility, chemotaxis, and flagellar structure of *R. sphaeroides* have been the subject of previous studies (4–6, 53). We are interested in extending the molecular characterization of flagellar proteins and in studying the mechanisms of flagellar export, assembly, and genetic regulation in this organism. We generated transposon flagellar mutants (Fla^-) in order to characterize some of the gene products involved in flagellar assembly.

Characterization of FlgE. In this work, we isolated and characterized a nonmotile mutant of *R. sphaeroides* that makes a polyhook structure instead of a normal flagellum. This report constitutes the first isolation of a polyhook mutant in a photosynthetic bacterium. This mutant was very useful for the isolation of the hook protein (FlgE), given that FlgE is present in a minute amount compared to that of flagellin (FliC) in wild-type cells.

Previous studies of *S. typhimurium* and *E. coli* have demonstrated that the organization of FlgE subunits in polyhooks appears to be identical to that of wild-type hooks (54, 56). In addition, wild-type and polyhook structures showed the same structural and biochemical characteristics (21, 22). This conclusion has been confirmed in these enteric bacteria, in which mutations in *fliK* do not affect the quaternary structure of hook subunits (1, 19). Therefore, the polyhook structure has been commonly used for the characterization of hook structure and the properties of FlgE monomers (47). In this study, we showed that the apparent molecular masses of the hook monomers of *R. sphaeroides* from both wild-type and polyhook mutant cells are the same and that the macroscopic organization of FlgE subunits is also conserved.

Structural analysis of the flagellum of wild-type *R. sphaeroides* by electron microscopy revealed a structure described as a straight hook (53). This feature was also observed with a polyhook mutant (Fig. 2c). Since the term straight hook is somewhat misleading, perhaps the term hook should be changed for a more general name like coupler or connector.

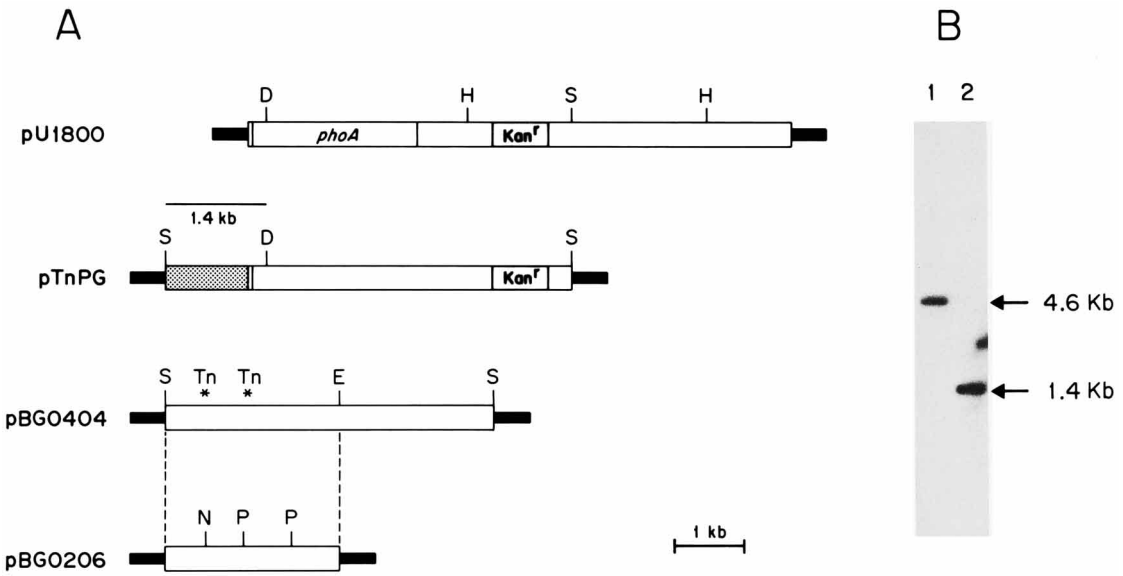

FIG. 6. Cloning strategy for the *fliK* gene. (A) Plasmids are described in Table 1. Plasmid pU1800 contains Tn*phoA*. Plasmid pTnPG contains a 5.7-kb DNA fragment (4.6 kb of Tn*phoA* DNA plus a 1.1-kb fragment of *R. sphaeroides* DNA [gray stippled box]). Plasmid pBG0404 is a 4.6-kb *Sal*I restriction fragment that contains the complete *fliK* gene. Asterisks above pBG0404 indicate the sites of Tn*phoA* insertion (Tn) into the chromosomes of two polyhook mutants. The direction of transcription of the *fliK* gene is from the *Sal*I 5′ end to the *Eco*RI restriction site in pBG0404. pBG0206 is a 2.4-kb *Sal*I-*Eco*RI subclone from pBG0404 used for sequencing the *fliK* gene. The restriction sites indicated were used for the construction of plasmids pBG0007 and pBG0108 (Table 1). Abbreviations: D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; N, *Not*I; P, *Pst*I; S, *Sal*I. (B) Southern blot of cosmid c140 DNA digested with *Sal*I (lane 1) and with a 1.4-kb (*Sal*I-*Dra*I) fragment from pTnPG as a probe (lane 2). A 4.6-kb *Sal*I fragment from cosmid c140 was identified and cloned to create plasmid pBG0404.

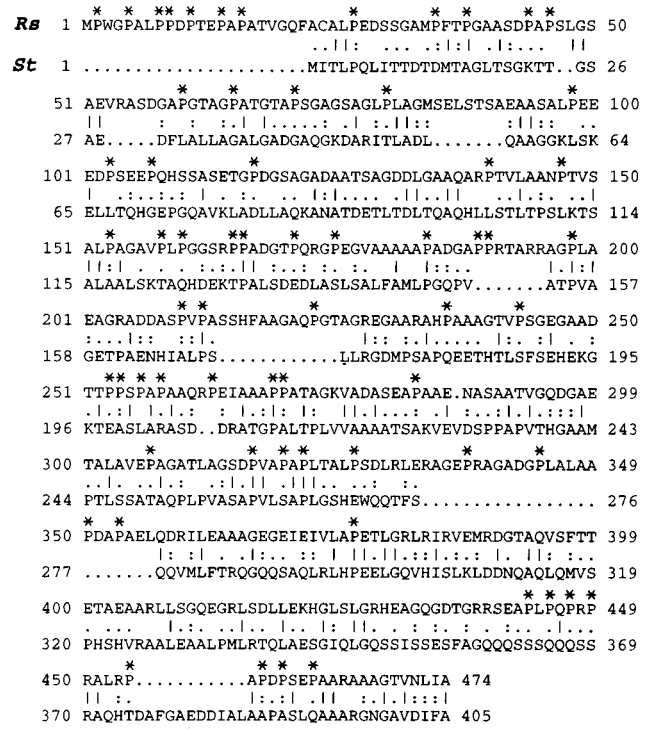

FIG. 7. Comparison of the deduced amino acid sequences of FliK proteins from *R. sphaeroides* (*Rs*) and *S. typhimurium* (*St*). Identical matches (vertical lines) and strongly (colons) and weakly (dots) conserved amino acid residues are indicated. Asterisks highlight the proline residues in the *Rhodobacter* sequence.

Furthermore, unlike the situation in the majority of flagellated bacteria studied to date, the HAPs in *R. sphaeroides* are clearly distinguishable structures. The different patterns in the arrangement of subunits in the hook and filament are also evident in this bacterium (Fig. 2b). The length of the *Rhodobacter* hook, ca. 100 nm, agrees with the observation that monotrichous flagellated bacteria, such as *Campylobacter* and *Caulobacter* spp., possess longer hooks than do multiflagellated bacteria. This feature has previously been related to the need for the greater torque that has to be applied to a single flagellum (43).

A comparison of the N-terminal sequence of the purified FlgE monomer with N-terminal sequences of FlgE proteins from different bacteria revealed that *R. sphaeroides* FlgE contains the conserved SGL motif as well as the hydrophobic heptad repeats that have been described previously (17). These repeats have been suggested to be important for the formation of coiled-coil structures that allow interactions between subunits, which are in turn important for the organization of the axial structure of the flagellum (37). Further sequencing is needed to identify the second, extended GNNIAN N-terminal motif (17) and other features of the protein. We recently cloned the *flgE* gene from *R. sphaeroides* (8), and DNA sequence analysis is in progress.

Identification of the *fliK* gene in *R. sphaeroides*. We cloned and sequenced a DNA fragment that is predicted to encode a protein that is homologous to FliK from *S. typhimurium*. This assumption was made based on the high proline content and the overall sequence similarity between these two proteins. In addition, mutations in *fliK* from *S. typhimurium* and in the *R. sphaeroides* ORF give rise to the same polyhook phenotype. Like FliK, the protein encoded by this ORF is responsible for hook length control.

We compared the C-terminal sequence of this ORF to those of other FliK sequences (Fig. 8) because this region is the most conserved among these proteins. This comparison revealed five highly conserved leucine residues and various conserved glutamine residues, whose functions will be tested.

In a recent study, we identified a 5.3-kb DNA fragment from wild-type *R. sphaeroides* 2.4.1. (7) that contained genes homologous to the *fliF* operon of *S. typhimurium* (*fliFGHIJK*). The orientation of the *Rhodobacter fliK* gene found in this work agrees with the previously described orientation for the *fliH*, *-I*, and *-J* genes from *R. sphaeroides* 2.4.1. and WS8 (7, 14).

We found an ORF upstream of *fliK* which does not correspond to *fliJ* (data not shown). This ORF has also previously been found in *Bacillus subtilis* (3) and *Vibrio parahaemolyticus* (34), but not in *E. coli* or *S. typhimurium*. There is a putative ORF with no obvious consensus promoter sequence downstream of *fliK*, suggesting that *fliK* is not the last gene in its operon.

Proposed models for hook length regulation. The hook must have a precise length in order to efficiently transmit torque to the filament (23). Several models have been proposed to explain the hook length control exerted by FliK (15, 25, 26, 57). These mechanisms involve switching export machinery and the participation of different proteins, i.e., FlhB, FlgM, and RflH. FlhB has previously been proposed to form part of the export apparatus, mediating the ordered export of proteins in response to the state of flagellar assembly (25). FlgM is involved in negative regulation of late gene expression (13, 24) until it is excreted from the cell (18). RflH has been recently proposed to participate with FlhB in negative regulation of FlgM export via a double-locked-gate model (26).

The discovery of a polyhook mutant of *R. sphaeroides* suggests that this bacterium also has export switching machinery that allows the export apparatus to change its substrate spec-

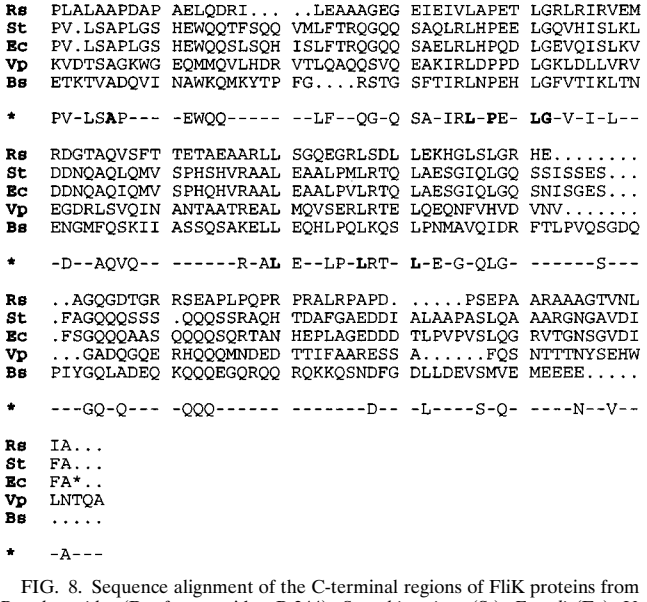

FIG. 8. Sequence alignment of the C-terminal regions of FliK proteins from *R. sphaeroides* (Rs; from residue P-344), *S. typhimurium* (St), *E. coli* (Ec), *V. parahaemolyticus* (Vp), and *B. subtilis* (Bs). The sequence marked with an asterisk represents a consensus in at least three sequences. Residues identical in all five sequences are shown in boldface. The alignment was performed with the PILEUP program (12). Dashes indicate residues that are not conserved in the consensus sequence; dots indicate gaps.

ificity. In addition, the expression of flagellar operons in *R. sphaeroides* should be coupled to flagellar assembly, possibly through an anti-sigma factor regulation mechanism. It must be stressed that *R. sphaeroides* is a uniflagellated bacterium with a straight hook, unidirectional flagellar rotation, and a swimming pattern different from that of enteric bacteria. Thus, it seems that the general mechanisms of flagellar export and regulation of flagellar genes are conserved among distantly related bacteria.

How the state of assembly of the hook is sensed remains to be answered. Research will be directed toward understanding this process.

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