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The Hook Gene (*flgE*) Is Expressed from the *flgBCDEF* Operon in *Rhodobacter sphaeroides*: Study of an *flgE* Mutant

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In this work we identified the *flgE* gene encoding the flagellar hook protein from *Rhodobacter sphaeroides*. Our results show that this gene is part of a flagellar cluster that includes the genes *flgB, flgC, flgD, flgE*, and *flgF*. Two different types of mutants in the *flgE* gene were isolated, and both showed a Fla$^-$ phenotype, indicating the functionality of this sequence. Complementation studies of these mutant strains suggest that *flgE* is included in a single transcriptional unit that starts in *flgB* and ends in *flgF*. In agreement with this possibility, a specific transcript of approximately 3.5 kb was identified by Northern blot. This mRNA is large enough to represent the complete *flgBCDEF* operon. FlgE showed a relatively high proline content; in particular, a region of 12 amino acids near the N terminus, in which four prolines were identified. Cells expressing a mutant FlgE protein lacking this region showed abnormal swimming behavior, and their hooks were curved. These results suggest that this region is involved in the characteristic quaternary structure of the hook of *R. sphaeroides* and also imply that a straight hook, or perhaps the rigidity associated with this feature, is important for an efficient swimming behavior in this bacterium.

Salmonella enterica serovar Typhimurium swims toward favorable environments in response to changes in the surrounding medium using its flagella; these appendages consist basically of a helical filament driven by a rotary motor. When flagella rotate in counterclockwise direction, the filaments coalesce in a bundle that functions as a propeller to push the bacterial cell body in a linear trajectory. On the contrary, when flagella reverse the sense of rotation the bundle is no longer stable, and the uncoordinated movement of each flagellum causes the cell to tumble. As revealed by early studies of electron microscopy, the flagellum consists of a filament, a curved hook, and a basal body (6). The filament and the hook are each composed of repeats of a single protein, flagellin and hook protein, respectively. These polypeptides do not share extensive similarity at the level of its primary sequence but both have the ability to self-assemble, and the resulting structures are capable of displaying polymorphic transitions; this capability has been suggested to be important in the motility of certain species of bacteria (12, 16, 22).

The structure of the filament has been the subject of extensive study during the last few decades, and various structural models have been proposed (18, 21, 33, 37). In contrast, the hook structure has been less well characterized; however, since the hook protein shares important features with flagellin, it has been suggested that hook and flagellin subunits have a similar folding pattern (20, 34, 35, 36).

The detailed knowledge about the structure and function of the flagellum in enterobacteria contrasts strongly with the limited data on these aspects that exist for other bacterial groups. However, it seems clear that as far as structure and function are concerned, a general pattern is shared by a great number of bacteria; some variations of this pattern have been introduced to allow the adaptation of certain bacteria to their particular habitat.

Rhodobacter sphaeroides, is a purple nonsulfur photosynthetic bacterium that swims using a single subpolar flagellum that rotates unidirectionally. This rotation is interrupted periodically by stop events; during these periods, the flagellar helix relaxes and Brownian motion allows changes in the swimming direction (1).

The function and the structure of the flagellum of *R. sphaeroides* show interesting features. For instance, the motor rotates only in one direction; during the stop periods, the flagellar helix progressively relaxes into a coiled form, and the hook is actually a straight structure (1, 8, 29). As far as this last characteristic is concerned, there is no information about the importance of the straightness of the hook in the proper functioning of the flagellum; in addition, it is not known to what extent this structure is capable of suffering polymorphic interconversions. In this regard, the study of the hook of *R. sphaeroides* might give important information about the molecular bases that underlie the straightness of this structure.

Previously, we reported a *fliK* mutant from *R. sphaeroides* having an unusually long hook. This strain showed a similar phenotype to that of a polyhook mutant in *S. enterica* serovar Typhimurium. Purified hooks from the *fliK* mutant were shown to be straight and composed of a single protein, which presumably was the product of the *flgE* gene (8). In this work we identify the *flgE* gene from *R. sphaeroides* as part of a transcriptional unit that includes some genes encoding proteins involved in the basal body formation. Our data suggest that the *flgBCDEF* operon is expressed as a single mRNA, whose expression is dependent on a sigma-54 promoter identified upstream of *flgB*. Regarding the hook structure, we show evi-

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
JM103	*hsdR4* Δ(*lac-pro*) F′ *traD36 proAB lacI*^{q} ZΔM15	3
S17-1	*recA endA thi hsdR* RP4-2-Tc::Mu::Tn*7*; Tp^r Sm^r	27
R. sphaeroides		
WS8	Wild type; spontaneous Nal^r	30
LC1	WS8 *flgE::aadA*; Spc^r Nal^r	This work
TE1	WS8 Tn*phoA* derivative, *flgE*::Tn*phoA*; Km^r Nal^r	This work
Plasmids		
pTZ19R	Cloning vector; Ap^r; pUC derivative	Pharmacia
pWM5	Plasmid carrying the omega-Spc cassette	17
pJQ200mp18	Suicide vector used for gene replacement in gram-negative bacteria	24
pRK415	pRK404 derivative, used for expression in *R. sphaeroides*	13
pU1800	pSUP203 derivative carrying Tn*phoA*; Cm^r Tc^r Km^r	19
pRS2000	2.0-kb fragment carrying *flgE* and *flgE* obtained by PCR, cloned into pRK415	This work
pRS2001	pRK415 carrying the *flgEΔ1* allele	This work
pRS4300	4.3-kb *Pst*I fragment from WS8 cloned into pTZ19R	This work
pRS4303/4303	4.3-kb *Pst*I fragment from pRS4300 subcloned into pRK415/reversed direction.	This work
pRS5600	5.6-kb *Sal*I fragment cloned into pTZ19R containing 4.6-kb of Tn*phoA* plus 1 kb of TE1 DNA flanking the site of transposon insertion; Km^r Ap^r	This work

dence that a region near the N terminus of FlgE that has a high proline content is important in generating the characteristic straight hook as well as for normal swimming. Therefore, we propose that in this bacterium a straight hook, or perhaps its associated rigidity, is required for a proper swimming behavior.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are described in Table 1. The plasmid pRS2000 carrying the *flgE*$^+$ and *flgF*$^+$ genes was constructed by PCR using the following oligonucleotides: 5′-CCAAGCTTCAATGTCGCCGCCGACACCGTC-3′, 5′-GCTCTAGACTCAC TCGGGCGGACGCAGGAG-3′. The first oligonucleotide was primed 30 bp upstream of the initiation codon of *flgE*. A *Hin*dIII recognition site was included at the 5′ end. The second oligonucleotide carried an *Xba*I restriction site at the 5′ end and is complementary to the sequence located near the stop codon of *flgE*. The amplification product was sequenced and cloned in pRK415 under control of the plasmid promoters.

Media and growth conditions. *R. sphaeroides* cell cultures were grown photoheterotrophically in Sistrom medium (28) under continuous illumination at 30°C in either liquid or solid medium. Aerobic growth conditions were achieved in the dark with strong shaking. Motility plates were prepared using 1% tryptone, 0.7% NaCl, and 0.3% Bacto-Agar. Strains of *E. coli* were grown in Luria-Bertani medium (3). When needed, antibiotics were added at the following concentrations: spectinomycin, 10 μg/ml; gentamicin, 30 μg/ml; kanamycin, 25 μg/ml; and tetracycline, 1 μg/ml. For *E. coli*, the following antibiotics were used: ampicillin, 100 μg/ml; tetracycline, 15 μg/ml; gentamicin, 30 μg/ml; and spectinomycin, 100 μg/ml.

Recombinant DNA techniques. The isolation of chromosomal DNA was performed as described elsewhere (3). Plasmid DNA preparations were carried out with Qiagen Mini or Midi Column Plasmid Purification Kits (Qiagen, Inc., Santa Clarita, Calif.). DNA amplification was carried out with *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) and 0.5 μM concentrations of the appropriate oligonucleotides; the reaction was performed for 30 cycles in a GeneAmp PCR system (Perkin-Elmer, Foster City, Calif.). Sequencing was carried out using the Thermosequenase kit (Amersham, Piscataway, N.J.) on single- or double-stranded clones. Southern hybridization was carried out using the PhotoGene system from Life Technologies (Rockville, Md.).

Transposon mutagenesis of *R. sphaeroides*. pU1800 plasmid harboring the transposon Tn*phoA* was mobilized into *R. sphaeroides* WS8 from *E. coli* S-17 by diparental mating (4). Transposon mutants were selected on agar plates containing kanamycin. Single independent colonies were tested for loss of motility by using swarm plate assays. Cells were also analyzed by dark-field microscopy. Ten independent mutants were selected for further characterization. The Tn*phoA* insertion site of each mutant was cloned as a kanamycin-resistant *Sal*I-fragment into pTZ19R. The chromosomal DNA flanking the Tn*phoA* was sequenced.

DNA analysis. Sequences were analyzed and compared with the protein database by using the BLAST server at the National Center for Biotechnology Information (Bethesda, Md.), as well as the Genetics Computer Group software package. Predictions of secondary structure were done using the PSA server of the Biomolecular Engineering Research Center of Boston University.

Electron microscopy. Bacterial cell suspensions were applied on Formvar-coated grids. Samples were negatively stained with 1% uranyl acetate and observed with a JEM-1200EXII electron microscope (Jeol, Tokyo, Japan).

Motility assays. A 5-μl sample of a stationary-phase culture was placed on the surface of swarm plates and incubated aerobically in the dark. The swarming capability was recorded as the ability of bacteria to move away from the inoculation point after 36 to 48 h. The motility of free-swimming bacteria was evaluated in an aliquot from aerobic or anaerobic cultures placed directly between a slide and a coverslip. The samples were observed with an Olympus microscope adapted for high-intensity dark-field illumination or using a Nikon microscope with differential interference contrast (DIC).

Construction of an *flgE::aadA* mutant. To interrupt *flgE* with a selectable marker, we obtained an internal portion of the omega-Spc cassette by PCR using the following oligonucleotides: 5′-GGAATTCCCTGAAGCGAGGGCAGAT C-3′ and 5′-GGAATTCTCATGATATATCTCCCAAT-3′. An *Eco*RI recognition site was included at their 5′ ends. The PCR product obtained using these oligonucleotides excludes the transcriptional termination signals present in the flanking regions of the original cassette. The amplification product was cloned into the unique *Eco*RI site of pRS4303 located after amino acid 40 from the predicted FlgE sequence. The 5.5-kb *Pst*I fragment carrying *flgE*::*aad* was subcloned into pJQ200mp18 (25). The resulting plasmid was introduced by transformation into *E. coli* S17-1 and subsequently transferred to *R. sphaeroides* by conjugation. Since pJQ200 cannot replicate in *R. sphaeroides*, the double recombination event was selected directly on Luria-Bertani (LB) plates in the presence of spectinomycin and 5% sucrose.

Construction of the *flgEΔ1* allele. To obtain the *flgEΔ1* allele, two independent PCRs flanking the region to be deleted were carried out. The region encoding the N terminus of FlgE was amplified in one reaction using the oligonucleotide 5′-CGTCTAGAGAGCGTCACGGCCGCCTCGTC-3′, which primed at the C-terminal of *flgD*. This oligonucleotide also carried an *Xba*I recognition site at the 5′ end. The reverse oligonucleotide used in this reaction primed the sequence located immediately upstream of the deletion start point (5′-GTGACGTCCCC GACGGCGTCCTCGGTGGCGAAG-3′). At the 5′ end of this oligonucleotide, an *Aat*II recognition site was included; the sequence of this site was designed to be in frame with the *flgE* open reading frame (ORF). The PCR product was cloned in pTZ19R and sequenced to confirm that no errors were introduced during the amplification reaction. The other PCR was carried out using the forward oligonucleotide 5′-GTGACGTCATCTACACCCGCGCGGGCGCC-3′, priming the sequence downstream of the end of the deletion site and carrying an *Aat*II recognition site at the 5′ end. The reverse primer (5′-GGGGTACCT CCATGATCCGCCTCAGCTGC-3′) carried a *Kpn*I restriction site at the 5′ end and was complementary to the sequence located near the *flgE* stop codon. The

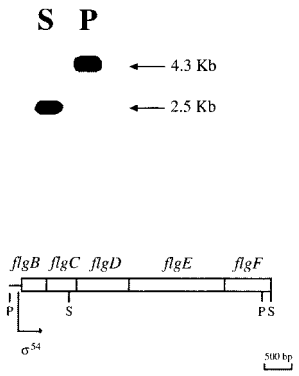

FIG. 1. Genomic Southern blot of wild-type WS8 from *R. sphaeroides*. Chromosomal DNA digested with *Sal*I (S) or *Pst*I (P) was probed against a 1.2-kb *Dra*I-*Sal*I fragment derived from pRS5600 carrying the incomplete *flgE* and *flgF* genes. The organization of the *flg* genes identified in this work, with each ORF represented as an open box, is presented graphically below the blots. The restriction enzyme sites shown are *Sal*I (S) and *Pst*I (P). An arrow indicates the direction of transcription. A sequence similar to that of the sigma-54 consensus promoter located upstream the coding region of *flgB* is also indicated (σ^{54}).

product of this reaction was also cloned in pTZ19R and sequenced. To join these fragments, the plasmid carrying the sequence corresponding to the 5′ end of the *flgE* gene was digested with *Xba*I and *Aat*II, and the released insert was gel purified and subsequently cloned in the plasmid carrying the 3′ end of *flgE*, previously digested with *Xba*I and *Aat*II. The resultant plasmid carrying the complete *flgEΔ1* allele was sequenced at the junction point to confirm the correct insertion.

RNA isolation and Northern blot. Total RNA was isolated from *R. sphaeroides* cells grown heterotrophically as described previously (3). For Northern blots, 20 μg of the sample was separated electrophoretically on a 1% agarose-formaldehyde gel. RNA was transferred onto a nitrocellulose membrane with a pore size of 0.45 μm and then UV cross-linked to the filter by exposure to 120 J of UV irradiation. Filters were hybridized with denatured DNA probes for at least 18 h. The DNA probe was labeled with [α-^{32}P]dCTP by use of random primers using a commercial kit purchased to GIBCO-BRL.

Nucleotide sequence accession number. The DNA sequences of the *R. sphaeroides flgB, flgC, flgD, flgE*, and *flgF* genes have been deposited in GenBank under accession number AF133240.

RESULTS

Isolation of a strain carrying the allele *flgE*::Tn*phoA*. To identify the *flgE* gene from *R. sphaeroides*, we screened a bank of mutants unable to swim. This bank was obtained by transposon mutagenesis of the wild-type strain WS8. Since the transposon, Tn*phoA*, carries the *npt*II gene conferring kanamycin resistance; after mutagenesis, individual colonies were selected on LB-kanamycin plates. The colonies obtained were subsequently tested for motility on swarm plates. A set of 10 independent nonmotile mutants was isolated.

In order to identify the insertion point of the transposon in each mutant, chromosomal DNA was purified and digested with *Sal*I. A DNA fragment carrying the *npt*II gene from the transposon, as well as chromosomal DNA adjacent to the insertion site was cloned in pTZ19R. The sequence from these clones was determined and compared against the database from the National Center for Biotechnology Information.

The sequence obtained from the fragment derived from one of these mutants, the TE1 mutant, was found to be similar to the 3′ end of *flgE* and to the 5′ end of *flgF* from several bacterial species. This clone was named pRS5600.

Cloning of the wild-type *flgE* gene. To clone a DNA fragment carrying the complete *flgE* gene, chromosomal DNA digested with either *Sal*I or *Pst*I was hybridized with a 1.2-kb *Dra*I-*Sal*I fragment from pRS5600. A 4.3-kb *Pst*I fragment and a 2.5-kb *Sal*I fragment were clearly detected (Fig. 1). The 4.3-kb *Pst*I fragment was cloned in pTZ19R, this clone was named pRS4300. The complete sequence of this fragment revealed the presence of five ORFs that showed strong similarity to *flgB, flgC, flgD, flgE*, and *flgF* genes identified in other bacterial species (Fig. 1). Since this fragment carried the *flgF* gene truncated by the *Pst*I site, the sequence was completed using an overlapping *Sal*I fragment, carrying the *flgF* stop codon besides other flagellar genes in process to be characterized (B. González-Pedrajo, J. De la Mora, T. Ballado, L. Camarena, and G. Dreyfus, Genbank accession no. AF205139).

All of these ORFs start with the most common triplet ATG and end with TGA. The start and stop codons for these ORFs are almost contiguous, suggesting that they conform to a single transcriptional unit. In accordance with this possibility, the sequence analysis of the complete fragment does not show any potential coding region in the first 260 nucleotides; instead, a sequence similar to that of the σ^{54} consensus promoter was identified (TGGCAN$_6$TTGCA).

The amino acid sequence predicted for these five ORFs was compared with their counterparts present in other bacteria. As it can be observed in Table 2, the highest degree of identity was obtained when these ORFs were compared to those from *S. enterica* serovar Typhimurium.

The N(I/L)AN motif was found in the N terminus from all the predicted amino acid sequences with the exception of FlgD. An additional **GF(R/K)** motif shared only by FlgE and FlgF was also identified (Fig. 2). In the C terminus a EE(M/L)VX$_8$(Y/F) motif was found in FlgC, FlgE, and FlgF, which are all axial proteins (Fig. 2). A sequence that weakly resembles this consensus can also be detected in FlgB, whereas FlgD does not show any similarity to these axial proteins. Homma et al. (10, 11) have suggested that these conserved characteristics could play an important role in quaternary interactions that take place during assembly.

The tendency to form coiled-coil structures was calculated using the algorithm of Lupas et al. (15); except for FlgD, all of these regions predicted a propensity to form coiled coils. The strongest probability to form coiled coils was predicted for the C termini of FlgB, FlgC, and FlgE.

Characterization of the mutant strain TE1. From the selection procedure used to isolate the TE1 mutant, we learned that this mutant is unable to form a swarm ring on soft agar plates

TABLE 2. Comparison of encoded proteins of *R. sphaeroides* flagellar genes with those of other bacterial species

Gene product	% Similarity/% identity[a]			
	B. burgdorferi	Serovar Typhimurium[b]	*S. meliloti*	*A. tumefaciens*
FlgB	26.9/20.6	50.7/41.4	29.3/22.2	27.5/20.4
FlgC	38.6/27.7	46.2/37.3	36.7/27.2	33/26.4
FlgD	31.1/26	37.1/33	–	–
FlgE	35.9/28.9	38.8/32.1	37.0/29.1	–
FlgF	–	37.9/28.9	34.6/26.3	32.5/24.6

[a] –, Not available in the data base.
[b] *S. enterica* Serovar Typhimurium.

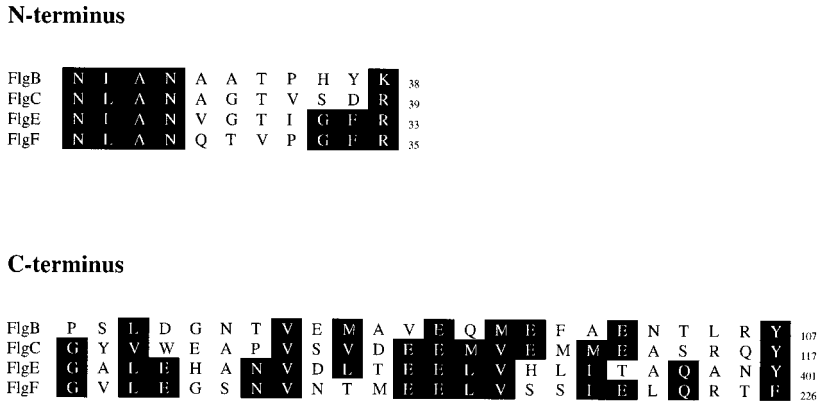

FIG. 2. Comparison of the N-terminal and C-terminal regions of axial proteins identified in this work. Conserved residues in the sequences are shaded.

(Fig. 3A). Also, according to the results obtained from the sequence analysis, we anticipated a Fla$^-$ phenotype for this mutant. The observation of TE1 cells by electron microscopy confirmed this expectation (data not shown). To recover TE1 cell motility, the 4.3-kb *Pst*I fragment was cloned in a direct or reverse orientation from the pRK415 promoters; the resulting plasmids, pRS4302 or pRS4303, respectively, were both unable to complement TE1 cells (Fig. 3A). This result can be ascribed to the polar effect that the transposon exerts on the expression of the genes located downstream of *flgE*.

Further studies showed that TE1 cells could be complemented using a DNA fragment carrying only *flgE*$^+$ and *flgF*$^+$ genes. This fragment was obtained from a specific PCR reaction, whose product was cloned into pRK415 (pRS2000). As expected, motility was recovered when the fragment was expressed from the promoters of pRK415 (Fig. 3A). This result supports the idea that *flgF* might be the last gene from the putative operon. We did not detect any possible hairpin structure corresponding with a hypothetical mRNA terminator downstream of *flgF*. However, the possibility remains that a ρ-dependent terminator could accomplish this function.

Isolation of a strain carrying a nonpolar mutation in *flgE*. To confirm that the inability of the pRS4302 plasmid to complement the TE1 mutant was due to the polar effect of the Tn*phoA* insertion, we decided to isolate a nonpolar *flgE* mutant. For this purpose, the omega cartridge was chosen to interrupt the coding region of *flgE*. As has been reported previously (17), this cassette carries strong terminator signals flanking the resistance marker; therefore, we removed these regions by PCR amplification. The PCR product was cloned in the *Eco*RI site present in the first half of *flgE*. The allele *flgE*::*aadA* was transferred to the chromosome of the wild-type strain by homologous recombination (see Materials and Methods). A mutant was isolated, and it was confirmed by Southern blotting that a double-recombination event occurred in this strain (data not shown). This mutant was named LC1 and, as expected, it showed Fla$^-$ phenotype (data not shown) and is unable to form a ring in swarm plates (Fig. 3B).

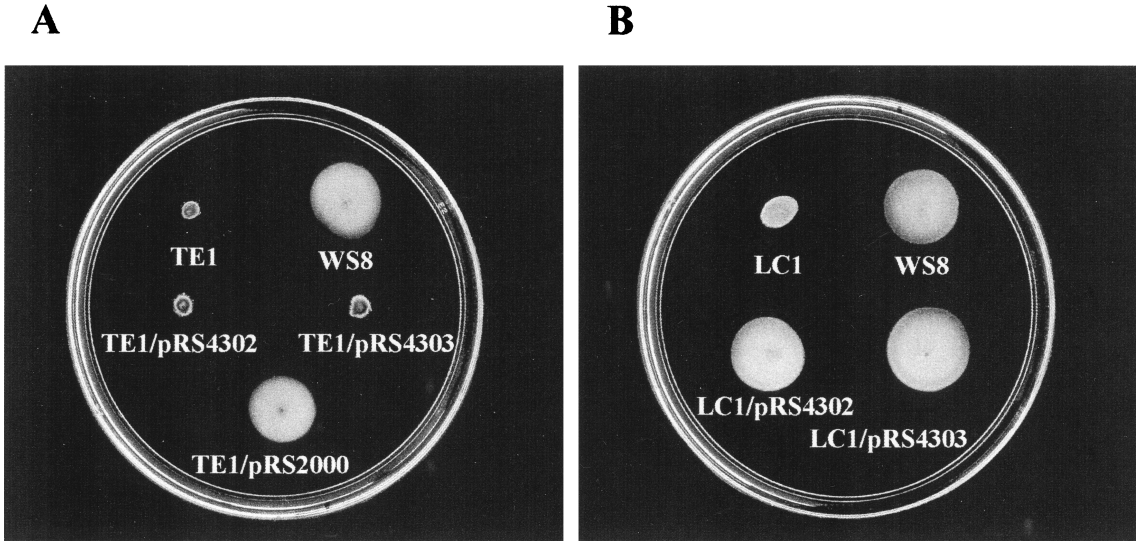

FIG. 3. (A) Swarming assay of wild-type WS8, TE1 strain, and TE1 carrying different plasmids. (B) Swarming assay of wild-type WS8, LC1 strain, and LC1 complemented with pRS4302 and pRS4303.

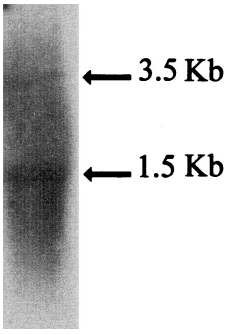

FIG. 4. Northern blot analysis of *flgE* transcripts. Total RNA extracted from WS8 was probed with a ^{32}P-labeled *flgE* fragment as described in Materials and Methods.

The pRS4302 and pRS4303 plasmids, which had failed to complement TE1 cells, were able to promote full motility in LC1 cells (Fig. 3B). The fact that the 4.3-kb *Pst*I fragment was able to complement LC1 cells independently of the pRK415 promoters allows us to suggest that a promoter responsible for *flgE* expression is present in this fragment.

Characterization of the *flgBCDEF* operon. To obtain physical evidence supporting the hypothesis that the *flgB, flgC, flgD, flgE*, and *flgF* genes form an operon, we carried out a Northern blot hybridization using total RNA isolated from WS8 cells and an internal *flgE* fragment as a probe. Surprisingly, two different transcripts were detected (Fig. 4). The larger and less-abundant transcript (3.5 kb) is long enough to represent the complete mRNA of the *flgBCDEF* operon. The presence of this mRNA strongly supports the idea that *flgE* is expressed as a part of this operon. The smaller mRNA (1.5 kb) may represent specific cleavage of the complete transcript and/or an independent mRNA population expressed from an internal promoter within this transcriptional unit. This result will be further discussed in the next section.

Analysis of the FlgE sequence. The conceptual translation of the *flgE* gene predicts a product of 42,660 Da. The N terminus of FlgE from *R. sphaeroides* ($FlgE_{Rs}$) shows the sequences LSGL and NIANXXTXXGFR that are conserved in all of the FlgE proteins known so far (data not shown). An interesting feature of $FlgE_{Rs}$ that contrasts with FlgE from *S. enterica* serovar Typhimurium ($FlgE_{Se}$) is the presence of two short insertions of 7 and 6 amino acids that are located after amino acids 46 and 90, respectively, from the sequence of *Salmonella* sp. (Fig. 5A). This last insertion, together with the four preceding amino acids and the two amino acids following it, presents an unusually high content of proline residues, i.e., 4 of 12 amino acids are prolines. In fact, $FlgE_{Rs}$ shows a 5.4% content of proline residues in contrast to 2.9 and 2.5% observed for FlgE proteins from *Salmonella* sp. and *Sinorhizobium meliloti*, respectively. These proline residues are distributed throughout the $FlgE_{Rs}$ sequence and are not only confined to the variable region, which is located between residues 148 to 260 from $FlgE_{Se}$ (34).

To investigate whether this proline-rich region located near the N terminus of $FlgE_{Rs}$ was in some way required for proper function of the hook, we deleted and modified this region, as depicted in Fig. 5B, to yield *flgEΔ1*. This allele was cloned in pRK415 under control of the vector promoters (pRS2001 plasmid) and then transferred to LC1 cells (*flgE*::*aadA*) by conjugation. When free-swimming cells were analyzed directly under the microscope, we observed that most of the exconjugants showed an atypical swimming behavior; illustrated in Fig. 6A. The swimming paths of the mutant cells compared with those of the wild-type cells show a corkscrew trajectory that contrasts with the slightly curved trajectory of the wild-type cells. In a swarm plate, the cells expressing FlgEΔ1 showed a slight re-

FIG. 5. (A) Sequence alignment of the FlgE proteins from *R. sphaeroides* and *S. enterica* serovar Typhimurium starting from residue number 39 and extending up to residue 110 from $FlgE_{Rs}$. The residues that are similar were shaded. The sequence from *R. sphaeroides* shows two insertion sites that are absent in *S. enterica* serovar Typhimurium FlgE. (B) The region of FlgE was modified to obtain $FlgE_{Rs}\Delta1$. The residues GFF are placed in an alignment equivalent to those in panel A in order to facilitate its comparison. The amino acid sequences of $FlgE_{Rs}$ and $FlgE_{Se}$ are included.

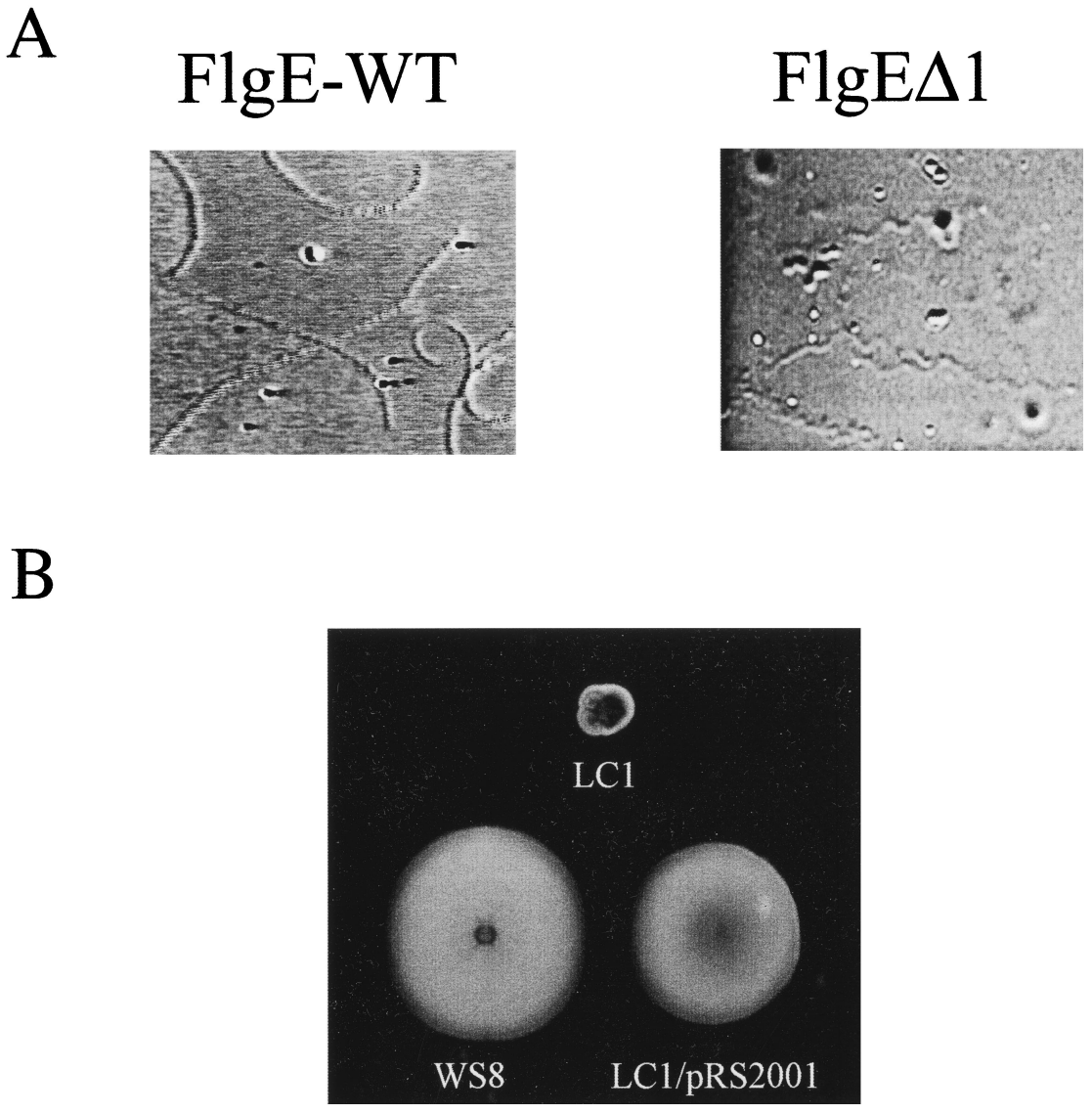

FIG. 6. (A) Swimming paths of mutant cells expressing FlgEΔ1 protein and of wild-type cells. The paths were obtained using Adobe Photoshop v. 4. The images were obtained from individual frames imported from videotapes. (B) Swarming assays of wild-type WS8, LC1 strain, and LC1/pRS2001 expressing FlgEΔ1 protein.

duction (ca. 15 to 20%) in the ring size compared with that produced by wild-type cells (Fig. 6B). To determine if the quaternary structure of the hook was conserved in the mutant cells, sheared filaments from a culture of LC1/pRS2001 cells were analyzed by electron microscopy. A large amount of filament fragments were detected, some of them had the hook still attached. We observed that in contrast with the straight structure of the wild-type hooks, most of the hooks assembled with FlgEΔ1 were curved but conserved the distinctive cross-hatched helical pattern characteristic of this structure (Fig. 7A). As a control, the same procedure was done for wild-type cells, and only straight hooks were detected (Fig. 7B).

DISCUSSION

In this work we identified the gene that encodes the hook protein from *R. sphaeroides*, as well as the genes encoding for the proximal rod proteins FlgB, FlgC, and FlgF and the scaffolding protein FlgD.

In addition, we showed three lines of evidence supporting the idea that *flgE*, together with the other four *flg* genes reported in this work, form an operon. First, the fact that TE1 cells (*flgE*::Tn*phoA*) were complemented with a plasmid carrying both *flgE*$^+$ and *flgF*$^+$ genes, whereas LC1 cells (*flgE*::*aadA*) were complemented with only the *flgE*$^+$ gene, suggests that the insertion of Tn*phoA* in *flgE* exerts a polar effect on the expression of the genes located downstream. Therefore, *flgF* must be the last gene of this operon. Second, since LC1 cells were complemented with the 4.3-kb *Pst*I fragment independently of the vector promoters, the presence of a promoter responsible for the expression of *flgE* was suggested. We ascribed this promoter function to the first 260-bp of this fragment because a sequence similar to the σ^{54} consensus promoter was identified. In fact, we recently showed evidence that

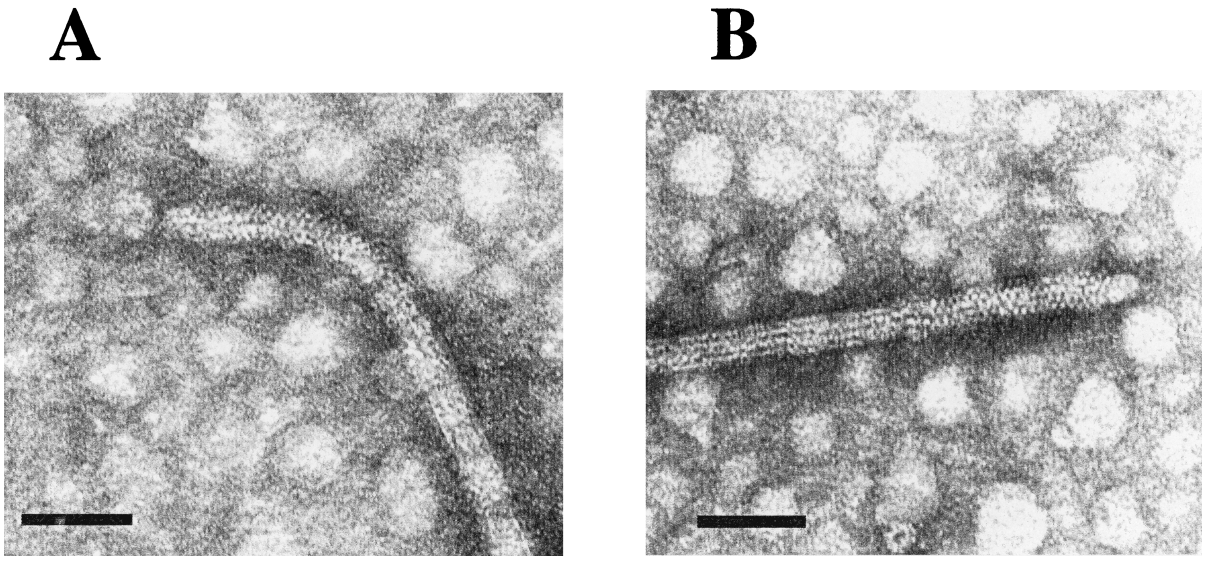

FIG. 7. Negatively stained electron micrographs of hook structures. (A) Sample from cells expressing FlgEΔ1 protein. (B) Sample from wild-type cells. Bar, 50 nm.

this sequence is indeed a functional σ^{54} promoter (23). Finally, in a Northern blot experiment using a fragment from the *flgE* gene as a probe, we found a 3.5-kb mRNA. The detection of this large mRNA supports the idea that *flgE* is transcribed as part of a polycistronic messenger. The presence of an additional 1.5-kb mRNA on the Northern blot suggests the possibility that an internal promoter is also used to express *flgE*. Alternatively, this small mRNA could be produced by cleavage of the 3.4-kb transcripts; this possibility is supported by the fact that the 1.5-kb mRNA is the strongest signal on the blot; however, no evidence of a consensus promoter sequence was found within the genes located upstream of *flgE* (data not shown). The processing of large mRNAs has been already reported for the *puc* and the *puf* messengers in *R. capsulatus* and *R. sphaeroides*. This process seems to control the stoichiometry of the structural proteins encoded by the primary transcripts of these operons (9, 14, 38). It remains to be investigated if specific mRNA cleavage might occur in the *flgBCDEF* transcript of *R. sphaeroides*.

The results summarized above allow us to propose that *flgBCDEF* genes are transcribed as an mRNA of 3.5 kb. This transcript seems to be synthesized from the σ^{54}-dependent promoter located upstream of *flgB* and ends downstream of *flgF*.

As shown in Table 2, FlgB and FlgC proteins showed the highest homology value when compared with their counterparts from enteric bacteria, such as *S. enterica* serovar Typhimurium (γ subclass) In contrast, a low value was found when they were compared with their counterparts found in α subclass of *Proteobacteria*, such as *Agrobacterium tumefaciens* and *S. meliloti*. Since *R. sphaeroides* also belongs to the α subclass of *Proteobacteria*, this result was unexpected. By the same token, we noticed that the order of the genes *flgBCDEF* in *R. sphaeroides* is similar to that found in these enteric bacteria, whereas *S. meliloti* and *A. tumefaciens* show a different gene order, which is similar between them (5, 31). We believe that an α-proteobacterium may exist that has a high degree of similarity to FlgB and FlgC from *R. sphaeroides* and that could perhaps show the same gene order.

We observed that, regarding the hook structure, the primary structure of $FlgE_{Rs}$ is highly similar to that of $FlgE_{Se}$; however, as mentioned in the previous section, $FlgE_{Rs}$ shows a high proline content, in particular in two small insertions located near the N-terminal region of this protein. Since it has been reported that proline residues are involved in the structural rigidity in some proteins (32), we removed one of these insertions in order to investigate its role in the structure of the hook.

The hook assembled with FlgEΔ1 subunits was observed as a curved structure. This result indicates that this region does not seem to be important for the export or assembly of FlgE monomers; instead, it seems to be involved in the straightness of the hook structure. The possibility that artifacts were introduced when samples were prepared was discarded since under the same conditions of temperature and pH the samples from wild-type cells showed straight hooks.

In *R. sphaeroides*, the filament of free-swimming cells shows polymorphic transitions; two polymorphs, coiled and helical, have been associated with periods of stop and swimming respectively (1). A third waveform has been recently reported (2), in this case, when the flagellum rotated rapidly a straight filament or perhaps a low-amplitude helix was identified. In addition, the filament also suffers polymorphic interconversions in vitro, when the pH or the ionic strength is changed (26). For *E. coli*, a strain carrying a point mutation in the *flgL* gene, encoding the HAP3 protein, is unable to form swarm rings in 0.28% agar. In this condition, the filaments undergo torque-induced transformations to straight forms that impair motility (7). Therefore, the ability to control flagellar transitions seems to be important for proper flagellar function. It will be interesting to study whether the filament of the strain expressing the FlgEΔ1 protein undergoes abnormal transitions during swimming.

A mutant strain producing straight hooks in *Salmonella* has been reported. In this strain, the export of FlgM was depen-

dent on the NaCl concentration. Observation of the swimming behavior of this strain at a concentration of NaCl allowing good flagellation suggested that the function of the hook had deteriorated, since the flagellar bundles were not as tight as those of the wild-type cells (25). In the case of *R. sphaeroides*, a strain showing a curved hook produced normal amounts of flagellin, but the swimming behavior both in swarm plates as well as in liquid medium was altered. Although it is not possible to be certain that this anomalous behavior is directly related to the presence of a curved hook; it seems clear that this atypical swimming behavior is the consequence of the deletion of the proline-rich region from the N terminus of FlgE. This mutation, in addition to causing the assembly of a curved hook, could also affect the hook intrinsic flexibility or its capability to correctly transmit torque. We believe that the study of hook shape mutants in a uniflagellated bacterium may help clarify the role of the hook (the so-called "universal joint") in the swimming action of this microorganism.

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T.B. and L.C. contributed equally to this work.

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