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Isolation, Characterization, and Complementation of a Paralyzed Flagellar Mutant of *Rhodobacter sphaeroides* WS8

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A paralyzed *Rhodobacter sphaeroides* mutant strain (PARA1) was isolated by a motility screening procedure following mutagenesis of wild-type *R. sphaeroides* WS8-N with the transposable element Tn*phoA* (Tn*5* IS*50*$_L$::*phoA*). PARA1 synthesized a wild-type level of flagellin, as detected by Western immunoblotting with antiflagellar antiserum. Flagellar staining showed that flagellin was assembled into apparently normal external flagellar filaments. Electron micrographs of basal body structures from PARA1 showed that some ring structures that were present were similar to those in wild-type *R. sphaeroides* WS8-N. PARA1 cells were nonmotile under all growth conditions. No pseudorevertants to motility were seen when PARA1 was grown in the presence of kanamycin to select for the presence of the transposon. The presence of the single copy of Tn*phoA* in the PARA1 chromosome was demonstrated by Southern blotting. Western blotting of cytoplasmic, periplasmic, and membrane fractions of PARA1 with anti-alkaline phosphatase antiserum showed that the transposon had been inserted in-frame into a gene encoding a membrane protein. A *SalI* restriction endonuclease fragment was cloned from the chromosome of PARA1; this fragment contained a portion of the transposon and *R. sphaeroides* DNA sequence 5′ of the site of insertion. This flanking *R. sphaeroides* DNA sequence was used to probe an *R. sphaeroides* WS8 cosmid library. A cosmid designated c19 hybridized to the probe, and a *SalI* restriction endonuclease fragment derived from this cosmid restored wild-type motility to PARA1 when introduced into this mutant strain by conjugation. The significance of this finding in a bacterium with unidirectionally rotating flagella is discussed.

The purple, nonsulfur bacterium *Rhodobacter sphaeroides* has a single lateral flagellum which rotates clockwise, powering the organism at swimming speeds of up to 100 μm s^{-1} (2). *R. sphaeroides* grows chemoheterotrophically, phototheterotrophically, and anaerobically in the dark using, for example, dimethyl sulfoxide as a terminal electron acceptor (7) and is motile under all of these growth conditions. Unlike most other bacterial species, *R. sphaeroides* moves in a series of runs and stops, being reoriented by Brownian motion during stops (2). Flagellar rotation in this and other flagellate bacteria is thought to be caused by the interaction of ions, likely to be protons, with some component of the flagellar basal complex (13). The mechanism involved in converting the electrochemical ion gradient into rotational motion in the flagellar motor is currently unknown.

The swim-stop-swim behavior of *R. sphaeroides* is modulated in response to tactic stimuli, such as changes in light intensity, oxygen tension, or concentrations of particular chemicals (18, 19). The net effect is a change in flagellar rotation speed and stopping frequency, resulting in a net migration of *R. sphaeroides* toward favorable stimuli. *R. sphaeroides* does not have the methyl-accepting chemotaxis system (19) which facilitates the motility response to stimuli in many other bacteria, such as *Escherichia coli*, with bidirectionally rotating flagella (9). Results from our laboratory suggest that the change in flagellar behavior in *R. sphaeroides* in response to different tactic stimuli is caused by a metabolic signal (18). This has also been suggested for some tactic responses in *E. coli* (11).

Flagellar action and its modulation in *R. sphaeroides* therefore provide a system with which to investigate flagellar rotation and its attenuation without the additional complexity of a change in the direction of rotation, which is seen in other bacteria. A characterization of flagellar components involved in rotation also can lead to an understanding of how metabolites can influence flagellar rotational behavior in the absence of a dedicated multiprotein methyl-accepting chemotaxis system.

We have undertaken the characterization of the nature of proteins involved in the high-speed rotation of the *R. sphaeroides* flagellar motor within the cytoplasmic membrane. To this end, we used transposon mutagenesis coupled with screening for motility to isolate nonmotile mutants and to identify the defective gene in these mutants by complementation with wild-type DNA. We report the isolation of a paralyzed mutant of *R. sphaeroides* (PARA1) defective in a membrane protein and the isolation of a wild-type gene which restores motility in *trans* to PARA1. This is the first report of a motility lesion in *R. sphaeroides*, a bacterium with unidirectionally rotating flagella. The isolation of the wild-type gene and the characterization of the protein it encodes open the way for studies on the mechanism of flagellar rotation and its stopping and starting and for comparative studies on the mechanisms of flagellar rotation in all bacteria.

MATERIALS AND METHODS

Bacterial strains and growth. The bacterial strains and plasmids used in this work are described in Table 1. *R. sphaeroides* strains were grown in Sistrom's medium A as previously described (12); anaerobic photosynthetic growth was carried out in a 3% CO_2–97% N_2 atmosphere in an

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Source or reference
Strains		
E. coli S17-1	Pro$^-$ Res$^-$ *recA*; integrated plasmid RP4-Tc::Mu-Kn::Tn*7*	5
R. sphaeroides WS8-N	Wild type; spontaneous Nal^r	21
PARA1	WS8-N Tn*phoA* derivative; nonmotile; Kn^r; Nal^r	This study
Plasmids		
pUI800	pSUP203 derivative carrying Tn*phoA*; Cm^r; Tc^r; Kn^r	17
pKN2	3.3-kb internal *Hin*dIII fragment of Tn*5* carrying Kn^r cloned into pBR322; Kn^r; Ap^r	C. N. Hunter
c19	pLA2917 derivative carrying 20 kb of *R. sphaeroides* WS8 DNA, which restores motility to PARA1; Kn^r	This study
pRES1	9.6-kb *Sal*I fragment cloned into pUC18 containing 4.6-kb Tn*phoA* Inc ISL and *phoA* plus 5 kb of PARA1 DNA flanking the site of transposon insertion; Kn^r; Amp^r	This study
pRES23a	5.5-kb *Sal*I fragment from c19 cloned into pRK415-1; Tc^r	This study
pRES23b	Like pRES23a, with the fragment in the opposite orientation in pRK415-1; Tc^r	This study
pRES26	3-kb *Sal*I-*Bgl*II subclone from pRES23a in pRK415-1; Tc^r	This study
pRES27	2-kb *Sal*I-*Eco*RV subclone from pRES26 in pRK415-1; Tc^r	This study

illuminated anaerobic cabinet (Don Whitely Mark III). Antibiotic-resistant strains of *R. sphaeroides* and *E. coli* were grown as described previously (5, 20).

Genetic and molecular biology techniques. Tn*phoA* DNA was mobilized into *R. sphaeroides* WS8-N from the donor *E. coli* S17-1 on a suicide plasmid by diparental conjugation (17). Minipreparations of plasmid DNA for screening were made by the alkaline lysis technique (15). Total *R. sphaeroides* DNA and highly purified plasmid DNA were prepared as previously described (6). The *R. sphaeroides* cosmid gene library was prepared by the method of S. C. Dryden and S. Kaplan (cited in reference 17). In this procedure, partial digestion of *R. sphaeroides* WS8 total DNA was performed with the enzyme *Mbo*I to yield 15- to 25-kb restriction fragments which were ligated into the broad-host-range cosmid vector pLA2917 (1). The cosmids were packaged into lambda heads with a commercial packaging system (Stratagene Gigapack) and used to infect *E. coli* S17-1. Recombinant cosmids were then introduced into *R. sphaeroides* strains by conjugation from *E. coli* S17-1. Conjugation was also used to transfer cosmid subclones in plasmid pRK415-1 (10) into *R. sphaeroides* strains in complementation experiments. Restriction digestion and labeling of DNA were performed with enzymes from Bethesda Research Laboratories in accordance with the manufacturer's protocols. Southern hybridization was carried out as previously described (20); [α-^{32}P]dCTP used for DNA probe labeling was obtained from Amersham International, as was the Hybond-C used to immobilize the DNA for hybridization.

Motility analysis. Tryptone-yeast extract swarm plates (3) used for the initial screening of putative mutants were composed of 0.03% (wt/vol) Bacto-Peptone, 0.03% (wt/vol) yeast extract, and 0.3% (wt/vol) Bacto-Agar (Difco Laboratories). Colonies from solid-agar plates were stabbed into the swarm plates, incubated aerobically for 48 h at 30°C, and examined for swarming of the bacteria away from the site of inoculation. Photosynthetically grown liquid cultures were examined by phase-contrast microscopy for motility.

Western immunoblot analysis. *R. sphaeroides* cell lysates were prepared as previously described, with the omission of the 200,000 × *g* centrifugation step (17, 20). *R. sphaeroides* cells were fractionated into cytoplasmic, periplasmic, and membrane preparations by the method of Tai and Kaplan (22). Protein was determined by the Lowry method, as modified by Markwell et al. (16). Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 12% slab gels, and polypeptides were transferred to Hybond-C with a Bio-Rad Transblot cell (20). Western blots were incubated with polyclonal antisera raised against purified flagellar filaments by the method of Block and coworkers (4). In addition, a polyclonal antiserum against *E. coli* alkaline phosphatase (17) (a gift from T. J. Donohue) was used to look for an in-frame fusion of Tn*phoA* into a protein in strain PARA1. Visualization of antibody-antigen complexes was achieved with goat anti-rabbit secondary antiserum conjugated to alkaline phosphatase and with the Nitro Blue Tetrazolium–5-bromo-4-chloro-3-indolyl phosphate indicator system (Sigma Chemical Co.).

Preparation of "intact" *R. sphaeroides* flagella. The method of Hahnenberger and Shapiro (8) was used to isolate flagella with the basal bodies intact. Flagella from PARA1 cells were compared with those from WS8-N cells. The preparations were negatively stained and examined by electron microscopy.

RESULTS

Isolation of PARA1. PARA1 was isolated at a frequency of 7×10^{-4} as a kanamycin-resistant colony which failed to form a diffuse swarm on a semisolid tryptone-yeast extract plate. Examination of PARA1 cells by phase-contrast microscopy revealed this culture to be nonmotile. Western blot analysis of total cell protein of PARA1 probed with antiflagellar antiserum revealed the presence of a protein of 55 kDa which cross-reacted with the antiflagellar antiserum. This molecular mass was identical to that for a wild-type WS8-N control (Fig. 1a). To determine whether the flagellin present was in the form of monomers or was part of an assembled flagellar structure, we prepared whole cells and intact flagella from PARA1 and wild-type WS8-N and examined them as negatively stained preparations under the electron microscope. Flagellar filaments could clearly be seen protruding from the cells, and hook and ring structures could be identified in the flagellar preparations (Fig. 2). Fewer intact basal ring structures were isolated from PARA1 than from wild-type WS8-N, and comparisons between those of the wild type and the mutant seemed to suggest that the mushroom-shaped M ring most distal from the filament was missing from PARA1 flagella.

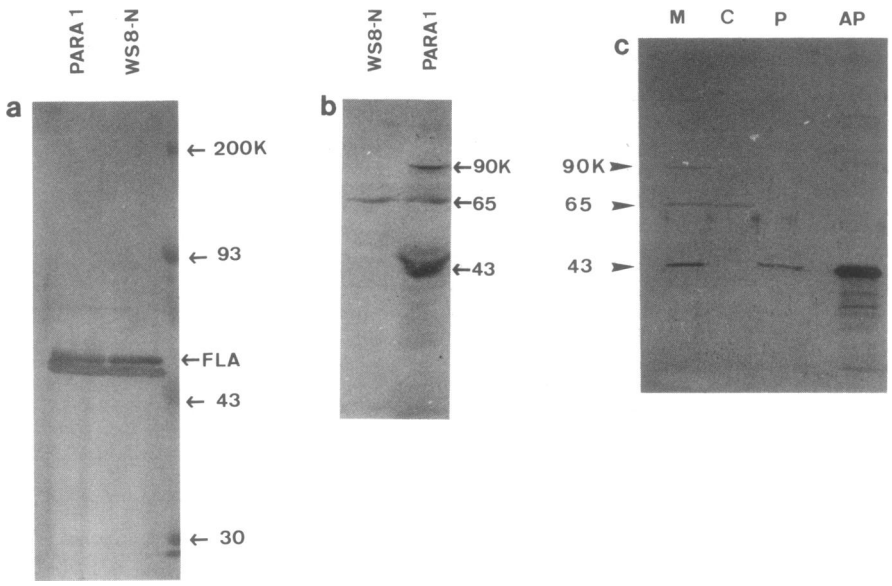

FIG. 1. (a) Western blot of whole-cell lysates from equal numbers of PARA1 and WS8-N cells probed with antiflagellar antiserum. The upper, cross-reacting band of 55 kDa (marked FLA) represents the flagellin. (The lower, cross-reacting band has no flagellar association, as it is present in nonflagellated cells also.) (b) Western blot of whole-cell lysates from equal numbers of PARA1 and WS8-N cells probed with anti-alkaline phosphatase antiserum. (c) Western blot of membrane (M), cytoplasmic (C), and periplasmic (P) extracts from PARA1 and purified alkaline phosphatase (AP) (Sigma) probed with anti-alkaline phosphatase antiserum. K, Kilodaltons.

Studies on the site of the Tn*phoA* insertion. (i) Western blotting. Western blotting against total cell lysates from PARA1 and WS8-N with anti-alkaline phosphatase antiserum (Fig. 1b) revealed that there were three immunoreactive protein bands in PARA1 of 43, 65, and 90 kDa. One of these, the 65-kDa protein, was also seen in extracts of wild-type WS8-N cells, suggesting that it may represent an endogenous *R. sphaeroides* phosphatase. The 43- and 90-kDa proteins were, however, unique to PARA1, showing that alkaline phosphatase was being made in this strain. Western blotting with anti-PhoA antiserum against PARA1 cell fractions revealed that the 90-kDa cross-reacting protein band was derived from the membrane fraction (Fig. 1c).

(ii) Southern blotting. The transposon was localized in the chromosome of PARA1 by Southern blotting with pKN2 (Table 1) as a probe. This probe hybridized to bands of 5.2 and 4.0 kb in *Bam*HI restriction endonuclease digests of PARA1 chromosomal DNA (Fig. 3a). There is a single site for *Bam*HI in transposon Tn*phoA*, so the two bands represented the distance in kilobases from that *Bam*HI site to the next *Bam*HI sites in the PARA1 DNA 5′ and 3′ of the site of insertion of the transposon. This result confirmed that there had been a single insertion of the transposon into the chromosome in PARA1.

Cloning of the wild-type motility gene. DNA upstream of the site of insertion of Tn*phoA* in PARA1 was cloned into pUC18 (23) as a 9.6-kb *Sal*I restriction fragment from total PARA1 DNA to yield plasmid pRES1. This clone contained the Kn^r and *phoA* genes along with *R. sphaeroides* DNA upstream of the *phoA* gene. DNA prepared from plasmid pRES1 was subjected to restriction analysis. A 5.0-kb fragment of *R. sphaeroides* DNA was isolated from this clone and used in Southern hybridization to probe the *R. sphaeroides* WS8 genomic cosmid library. One cosmid, c19, hybridized to this probe. This cosmid clone was conjugated into PARA1, and exconjugants were examined for motility. Motility was restored to PARA1 by cosmid c19. Highly purified DNA from this cosmid clone was prepared and subjected to further restriction analysis and Southern hybridization with the pRES1 probe. A 5.5-kb *Sal*I restriction fragment from cosmid c19 which hybridized to the probe was identified. This fragment was cloned into pUC18, and the site of insertion of the transposon into this fragment in the PARA1 chromosome was confirmed by restriction map comparisons with clone pRES1. The 5.5-kb *Sal*I restriction fragment was introduced into PARA1 as a clone (pRES23a) in plasmid pRK415-1 (10), and exconjugants were tested for motility. Wild-type motility was restored to PARA1 by the 5.5-kb clone. The 5.5-kb insert was cloned into pRK415-1 in an orientation opposite to that in pRES23a to yield pRES23b. This plasmid also restored motility when conjugated into PARA1. A 3-kb *Sal*I-*Bgl*II restriction fragment subcloned from pRES23a into pRK415-1 to yield pRES26 restored motility to PARA1 after introduction by conjugation, but a 2-kb *Sal*I-*Eco*RV restriction fragment (pRES27) did not (Fig. 3b).

DISCUSSION

Isolation of mutant PARA1 has shown the utility of a transposon mutagenesis approach for identifying *R. sphaeroides* mutants defective in flagellar function. Since the flagellum cannot be purified in an active form, this approach is essential to determine the function of motility proteins. Selective deletion of genes encoding motility proteins coupled with examination of the primary sequences of the gene products allows the prediction of the nature and the location of the proteins. This prediction can then be reconciled with the effect that deletion of the gene products has on the motile behavior of the bacterium.

The swarm plate and microscopic analysis confirmed that PARA1 was a nonmotile derivative of WS8-N. The finding by Western blotting that a wild-type level of flagellar filament protein was present in PARA1 coupled with the observation

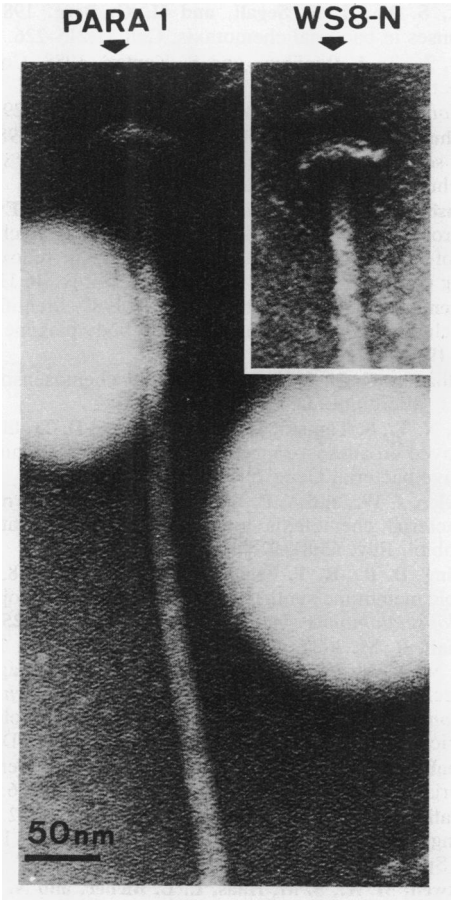

FIG. 2. Electron micrograph of intact flagella isolated from PARA1 and WS8-N cells by the method of Hahnenberger and Shapiro (8). Samples were negatively stained with 1.5% uranyl acetate solution. The WS8-N flagellar basal body consists of the domed M ring, the small, extra E ring, and paired P and L rings connected to the characteristically straight, narrow hook structure. The PARA1 flagellar basal body is missing structures above the P and L rings, including the domed M ring.

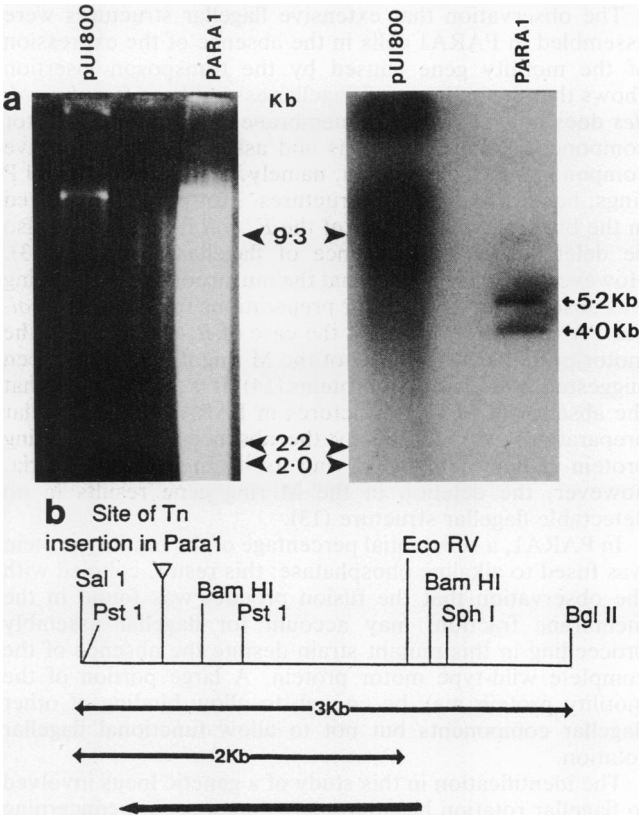

FIG. 3. (a) Southern blot of total DNA from PARA1 cut with *Bam*HI and uncut pUI800 DNA probed with the 3.5-kb internal *Hin*dIII fragment from the transposon (from pKN2). (b) Restriction map of the 3-kb DNA fragment which restores motility to PARA1 in *trans*. The site of insertion of Tn*phoA* into the chromosome in PARA1 is shown; the orientation of Tn*phoA* in PARA1 is such that the *phoA* gene lies at the *Bgl*II end of the fragment. The direction of transcription of the motility gene interrupted by Tn*phoA* is from the *Bgl*II to the *Sal*I restriction sites (shown by the bottom arrow).

of substantially intact flagellar structures in preparations of PARA1 under the electron microscope suggested that PARA1 was a mutant with paralyzed flagella. The presence of a single copy of Tn*phoA* in the PARA1 chromosome and the complementation of this mutant by a wild-type DNA fragment flanking the site of transposon insertion confirmed that the phenotype of PARA1 was due to the insertion of Tn*phoA*.

Sequence analysis of DNA flanking the site of transposon insertion is under way and will determine the nature of the primary structure of the protein encoded by the gene interrupted in PARA1. Evidence from the immunological localization of the alkaline phosphatase fusion product to the membrane fraction of PARA1 cells suggests that the protein is either membrane bound or associated. This suggestion correlates well with the observation that flagella from PARA1 lacked the M ring, which is generally believed to be a membrane protein. The observation of monomers of the 43-kDa protein which cross-reacted with anti-PhoA antiserum in membrane and periplasmic fractions indicates that there was some liberation of nonfused alkaline phosphatase from PARA1 (17) and that the protein was exposed to the periplasm. The fusion product had a molecular mass of 90 kDa, indicating that the portion of the motility protein to which the alkaline phosphatase was fused was at least 45 kDa and that the wild-type protein must therefore be larger. These indications correlate well with the complementation data: the 3-kb *Sal*I-*Bgl*II clone which restored motility to PARA1 would encode a wild-type protein of up to 90 kDa, assuming that virtually all of its DNA was a coding sequence; the finding that the 2-kb *Sal*I-*Eco*RV clone did not complement PARA1 suggests that one end of the coding sequence of the motility gene lies between the *Bgl*II site and the *Eco*RV site.

The identification of an alkaline phosphatase fusion protein in PARA1 produced as a result of the insertion of Tn*phoA* into the motility gene is evidence that the 5′ start of the coding sequence lies between these two restriction sites and that transcription proceeds from the *Bgl*II site to the *Sal*I site. This evidence was confirmed by preliminary sequence analysis across the site of transposon insertion in clones from the PARA1 chromosome with primers made against *phoA*, transposon, and vector sequences. The region of DNA from the transposon insertion site to the proximal *Sal*I site shown in Fig. 3b does not contain any translational start sequences but does have an in-frame stop sequence (data not shown).

The observation that extensive flagellar structures were assembled in PARA1 cells in the absence of the expression of the motility gene caused by the transposon insertion shows that the pathway of flagellar assembly in *R. sphaeroides* does not require early membrane insertion of all motor components before synthesis and assembly of the passive components of the flagellum, namely, the grommet L and P rings, hook, and filament structures. Mot proteins involved in the bidirectional rotation of the *E. coli* flagellum can also be deleted without hindrance of flagellar assembly (13). However, the observation that the mushroom-shaped M ring was absent in intact flagellar preparations from *R. sphaeroides* PARA1 suggests that in the case of *R. sphaeroides*, the motor proteins may be part of the M ring itself, as has been suggested for *E. coli* Mot proteins (14). It is also possible that the absence of M ring structures in PARA1 intact flagellar preparations was caused by the absence of a connecting protein rather than the M ring itself. In enteric bacteria, however, the deletion of the M ring gene results in no detectable flagellar structure (13).

In PARA1, a substantial percentage of the motility protein was fused to alkaline phosphatase; this result, coupled with the observation that the fusion product was found in the membrane fraction, may account for flagellar assembly proceeding in this mutant strain despite the absence of the complete wild-type motor protein. A large portion of the motility protein may be enough to allow binding of other flagellar components but not to allow functional flagellar rotation.

The identification in this study of a genetic locus involved in flagellar rotation begs an interesting question concerning the clustering of other flagellum-associated genes in this area of the chromosome. In *E. coli*, the Mot proteins are encoded by two genes, *motA* and *motB*, which lie in the mocha operon, which also includes genes the products of which are components of the methyl-accepting chemotaxis sensory system of that bacterium (13). As mentioned above, *R. sphaeroides* does not have this chemotactic sensory system, so it will be interesting to examine the DNA flanking the motility gene in cosmid c19 for other genes the products of which may be involved in taxis or motility.

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