Isolation, Characterization, and Cellular Insertion of the Flagella from Two Strains of the Archaebacterium

* Methanospirillum hungatei *

G. SOUTHAM,1 M. L. KALMOKOFF,2 K. F. JARRELL,2* S. F. KOVAL,3 AND T. J. BEVERIDGE1

Department of Microbiology, College of Biological Science, University of Guelph, Guelph, Ontario N1G 2W1,1
Department of Microbiology and Immunology, Queen’s University, Kingston, Ontario K7L 3N6,2 and Department of Microbiology and Immunology, University of Western Ontario, London, Ontario N6A 5C1,3 Canada

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In high (45 mM)-phosphate medium, Methanospirillum hungatei strains GP1 and JF1 grew as very long, nonmotile chains of cells that did not possess flagella. However, growth in lower (3 or 30 mM)-phosphate medium resulted in the production of mostly single cells and short chains that were motile by means of two polar tufts of flagella, which transected the multilayered terminal plug of the cell. Electron microscopy of negatively stained whole mounts revealed a flagellar filament diameter of approximately 10 nm. Flagellar filaments were isolated from either culture fluid or concentrated cell suspensions that were subjected to shearing. Flagellar filaments were sensitive to treatment with both Triton X-100 and Triton X-114 at concentrations as low as 0.1% (vol/vol). The filaments of both strains were composed of two flagellins of M, 24,000 and 25,000. However, variations in trace element composition of the medium resulted in the production of a third flagellin in strain JF1. This additional flagellin appeared as a ladderlike smear on sodium dodecyl sulfate-polyacrylamide gels with a center of intensity of M, 35,000 and cross-reacted with antisera produced from filaments containing only the M, 24,000 and -25,000 flagellins. On sodium dodecyl sulfate-polyacrylamide gels, all flagellins stained by the thymol-sulfuric acid and Alcian blue methods, suggesting that they were glycosylated. This was further supported by chemical deglycosylation of the strain JF1 flagellins, which resulted in a reduction in their apparent molecular weight on sodium dodecyl sulfate-polyacrylamide gels. Heterologous reactions to sera raised against the flagella from each strain were limited to the M, 24,000 flagellins.

In eubacteria, flagella are organized into three functional regions (filament, hook, and basal body complex) (30). The basal body consists of a series of stacked rings (generally two in gram-positive eubacteria and four in gram-negative eubacteria) that anchor the flagellum to the cell envelope and cause its rotation. Energy for rotation is derived from either an electrochemical proton (32) or sodium (37) gradient. Additional basal body-associated structures have been described in some species (8, 9, 13). Both the hook and filament are helical assemblies of many copies of single proteins (hook protein and flagellin, respectively). However, multiple flagellins have been reported in several eubacteria (5, 20, 29, 43) and the two species of archaebacteria examined to date (1, 2, 21). The hook appears to function as a flexible coupling between the filament and basal complex, with the filament propelling the cell through the liquid menastrum (20, 30).

Flagella of archaebacteria have been extensively studied either biochemically or ultrastructurally. From a limited number of observations, the occurrence of multiple flagellins in archaebacterial flagella appears to be more common than in eubacterial flagella (1, 21). In Halobacterium halobium, recent evidence indicates there are five different but highly homologous genes (16) coding for the flagellins, which are sulfated glycoproteins (1, 46). All five genes are expressed, and the five gene products have been shown to be integrated into the flagella (15), although their distribution has not been determined. Among the methanogens, Methanococcus voltae (21) and Methanogenium marisnigri and Methanococcus jannaschii (M. L. Kalmokoff, K. F. Jarrell and S. F. Koval, unpublished data) have two flagellins.

*Methanospirillum hungatei* is motile, can grow either as single cells or in chains of cells, and possesses a complex cell envelope; thus, this archaebacterium is a unique microorganism in which to study locomotory properties. These cells possess an encompassing cell wall, which in turn is bounded by a resilient sheath structure and two spacer plugs, one at each pole of the cell (4, 23, 38, 40, 47). It was of interest to determine whether the flagella would span all of these layers and still maintain movement without some architectural modification to either its component parts or the insertion site within the cell envelope. Since both the sheath and spacer (or end) plug have a paracrystalline structural format (36, 41), the existence of holes for flagella may introduce localized crystal defects that could affect the integrity of each structural layer. In addition, the plasma membrane possesses novel diether- and tetraether-linked phospholipids (26), which chemically bond the bilayer together. Therefore, lipid packing and movement within the membrane are affected by more than hydrophobic forces. Thus, these characteristics made it important to study the structural organization and insertion site of *M. hungatei* flagella into this novel and interesting cellular envelope.

In this paper, we report on the purification, biochemical characterization, and insertion of flagella in two strains of *M. hungatei*.


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* Corresponding author.
MATERIALS AND METHODS

Bacteria and growth conditions. M. hungatei GP1 (34) and JF1 (14) were obtained from G. D. Sporrt (National Research Council, Ottawa, Canada). Both strains were maintained at 37°C in JMA medium (18). For isolation of flagella, cells were grown in either SA medium (3 mM phosphate) (33) or JMA medium (30 mM phosphate) at 37°C in 1-liter bottles modified to accept serum stoppers. Cultures were pressurized on a daily basis with CO₂-H₂ (1:4, vol/vol). Where indicated, long filaments of both strains were obtained after growth in an elevated-phosphate medium (PA medium; 45 mM phosphate) (33).

Isolation of flagella filaments. Flagellar filaments were prepared by two methods. Cells (3 to 4 liters) were grown as described above and harvested after exponential growth by centrifugation (6,000 × g, 4°C, 15 min). The cells were gently suspended in 150 ml of 0.02 M Tris hydrochloride (pH 7.5) containing 0.15 M NaCl and sheared for 90 s in a Waring blender. Whole cells and large debris were removed by low-speed centrifugation (6,000 × g, 4°C, 30 min), followed by further clarification at 16,000 × g for 4 h. The supernatant was then centrifuged at 80,000 × g at 20°C for 90 min to pellet the flagellar filaments. The filaments were also recovered from the spent culture fluid by the same differential centrifugation procedure after an initial concentration of the spent culture fluid with an Amicon filtration cell (model 402; Amicon Corp., Lexington, Mass.) with an XM300 membrane (M₉, 300,000 cutoff).

Preparation of polyclonal antisera. Sheared flagella from each strain of M. hungatei composed only of the M₉, 24,000 and -25,000 flagellins were used to produce polyclonal antiserum. New Zealand White rabbits were first subjected to a preimmune bleed and then injected subcutaneously with 1.0 ml of a mixture of sheared flagella (containing 100 μg of protein) and Freund incomplete adjuvant (GIBCO Laboratories, Grand Island, N.Y.) at a ratio of 1:1. This subcutaneous injection was repeated on day 4. A 0.5-ml sample of the antiserum preparation was injected intramuscularly on days 8 and 15, and 0.5 ml of sheared flagella only (100 μg; no adjuvant) was injected intravenously on day 28. On day 31, animals were bled (approximately 30 ml). The blood was held at room temperature for 1 h and then incubated at 37°C for 1 h to allow for clotting and separation of serum. The serum was clarified of remaining cells by centrifugation in an Eppendorf microfuge for 1 min at 14,000 rpm.

Western immunoblotting. The immunological relationship between the flagellins of strains JF1 and GP1 and their respective homologous and heterologous antisera were determined by Western blotting (7, 44). Sera was used at a dilution of 1:150. Bound antibody was detected with goat anti-rabbit F(ab')₂ immunoglobulin G alkaline phosphatase-conjugated antibody (Jackson Immunoresearch Laboratories Inc.) and was visualized by using Nitro Blue Tetrazolium (28).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (27) as previously described (21). For good separation of the M₉, 24,000 and -25,000 flagellins, 30-cm-long gels were run. For rapid analysis of samples, a minigel apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) was routinely used. The protein molecular weight markers (Bio-Rad Laboratories Ltd., Mississauga, Canada) used were lysozyme (M₉, 14,400), soybean trypsin inhibitor (M₉, 21,500), carboxy anhydrase (M₉, 31,000), ovalbumin (M₉, 42,700), bovine serum albumin (M₉, 66,200), and phospholipase B (M₉, 97,400).

Glycoprotein staining. Staining for glycoproteins was carried out directly on SDS-PAGE gels by using both thymol-sulfuric acid (35) and Alcian blue (45) stains. Solubilized Methanogenium marisnigri cells were used as a positive control, because this organism has a glycosylated surface protein (M₉, 143,000 [23]) that could easily be detected on gels.

Deglycosylation of flagellins. The chemical deglycosylation method of Edge et al. (12) employing treatment of the flagellins with trifluoromethanesulfonic acid was used. The lyophilized sample (3 mg) was treated for 30 min at room temperature, and the protein was recovered by ether precipitation and analyzed by SDS-PAGE.

Protein determination. Flagella preparations were assayed for protein content by the modification of the Lowry et al. procedure described by Markwell et al. (31).

Electron microscopy. Material for negative staining was prepared by drying a sample of cells or purified flagella onto a Formvar carbon-coated copper grid before staining with either 1% (wt/vol) uranyl acetate or 1% (wt/vol) ammonium molybdate. Grids were examined at 60 kV on a Philips EM 300 electron microscope.

Preparation of material for thin sectioning was as follows. Cells were fixed with glutaraldehyde (1%, vol/vol) while in the culture medium and then enrobbed in Noble agar. Samples were next treated with 2% osmium tetroxide followed by 2% uranyl acetate, subjected to an ethanol-propylene oxide dehydration series, and embedded and cured in Epon 812. Sections (cut with a Reichert-Jung Ultracut E ultramicrotome) were collected on Formvar carbon-coated grids, poststained with uranyl acetate (7 min) and then lead citrate (10 min), and examined at 60 kV on a Philips EM 300 electron microscope.

Light microscopy. A small drop of motile cells was applied to a clean microscope slide. A cover slip was placed over the drop; after 5 min of incubation at 22°C to allow for the flagella to bind to the glass surfaces, 2 drops of Ryu stain (24) were applied to opposite edges of the cover slip. After a further 5 min of incubation to allow for capillary mixing of the stain, the slide was examined by light microscopy. In a separate experiment, cells were examined for rate of motility by using an ocular micrometer scale. No steps were taken to prevent the exposure of the cells to the atmosphere.

RESULTS

Both M. hungatei GP1 and JF1, when cultivated in either SA or JMA medium (3 or 30 mM phosphate, respectively), produced short chains and single cells. These short filaments of cells were motile and swam at about 8 μm/s, with the cell filaments rotating. Cells were not observed to reverse the direction of swimming under our conditions, in which no attempt was made to exclude oxygen during microscopy. Ryu staining of these preparations showed the presence of some flagella on cells and many free filaments (data not shown). In the stationary phase, cultures contained much longer chains of cells with fewer, if any, flagella, and cells exhibited limited motility. In addition, when cultures were grown in medium containing a higher phosphate level (45 mM, PA medium), only long chains of cells formed throughout all growth phases and no flagella were observed either on
cells or free within the culture fluid. Hence, the chain length of cells and degree of motility may be inversely related.

Examination of negatively stained whole cells by electron microscopy indicated that the flagella appeared to be inserted through the end plug and did not project through the sheath (Fig. 1A). The flagella filaments did not form broad helical curves and were approximately 10 nm in diameter which corresponds to that reported recently by Cruden et al. (10). Careful examination of thin sections enabled us to find good examples of flagella that transected the end plug in both strains (Fig. 2). The end plug is composed of four to five macromolecular layers (Fig. 2): the bacterial wall, a poorly staining region, and two to three darkly stained layers that are plugs with hexagonal symmetry (36). Attempts to isolate intact flagella by detergent treatment of spheroplasts (21) were unsuccessful. Subsequent treatment of spheroplast membrane preparations (39) or isolated flagellar filaments with 0.1% (vol/vol) Triton X-100 or Triton X-114 confirmed that chemical deglycosylation of the flagellins with trifluoromethanesulfonic acid resulted in a marked decrease in activity occurring at Mr 35,000 (Fig. 4, lane 2) or in combination with the Mr-24,000 flagellin of each strain; results are shown for strain JF1 (Fig. 4, lane 4). The heterologous reaction with strain GP1 was identical (data not shown). Again, the Mr-35,000 flagellin of strain JF1 reacted strongly with the homologous sera produced against filaments containing only the Mr-24,000 and -25,000 flagellins of this strain (Fig. 4, lanes 3 and 4).

DISCUSSION

The presence of polar tufts of flagella was observed on individual cells and chains possessing low numbers of cells with both M. hungatei GP1 and JF1, in agreement with the findings of Cruden et al. (10), who only studied strain GP1. Chains composed of two or three cells were motile, which suggested that some method of communication to coordinate flagellum rotation must exist between cells. This is an interesting observation, considering that cells within a filament are separated by a cell spacer ca. 400 nm in length (4) and raises interesting questions with regard to the method of communication for control of flagellar rotation.

The distinctly thinner filament diameter of the flagella of M. hungatei (10 nm) compared with those of most eubacterial flagellar filaments (20 to 24 nm [30]) has also been noted for other methanogens (19). We have demonstrated that the flagellar filaments insert through the end plug of both strains of M. hungatei. The end plug is composed of several proteinaceous matrices with hexagonal symmetry that possess pores of at least 15 nm (36). These pores are large enough to accommodate the flagellum filament (10 nm in diameter) and may act as a bushing for rotation.

Attempts to isolate intact flagella from spheroplasts produced by alkaline dithiothreitol treatment (39) were unsuccessful. It appears that the flagella are sensitive to treatment with either Triton X-100 or Triton X-114; thus, the structure reported earlier by us for the M. hungatei basal body (21), which agrees with that reported by Cruden et al. (10), may have been a serendipitous discovery. We are currently investigating the detergent sensitivity of the M. hungatei flagella to devise a method for isolation of intact flagella, including their basal bodies, in good yield. Since flagellar

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FIG. 1. (A) Electron micrograph of *M. hungatei* GP1 (negatively stained with uranyl acetate), showing multiple flagella at the pole of a filament. Bar, 0.5 μm. (B) Electron micrograph (negatively stained with uranyl acetate) of sheared flagellar filaments from *M. hungatei* JF1. Bar, 200 nm.
filaments from *Methanococcus voltae* (21) and a number of
eubacteria (11) are not sensitive to either detergent, it is
possible that glycosylation of the flagellins may influence
their stability in Triton detergents.

Both strains of *M. hungatei* share the characteristic of
multiple flagellins, as reported for several other archaeabac-
teria (*Methanococcus voltae, Methanococcus jannaschii,
*Methanogenium marisnigri, H. halobium*). Although the
flagellins from each strain have similar molecular weights,
cross-reactivity with heterologous sera raised against intact
filaments from each was weak and limited to the *M*,-24,000
flagellins. This suggests that the *M*,-24,000 and *M*,-25,000
flagellins of each strain may be different proteins rather than
a single protein modified by various degrees of glycosylation
and that the *M*,-24,000 flagellins from each strain bear some
relationship to each other. Cross-reactivity with heterolo-
gous sera was limited to Western blots only. Immunogold
labeling with heterologous sera did not decorate the flagellar
filaments (unpublished observation). The occurrence of mul-
tiple unrelated flagellins may not be uncommon in archaeabac-
teria, since the flagellins of *Methanococcus voltae* did
not appear closely related based on peptide mapping studies
(22). However, in *H. halobium* there are genes for five
closely related flagellins, all of which code for core proteins
of approximately *M* 20,000 (16).

The appearance of a third flagellin in strain JF1 appears to
be related to variations in the trace element composition of
the growth medium. This may be due to changes in divalent
cations, similar to the role of magnesium ions in determining

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**FIG. 2.** Thin section of *M. hungatei* JF1 showing insertion of several flagella through the end plug. Bar, 200 nm. The insert shows a high magnification of a single flagellum transecting the end plug of strain GP1. Bar, 20 nm.
comprising solely of the lower-molecular-weight flagellins. In addition, the Mr-35,000 protein reacted strongly with homologous sera produced with filaments containing only the Mr-24,000 and -25,000 flagellins, indicating some degree of relatedness among all of the flagellins. However, since the Mr-35,000 flagellin of strain JF1 did not react with heterologous strain GP1 antisera, it may represent either a modification of the Mr-25,000 flagellin or a related additional flagellin. Such a modification(s) in the Mr-35,000 flagellin may involve a higher degree of glycosylation than that found in the original form, since it ran as a smear (although with a distinct laddering effect) and not a tight band. This may also explain why chemical deglycosylation of all three flagellins (Mr, 35,000, 25,000, and 24,000) resulted in the appearance of only two major bands (Mr, 23,000 and 15,000).

The glycosylation of the *M. hungatei* flagellins is a very unusual characteristic so far shared only with *H. halobium* and possibly minor components of the periplasmic flagella of *Spirochaeta aurantia* (5). The function of the glycosylation and the effect it might have on the properties of the filaments is unknown at present. It is not likely that glycosylation plays a role in transport of the flagellins across the archaeabacterial cell envelope, since glycosylation of the flagellins in *H. halobium* is known to occur after transport outside of the cell (42). It has been suggested (3) that glycosylation of the flagellins in *H. halobium* allows the individual filaments that make up the flagellar bundle to slide more smoothly against one another, such that reversal of the direction of flagellar rotation does not disrupt the flagellar bundle. A similar function may be served by the glycoproteins associated with the periplasmic flagellar filaments of *S. aurantia*, i.e., to slide smoothly along membrane surfaces defining the periplasmic space. We have not observed reversal of the direction of swimming in either strain of *M. hungatei*. However, the filaments do occur in small bundles and as such may bear some similarity with *H. halobium*. What role, if any, the glycosylation might play in the unusual detergent sensitivity of the *M. hungatei* flagellites has yet to be determined.

To date, limited data have been published on flagella of the archaeabacteria. What is apparent at this point is that flagella in this kingdom appear to differ in several key features from those found in the eu细菌ia. First, flagellins (which may also be glycosylated) are common, whereas this is not usually the case in the eu细菌ia. Second, evidence is accumulating that suggests that flagellar biosynthesis in this kingdom may occur in a fashion very different from that of the eu细菌ia. In eu细菌ia, filament elongation is thought to occur by passage of the flagellin monomers up through an axial pore within the filament, followed by integration into the distal end of the growing filament (17, 30). Leader peptides are not found on eu细菌ial flagellins, with the exception of presumptive signal sequences on the flagellins of the internal flagella of *S. aurantia* (6). This model does not adequately explain recent findings concerning flagellar biosynthesis in the archaeabacteria. Sumper and co-workers (42) have reported that glycosylation of the flagellins in *H. halobium* occurs external to the cytoplasmic membrane, which would be difficult to envision with this model. Also, the significantly thinner diameter of archaeabacterial flagella may preclude the passage of flagellin monomers through an axial hole. Furthermore, the N termini of the flagellins from *H. halobium*, *M. hungatei* GP1, and *Methanococcus voltae* have been shown to be highly conserved, and preliminary evidence suggests the presence of a putative 12-amino-acid leader peptide not found on the mature flagellin (22). Finally, the N termini of the archaeabacterial flagellins bear no ho-

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**FIG. 4.** Flagellins of *M. hungatei* JF1. Because of the length of gel required to adequately separate the Mr-24,000 and -25,000 flagellins, only the relevant portion of the gel is shown. Size markers are indicated to the left. Lanes: 1, SDS-PAGE (12% polyacrylamide gel) of sheared flagellar filaments showing occurrence of Mr-35,000 flagellin with the Mr-25,000 and -24,000 flagellins; 2, Mr-35,000 flagellin occurring alone; 3, reaction of Mr-35,000, -25,000, and -24,000 flagellins with homologous antisera produced from filaments containing only the Mr-24,000 and 25,000 flagellins; 4, reaction of the Mr-35,000 flagellin alone with homologous antiserum; 5, Alcian blue-stained carbohydrates of JF1 flagellins; 6, reaction of the flagellins from strain JF1 with heterologous antiserum against strain GP1 flagellons.

the degree of glycosylation of flagella in *H. halobium* (42). We are currently investigating this finding in more detail; we have observed that the effects of small changes in divalent cation concentration in the medium affect growth, filament length, and flagellation in a complicated fashion. These trace element effects have not yet been studied in strain GP1.

Filaments composed entirely of the Mr-35,000 flagellin, or of the Mr-35,000 flagellin in combination with the Mr-24,000 and -25,000 flagellins, in strain JF1 were not distinctly different in electron microscopic appearance from those

**FIG. 5.** SDS-PAGE (12% polyacrylamide gel) demonstrating chemical deglycosylation of flagellins of *M. hungatei* JF1. (A) Lanes: 1, Mr-35,000 flagellin; 2, deglycosylation of Mr-35,000 flagellin ladder to a major band of Mr, 23,000; 3, Mr, 35,000, -25,000, and -24,000 flagellins; 4, deglycosylation of Mr-35,000, -25,000, and -24,000 flagellins resulting in two major bands of Mr, 23,000 and 15,000. (B) Enlargement of the Mr-35,000 flagellin smear from lane 3, showing ladderlike appearance.
mology to the N termini of eubacterial flagellins (16, 22). These findings, coupled with the unusual envelope types found within the archaea (23), indicate a possible common mechanism for flagellar biosynthesis in archaea, a mechanism that is distinct from that found in the eubacteria.

Crun et al. (10) published their findings during the preparation of the manuscript.

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LITERATURE CITED