Isolation and Ultrastructure of the Flagella of *Methanococcus thermolithotrophicus* and *Methanospirillum hungatei*

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Received 14 October 1988/Accepted 6 March 1989

The flagella of the archaeobacteria *Methanococcus thermolithotrophicus* and *Methanospirillum hungatei* enter the cells in regions with ultrastructure resembling that of the polar organelles found in a variety of eubacteria. Flagella of both organisms consist of a filament, a hook, and a basal body with two rings similar to those of gram-positive eubacteria. The integrity of the flagella of *M. thermolithotrophicus* is lost in the absence of high salt concentrations, and those of both organisms are unstable at high pH. The flagellar filaments of *M. hungatei* are composed of two flagellins of 24 and 26 kilodaltons.

Archaeobacteria are different from eubacteria in a number of significant ways (4). One of these differences is the complete lack of peptidoglycan in the cell walls of archaeobacteria and the variety of cell wall types within this group, including flexible or rigid envelopes of protein or glycoprotein and pseudomurein (14). Many methanogens are motile by means of flagella which superficially resemble those of eubacteria. Until recently, only the fact of motility and the presence of flagella have been mentioned in the formal description of species, e.g., *Methanospirillum* (11) and *Methanococcus thermolithotrophicus* (12). Doddema et al. (10) cataloged the presence of fimbriae as well as flagella in methanogens. Given the fundamental differences in cell wall structure (as well as differences in membrane lipids) between eubacteria and archaeobacteria, the antiquity of the separation of the two kingdoms, and the variety of cell walls within the archaeobacteria, it would not be unexpected to find fundamental differences between the flagella and mechanisms of motility in the two groups.

Studies of the flagella in the genus *Halobacterium*, an archaeobacterium with an osmotically sensitive cell wall, show that they are composed of three flagellins (2). The flagella pass through the cell wall to the cytoplasmic membrane, and the authors suggested on behavioral grounds that a single motor is involved in the rotation of the whole tuft of flagella rather than the individual assemblies found in eubacteria; if true, this would be a significant difference. More recent analyses suggest that the flagellins are sulfated glycoproteins which also occur in the membranes of these organisms (22).

Koval and Jarrell (16) reported that the flagella of *Methanococcus voltae* are inserted through the regularly structured (RS) protein cell wall into a region of the cell with ultrastructure very similar to that of the polar membrane region described by Murray and Birch-Andersen (18) in *Spirillum serpens*. Very recently, Kalmokoff et al. (13) isolated intact flagella from *M. voltae* and determined that the filaments are composed of two flagellins. No distinct hook (other than a slight thickening of the filament) was present, and the basal structure consisted of a small knob. Flagella from three other *Methanococcus* species had similar ultrastructure.

In this study we examine the ultrastructure and attachment of the flagella of two motile methanogens with different

wall types. *M. thermolithotrophicus* is halophilic and thermophilic. Its cell wall consists of a single layer of protein subunits, and it is osmotically sensitive (12). *Methanospirillum hungatei* cells are surrounded by a proteinaceous sheath which is extremely resistant to disruption (5, 6). Tufts of flagella are observed at the ends of the filaments which are plugged with several layers of protein. The plug material is different in structure from that of the sheaths (5).

(A preliminary report of this work has been presented [D. L. Cruden, R. Sparling, and A. J. Markovetz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, 136, p. 178].)

MATERIALS AND METHODS

Organisms. *M. thermolithotrophicus* (12) was obtained from K. O. Stetter, University of Regensburg, Regensburg, Federal Republic of Germany. *Methanospirillum hungatei* (11) GP 1 was obtained from G. D. Sprott, National Research Council of Canada, Ottawa, Ontario, Canada.

The cultivation techniques are based on those described by Balch et al. (4). The medium for *M. thermolithotrophicus* contained 4.5 mM KCl, 18 mM NH₄Cl, 0.7 mM CaCl₂, 1.5 mM K₂HPO₄, 500 mM NaCl, 1 μM NaSeO₄, 1 μM H₂WO₄, 10 ml of mineral elixir (8) per liter of medium, and 0.5 μg of resazurin per ml. The pH of the medium was adjusted to 5.9 before autoclaving under anaerobic conditions, and Na₂S and MgCl₂ were added to final concentrations of 2 and 25 mM, respectively. The medium for *Methanospirillum hungatei* contained 4.5 mM KCl, 7.5 mM NH₄Cl, 5 mM NaCl, 18 mM sodium acetate, 2 mM K₂HPO₄, 0.3 mM MgCl₂, 0.44 mM CaCl₂, 10 ml of mineral elixir (8) per liter of medium, 10 ml of vitamin mixture (23) per liter of medium, and 0.5 μg of resazurin per ml. The pH of the medium was adjusted to 7.0 before autoclaving under anaerobic conditions, and then Na₂S was added to 2 mM. Cultures were grown in 100 ml of medium in 500-ml stoppered serum bottles under approximately 200 kPa of H₂-CO₂ (4:1) at 37°C without shaking. In some experiments, *M. thermolithotrophicus* was grown at 60°C.

Purification of intact flagella from *M. thermolithotrophicus*. Actively motile cells were harvested from 15 bottles by centrifugation and suspended in approximately 50 ml of 0.1 M Tris buffer (pH 8.0) containing 500 mM NaCl and 0.1 M EDTA. A 1.5-ml volume of 20% Triton X-100 was added, and the suspension was stirred gently for 5 min, resulting in the lysis of most of the cells. Then 0.5 ml of 1.0 M MgCl₂ and a few milligrams DNase was added. After stirring for 20 min

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at 30°C the viscosity had decreased, and the mixture was centrifuged at $27,000 \times g$ for 2 h. The tan pellet was suspended in Tris-NaCl-Triton X-100, and the flagella were further purified in a CsCl gradient. CsCl (18 g) was added to 37 ml of the suspension of flagella, and the mixture was centrifuged for 18 h at $27,000 \times g$ in an SW28 swinging-bucket rotor. Fractions (2 ml) were removed beginning at the top of the tube, and the A_{260} and A_{280} determined. Protein-containing fractions were dialyzed overnight against Tris-NaCl. The purification was monitored by electron microscopy at each stage.

Purification of flagellar filaments. *Methanospirillum* or *Methanococcus* cells were harvested by centrifugation under aerobic conditions. *Methanococcus* cells were suspended in Tris-NaCl buffer (see above). *Methanospirillum* cells were suspended in 0.1 M potassium phosphate buffer (pH 7.2). Flagella were sheared by repeatedly forcing the suspension through a 22-gauge needle and homogenizing it in a Polytron homogenizer for 2 min. Cells were sedimented by centrifugation at $5,000 \times g$. The pellets were suspended in fresh buffer and centrifuged again. Supernatants were pooled and centrifuged at $29,000 \times g$ for 1 h (*Methanospirillum* cells) or 2 h (*Methanococcus* cells). Centrifugation in a CsCl gradient was performed as above, except that with *Methanospirillum* cells, 12 g of CsCl was added per 37 ml of flagellar suspension.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (17) with a running gel of 12.5% acrylamide. Molecular weight standards were obtained from Pharmacia, Inc. Gels were stained with Coomassie blue and then subjected to silver staining (Bio-Rad Laboratories).

Electron microscopy. Samples of actively motile cultures were fixed for transmission electron microscopy by the method of Kellenberger et al. (15) or with glutaraldehyde followed by osmium tetroxide with 0.15% ruthenium red (7). Preparations were embedded in Epon, sectioned with a diamond knife, and poststained with uranyl acetate and lead citrate (19). Flagellar preparations and whole cells were negatively stained with 2% phosphotungstic acid with bacitracin to aid spreading or with 4% uranyl acetate. Catalase crystals were used for calibration of magnification (24). Lengths of flagella and hooks were measured in photographic prints with a contour digitizer.

RESULTS

***Methanococcus* flagella.** *M. thermolithotrophicus* cells have loose tufts of up to 25 comparatively short (less than 4.5 μm) flagella (Fig. 1a). Motility is wobbly, with cells following irregular paths without abrupt changes in direction. Cells grown at 60°C rapidly lose motility when observed in wet mounts at room temperature, whereas cells grown at 37°C remain motile for at least 10 min, even without having the edges of the cover slips sealed to prevent the diffusion of O_2 into the preparation. In thin sections (Fig. 1b) an RS protein layer similar to that of *M. voltae* (16) can be observed. Thin sections of motile cells also have indications of polar regions with ultrastructure similar to that of *Spirillum* spp. (18) described by Koval and Jarrell (16) for *M. voltae*.

Cells of *M. thermolithotrophicus* lyse readily when 0.1% Triton X-100 is added to the preparation, and initial attempts at purification of flagella were made by using the method of DePamphilis and Adler (9). However, we discovered that the presence of 500 mM NaCl, which was required for the growth of the organism, was also required to maintain the

FIG. 1. Electron micrographs of *M. thermolithotrophicus*. (a) Negative stain of a single cell showing the characteristic loose clump of relatively short flagella. (b) Thin section of a cell showing the RS protein wall (arrows), cytoplasmic membrane, and polar organelle (arrowheads) in the region of insertion of the flagella. Bars, 0.2 μm .

integrity of the flagellar filaments. The dissociation of flagella in the absence of NaCl was reversible; addition of NaCl led to reassociation of filaments visible in negatively stained preparations within 2 h.

In more recent purification schemes for eubacterial flagella, 0.1 N NaOH is used to improve the separation of flagellar filaments from membranes which are solubilized at

FIG. 2. Isolated flagellar filament-hook-basal-body complexes from *M. thermolithotrophicus*. Panels a and d show pieces of cell membrane associated with the complex. Panel c shows two flagella with hooks of different lengths, with their basal bodies attached. Arrows in panels c and g indicate distal ends of hooks. Arrowheads in panel c indicate where helical striations in the filament are visible. Panels f, j, and k show isolated basal bodies (arrowheads). For panels a and i, bars = 100 nm; for panels b to h, j, and k, bars = 50 nm.

high pH (1). When a lysed suspension of *M. thermolithotrophicus* was exposed to conditions of high pH (pH 11), the flagellar filaments were completely dissociated within 1 h. No conditions of high pH were found under which membranes were solubilized while flagellar filaments remained intact. Therefore, the scheme which was adopted for purification of the flagellar filaments of *M. thermolithotrophicus* involved simply lysis of cells with Triton X-100, followed by differential centrifugation and centrifugation through a CsCl gradient in the presence of 500 mM NaCl. Flagella purified by this method are shown in Fig. 2a to i. The diameter of the flagellar filaments in preparations negatively stained with phosphotungstic acid is 12.6 ± 1.8 nm ($n = 50$). Intact filaments possess a hook which is 147 ± 51 nm long ($n = 34$; range = 72 to 265 nm) and 11.2 ± 0.9 nm wide ($n = 30$). Hooks and filaments may be differentiated as

easily by staining behavior as by differences in width and ultrastructure. In Fig. 2c, filaments with hooks of different lengths are attached by their basal assemblies. The filament has a helical fibrillar ultrastructure (best seen in Fig. 2c, f, and g), and a notch can often be seen on one side at its point of insertion into the hook (Fig. 2c and g, arrows). The hook terminates at a single pair of basal rings, which are 17.2 ± 4.9 nm in diameter ($n = 17$). The basal bodies occasionally may be attached to membrane fragments (Fig. 2a and d). The lower of the two rings appears to be thinner and is often less distinct than the one closer to the hook, but it may be associated with other poorly resolved but structured material (Fig. 2g).

In early purification attempts in which the method of DePamphilis and Adler (9) was used, when NaCl was not included in the buffers the presence of ammonium sulfate

and cesium chloride provided enough osmolarity to maintain some integrity of the flagellar filaments. This filament integrity was lost when the preparations were dialyzed. Electron micrographs taken to monitor the purification of these preparations revealed, on later close inspection, isolated, basal-body assemblies (Figs. 2f, j, and k). These assemblies are similar to those of gram-positive eubacteria in consisting of two disk-shaped rings, connected by a rod. One of the disks in the pair often contains the clawlike protrusions seen by Aizawa et al. (1) in *Salmonella typhimurium* (Fig. 2f, arrow-head).

***Methanospirillum* flagella.** In anaerobic wet mounts, *Methanospirillum* cells swim smoothly, often for as long as several minutes in one direction. Their speed is roughly inversely proportional to the length of the filament, although the velocity of a single cell may vary from 3 to 10 $\mu\text{m/s}$ over a short period.

Electron micrographs of negatively stained preparations from actively motile cultures show tufts of polar flagella at one or both ends of single-cell units, as well as multicellular filaments. Typically, there are 5 to 10 filaments in a tuft. The flagella are often up to 10 μm in length and show a sine wave form with a wavelength of $2 \pm 0.2 \mu\text{m}$ ($n = 7$) and an amplitude of $0.34 \pm 0.08 \mu\text{m}$ ($n = 8$). When observed in negatively stained preparations, if a cell unit has bipolar tufts of flagella, one tuft is usually extended, while the other curves back around the body of the cell. In our experience, *Methanospirillum* flagella are more easily broken during routine manipulation than are those of *Methanococcus* spp. or other bacteria.

In negatively stained preparations, the width of the flagellar filaments is $9.9 \pm 0.67 \text{ nm}$ ($n = 12$) (Fig. 3a). No helical substructure can be discerned in the filaments. Stain collects at the ends of the cells, so that insertion is difficult to see. However, in Fig. 3a the flagellar filaments extend through the plug material into the cell. At the ends of the cell filaments, short fine fibrils extend perpendicular to the smooth hoops of the cell sheath (Fig. 3a, arrowheads).

The width of the *Methanospirillum* flagella in thin sections (Fig. 3c to g) is $9.45 \pm 0.9 \text{ nm}$ ($n = 13$). Flagella penetrate the layers of plug material at the cell poles (85 to 100 nm) and then through the cytoplasmic membrane in a region which closely resembles the polar membrane found in *Spirillum* spp. (18). The polar region is electron transparent, with few ribosomes present. There is an electron-dense band approximately 25 nm from the cytoplasmic membrane, with diffuse striations between it and the cytoplasmic membrane and, usually, a second bilayer membrane close to the cytoplasmic membrane (Fig. 3d to f, arrowheads). This polar region is not present at the poles of cells without filaments (Cruden and Markovetz, unpublished data; also see micrographs in reference 5), and flagella do not pass through the cell sheath at the ends of internal cells. In occasional sections, however, ultrastructure similar to that of the polar region may be observed along the side of a terminal cell (Fig. 3e, arrows).

The procedure of Aizawa et al. (1) for the purification of flagella yielded no intact flagellar filaments from *Methanospirillum* spp. Similarly, cells disrupted with dithiothreitol at pH 9.0 and then subjected to treatment with 0.2% Triton X-100 did not yield intact filaments. By monitoring each step of the procedures by electron microscopy, we determined that the *Methanospirillum* flagella were unstable to 0.1 N NaOH for 1 h, as were the flagella of *M. thermolithotrophicus*. In addition, the *Methanospirillum* flagella were disrupted by 0.2% Triton X-100.

Figure 3b shows one of the few recognizable flagellar

fragments remaining in a preparation of cells disrupted with dithiothreitol followed by Triton X-100 for 15 min. The filament terminates in a hook which is 12.4 nm wide and 170 nm long. The filament is approximately 11.0 nm wide, which is wider than the filaments in culture medium or buffers, indicating that filament swelling may have occurred in the presence of dithiothreitol. The base plate consists of a single pair of rings connected by a rod.

Flagellar filaments were prepared after shearing from cells by differential and cesium chloride gradient centrifugation. A protein band at approximately three-quarters of the length of the centrifuge tube from the top correlated with the presence of flagellar fragments in electron micrographs. Increasing purity of preparations, as determined by their appearance in the electron microscope, correlated with the increasing prominence of two bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis corresponding to molecular weights of 24,000 and 26,000.

DISCUSSION

The ultrastructures of the flagella and associated hook-basal-body complex of *M. thermolithotrophicus* and *Methanospirillum hungatei* are similar, at least superficially, to those of gram-positive eubacteria, despite the differences in cell wall structures and other characteristics that separate the archaeobacteria from eubacteria. The basal structures in both organisms appear to consist of a single pair of ring-shaped base plates connected by a rod. The upper of the two rings is anchored in the cytoplasmic membrane. The rings are of different thicknesses, and there is some indication of the jeweler's claw mount-like projections on the proximal ring similar to those reported for *S. typhimurium* by Aizawa et al. (1). In both *M. thermolithotrophicus* and *Methanospirillum hungatei*, the hook length appears to be more variable than that of the same structure in eubacteria. The helicity of the filaments is visible in negatively stained preparations from *M. thermolithotrophicus*. It is not apparent in flagellar filaments from *M. hungatei*, possibly because of poor resolution combined with the small diameter of the filament. Shaw et al. (20) found that the pores in the end plates of *M. hungatei* cells have a diameter of 15 nm. Thus, they are large enough to accommodate the 10-nm diameter of the flagellar filament.

The susceptibility to disruption of the flagella of these two methanogens appears to be greater than that of the eubacterial flagella which have been extensively studied. The flagella of *S. typhimurium* are routinely purified by steps which require their stability at pH 11 for 2 h, and which require their stability in 1% Triton X-100 (1). Alam and Oesterhelt (3) recently reported that bundles of flagella shed by *Halobacterium* spp. were disrupted into individual flagellin molecules when the NaCl concentrations were decreased. The *Halobacterium* flagella consist of three flagellins of molecular weights of 23,500, 26,500, and 31,500. These are sulfated glycoproteins identical to those found in the cell membrane of this organism (22). The flagella of *M. voltae* are composed of two flagellins with molecular weights of 31,000 and 33,000 (13). As reported above, the *Methanospirillum* flagella consist of two flagellins with molecular weights of 24,000 and 26,000, whereas those of *M. thermolithotrophicus* are composed of at least two flagellins (Cruden and Markovetz, unpublished results). It is possible that the relative instability of the flagella of all these archaeobacteria is due to their assembly from two or more flagellins of different sizes. It remains to be determined whether flagella of all archaeobac-

FIG. 3. Electron micrographs of *Methanospirillum hungatei*. (a) Negatively stained cell preparation. Arrows indicate fibrillar material at the pole of the cell. (b) Isolated filament-hook-basal body. (c to g) Transmission electron micrographs showing flagella inserted through the plug material at the end of the filament into the cell. Arrowheads indicate the second bilayer membrane in the polar organelle. Arrows in panel e indicate the polar organelle along the side of the cell. Bars = 100 nm.

teria consist of two or more flagellins and whether the flagellins from methanogens contain only proteins or, like those from halobacteria (22), are substituted.

Kalmokoff et al. (13) reported that the basal-body structure of *M. voltae* and three other *Methanococcus* spp. (not including *M. thermolithotrophicus*) consists of a small knob and that there is no hook region other than a slight thickening of the filament adjacent to the basal knob. We observed distinct hooks, although of more variable lengths than those in eubacteria, and basal bodies similar to those of gram-positive eubacteria in both *M. thermolithotrophicus* and *Methanospirillum hungatei*. Flagellar filament diameter and filaments composed of two flagellins are in agreement in both studies. Kalmokoff et al. (13) also report the existence of basal bodies composed of two rings and attached to typical hook structures in flagella purified from *M. hungatei*. We would expect that the ultrastructures of the flagella of all the *Methanococcus* species would be generally similar. The differences in interpretation may be due to the stains used for electron microscopy of the flagellar preparations; we found that phosphotungstic acid routinely gave better resolution than uranyl acetate (which was used in the other study).

The serendipitous discovery of isolated basal bodies in preparations made under conditions of low osmolarity indicates that the hooks as well as the flagellar filaments of *M. thermolithotrophicus* are osmotically unstable. Use of these differential stabilities may provide a method for the purification of the component parts for further analysis.

The existence of structures resembling the polar organelle along the sides of bacteria with polar tufts of flagella as well as at the pole has previously been reported for swarmer cells of *Sphaerotilus natans* (21). The presence of a helically wound ribbonlike polar organelle in this organism was given as an explanation of the mode of inheritance of the flagellar apparatus. For *Methanospirillum* spp. there is a further problem of differentiation, since cells completely enclosed within the sheath do not have flagella or the polar apparatus, whereas the free ends of terminal cells do, even though plug material and cell septa still remain.

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