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Physical Characterization of the Flagella and Flagellins from *Methanospirillum hungatei*

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Flagellar filaments from *Methanospirillum hungatei* GP1 and JF1 were isolated and subjected to a variety of physical and chemical treatments. The filaments were stable to temperatures up to 80°C and over the pH range of 4 to 10. The flagellar filaments were dissociated in the detergents (final concentration of 0.5%) Triton X-100, Tween 20, Tween 80, Brij 58, *N*-octylglucoside, cetyltrimethylammonium bromide, and Zwittergent 3-14, remaining intact in only two of the detergents tested, sodium deoxycholate and 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS). Spheroplasting techniques were used to separate the internal cells from the complex sheath, S-layer (cell wall), and end plugs of *M. hungatei*. The flagellar basal structure was visualized after solubilization of membranes by CHAPS or deoxycholate. The basal structure appeared to be a simple knob with no apparent ring or hook structures. The multiple, glycosylated flagellins constituting the flagellar filaments were cleaved by proteases and cyanogen bromide. The cyanogen bromide-generated fragments of *M. hungatei* GP1 flagellins were partially sequenced to provide internal sequence information. In addition, the amino acid composition of each flagellin was determined and indicated that the flagellins are distinct gene products, rather than differentially glycosylated forms of the same gene product.

The structure of flagella from the domain *Bacteria* (35), such as those of *Escherichia coli* and *Salmonella typhimurium*, has been well studied (1, 17, 20, 34). In general, a flagellum consists of a filament, hook, and basal body. The filament constitutes the majority of the mass of a flagellum and is usually composed of many copies of a single protein, flagellin. The bacterial flagellar filament is thought to be assembled through addition of a flagellin monomer at the distal tip of the filament, with the monomer being transported through the hollow center of the filament (17, 34). Studies of *S. typhimurium* have shown that the termini of the flagellin monomer are essential for flagellar filament formation (1). The primary structures of all bacterial flagellins that have been studied have conserved N- and C-terminal domains (17, 34). These domains have been shown to be important in transport and assembly of the flagellins (17, 34).

The physical stability of flagellar filaments of bacteria is known in considerable detail. As early as 1964, the flagella of *S. typhimurium* were known to be reversibly depolymerized by several treatments, including heating to 50°C, exposure to pH 2.0, and treatment with urea and guanidine hydrochloride (27).

In contrast to those of bacteria, the flagella from members of the domain *Archaea* have not been well studied. The flagella from all archaea that have been studied are composed of multiple, often glycosylated flagellins (5, 15, 19, 33). The flagellin genes from *Methanococcus voltae* (18) and *Halobacterium halobium* (13) have been sequenced, and in addition, the N-terminal amino acid sequences of flagellins from several other methanogens (including *Methanospirillum hungatei* GP1 and JF1) have been published (8, 15). The primary sequence of the archaeal flagellins shares no homology with that of bacterial flagellins (8, 15, 18), and at least in the case of *M. voltae*, the flagellins are initially synthesized with a signal (leader) peptide (18). The archaeal flagellins do have significant homology to each other at the N terminus (8, 15, 18). Recently, we have reported that the flagellins from the archaea have some sequence and other similarities to the type IV pilin-transport superfamily found in bacteria (8).

M. hungatei is a mesophilic, motile, spiral-shaped prokaryote (11, 25). It is a structurally unique member of the archaea. Each individual cell is surrounded by an inner cell wall, and one or more cells are contained in a tube-like sheath to form a filament. Individual cells are separated within the filament by a cell spacer region, while filaments terminate in a multilayered end plug (4, 28). The filaments typically contain one to three cells. However, under certain growth conditions, *M. hungatei* can grow as long filaments (up to 900 μm) containing many cells (10, 24).

The production of flagella is also controlled by growth conditions in *M. hungatei*, specifically, the growth temperature and the calcium concentration in the medium (10). The flagella are composed of multiple glycosylated flagellins, three (with M_rs of 24,000, 25,000, and 35,000) in strain JF1 and two (with M_rs of 24,000 and 25,000) in strain GP1 (29). The flagellar filaments of *M. hungatei* and several other archaeal species are dissociated by treatment with low concentrations of Triton X-100. This sensitivity to dissociation by Triton X-100 is correlated with the glycosylation of the component flagellins, although the mechanism is unknown (9).

We report here an extensive investigation of the stability of *M. hungatei* flagella to detergents, temperature, pH, and chaotropic agents. In addition, significant primary sequence data obtained from microsequencing of cyanogen bromide-derived peptides are presented.

MATERIALS AND METHODS

Organisms and growth conditions. *M. hungatei* JF1 (11) and GP1 (25) were obtained from G. D. Sprott (National Research Council, Ottawa, Ontario, Canada). Liquid cultures were

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routinely grown at 37°C under a pressurized atmosphere of H_2-CO_2 (80:20, vol/vol), as previously described (29). The culture medium was JMA (29) modified by a reduction of the PO_4^{3-} concentration to 3 mM and an increase of the Ca^{2+} concentration to 1 mM. This modification provided a consistently higher yield of flagellar filaments (10).

Isolation of flagellar filaments. *M. hungatei* was grown in 20-liter carboys containing 5 liters of medium. Flagellar filaments were obtained from sheared cells by differential centrifugation as previously described (10, 29). In addition, flagellar filaments were also isolated from the culture supernatant by concentration, using a CH2 concentrator (Amicon Canada Ltd., Missisauga, Ontario, Canada) with an HP100 hollow fiber filter (100,000-molecular-weight cutoff), followed by centrifugation as described above.

Treatment of flagellar filaments. Isolated flagellar filaments in distilled water (dH_2O) (approximately 500 μg/ml) were mixed 1:1 with the appropriate solutions for a given treatment. Table 1 lists the treatments and final concentrations. The following detergents were used: Triton X-100 (Sigma Chemical Co., St. Louis, Mo.), Tween 80 (Sigma), Tween 20 (Sigma), sodium deoxycholate (Sigma), cetyltrimethylammonium bromide (CTAB) (Sigma), Brij 58 (Sigma), Zwittergent 3-14 (Calbiochem Co.), 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) (Calbiochem Co.), and *N*-octylglucoside (Boehringer Mannheim Canada, Laval, Quebec, Canada). For treatment of flagellar filaments at extremes of pH, isolated flagellar filaments (approximately 500 μg/ml) were mixed 1:1 with 40 mM solutions of glycine-HCl (pH 2.0), acetate-acetic acid (pH 4.0), methane-ethyl sulfonic acid (pH 6.0), sodium phosphate (pH 8.0), sodium bicarbonate (pH 10.0), or sodium phosphate (pH 12.0). Flagellar filaments were mixed 1:1 with 6 M urea, 3 M urea, 5 M guanidine · HCl, or 2.5 M guanidine · HCl to determine the effects of chaotropic agents on flagellar filament stability. All the reaction mixtures listed above (and appropriate controls) were incubated for 1 h at 37°C.

Isolated flagellar filaments in dH_2O (approximately 500 μg/ml) were incubated at 40, 60, 80, 90, and 100°C for 10 min to determine temperature stability.

Protease treatment of intact flagellar filaments was performed by addition of 50 μl of isolated flagellar filaments in dH_2O (approximately 500 μg/ml) to 50 μl of protease in phosphate-buffered saline followed by incubation at room temperature for 2 h. The proteases used and final concentrations were trypsin (Sigma) and pronase (Sigma) at 250 μg/ml and chymotrypsin (Sigma) at 150 μg/ml. All samples were examined by electron microscopy, and most were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Examination of treated filaments by SDS-PAGE. Control and treated filaments were centrifuged in an Eppendorf mini centrifuge (16,000 × *g*) for 30 min to recover intact filaments. We have previously shown that flagellar filaments pellet under these conditions, while filaments depolymerized into small fragments and monomers do not (9). The pellets were resuspended and examined by SDS-PAGE by the method of Laemmli (22), as previously described (10). The following molecular weight markers (Bio-Rad, Missisauga, Ontario, Canada) were used: lysozyme (18,500), soybean trypsin inhibitor (27,500), carbonic anhydrase (32,500), ovalbumin (49,500), bovine serum albumin (80,000), and phosphorylase *b* (106,000).

Electron microscopy. For protease-treated intact filaments, the samples were centrifuged in an Eppendorf centrifuge (16,000 × *g*) for 30 min and examined by electron microscopy. Samples were generally stained with 1% (wt/vol) uranyl acetate (Marivac Ltd., Halifax, Nova Scotia) for 30 s and examined in a Philips EM300 electron microscope operating at 60 kV or in a Hitachi H7000 transmission electron microscope operating at 70 kV. Some cell envelope preparations and spheroplasts were negatively stained with 2% (wt/vol) phosphotungstic acid (Marivac) or 2% (wt/vol) ammonium molybdate (Marivac) before examination.

TABLE 1. Summary of effects of treatments on the stability of flagellar filaments of *M. hungatei* GP1 and JF1

Treatment	Presence of intact filaments shown by[a]	
	SDS-PAGE	EM[b]
Detergent (0.5% final concn)		
Brij 58 (wt/vol)	−	−
CTAB (wt/vol)	−	−
Triton X-100 (wt/vol)	−	−
Zwittergent 3-14 (vol/vol)	−	−
Tween 20 (vol/vol)	−	−
Tween 80 (vol/vol)	−	−
N-Octylglucoside (wt/vol)	−	−
CHAPS (wt/vol)	+	+
Sodium deoxycholate (wt/vol)	+	+
pH		
2	−	−
4	+	+
6	+	+
8	+	+
10	+	+
12	−	−
Temp (°C)		
40	+	+
60	+	+
80	+	+
90	+/−	+/−
100	−	−
Chaotropic agents (final concn [M])		
Urea (1.5)	+	+
Urea (3)	−	−
Guanidine · HCl (1.25)	+	+
Guanidine · HCl (2.5)	−	−
Proteases (final concn [μg/ml])		
Trypsin (250)	ND[c]	+
Pronase (250)	ND	+
Chymotrypsin (150)	ND	+
EDTA (final concn [mM])		
1.0	ND	+
10.0	ND	+
100	ND	+

[a] +, presence; −, absence; +/−, substantial reduction but filaments still present.
[b] EM, electron microscopy.
[c] ND, not determined.

Spheroplast formation. Spheroplasts of *M. hungatei* were formed essentially as described by Sprott et al. (30, 31). Briefly, 100 ml of cells was centrifuged and the pellet was resuspended in 3.0 ml of 75 mM dithiothreitol–2.5 M sucrose–5 mM sodium phosphate (pH 8.7). The formation of spheroplasts was monitored by phase-contrast microscopy; generally, most cells became spheroplasts after 30 min to 1 h. In addition, cell envelope fragments were obtained by resuspending harvested cells in 50 mM $NaHCO_3$ (pH 9.5) and incubating the mixture for 1 h at 60°C (12).

Purification of flagellin monomer. Purified preparations of flagellin monomer were prepared by two methods, either by KBr banding and SDS dissociation or by differential centrifugation and Triton X-100 dissociation. Both methods produced preparations in which flagellin was the only visible band on Coomassie-stained SDS-PAGE gels. Banding was performed by loading the isolated flagellar filaments onto a KBr gradient (0.5 g of KBr for each ml of 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.5]) and then centrifuging at 151,000 × *g* (35,000 rpm) in an SW41 rotor for 20 h at 15 to 20°C. Flagella appeared as a white band near the bottom of the tube. The band was removed and dialyzed against dH_2O to remove the KBr. The flagellar filaments were then dissociated by addition of 0.1% (wt/vol) SDS. Dissociation by Triton X-100 was used to purify JF1 flagellin, which did not form a compact band in KBr gradients. Briefly, Triton X-100 was added to isolated flagellar filaments to 0.1% (vol/vol) and the samples were incubated for 1 h at 37°C and then subjected to centrifugation in an Eppendorf microcentrifuge (16,000 × *g*) for 45 min. The supernatant was loaded on a Sephadex G-100 gel filtration column. Flagellin was eluted with 100 mM ammonium acetate (pH 6.3). The fractions were assayed for flagellin by SDS-PAGE.

Cyanogen bromide and trypsin cleavage of flagellins. Purified flagellin monomer was digested by cyanogen bromide in formic acid as previously described (23). Briefly, 100 μl (approximately 100 μg) of flagellin in dH_2O was added to 800 μl of 100% formic acid. This mixture was flushed with N_2 gas for 2 min. A small piece of solid cyanogen bromide (Eastman Kodak, Rochester, N.Y.) (approximately 10 to 100 mg) was added. This mixture was incubated for 18 h at room temperature. The reaction mixture was then diluted by addition of 10 ml of dH_2O and lyophilized. The lyophilized material was resuspended in 50 μl of dH_2O, separated by SDS-PAGE, stained for 1 h at 60°C in 0.2% (wt/vol) Coomassie brilliant blue R250, and destained by diffusion. Unsuccessful attempts to digest the flagellins with trypsin, endoproteinase Lys-C, and endoproteinase Glu-C immobilized on solid supports in a microcolumn (U.S. Biochemicals) were made.

Purified flagellin monomers from strains JF1 and GP1 were digested with 800 μg of trypsin per ml in 0.05% sodium azide–0.5% SDS for 48 h at 37°C. Purified flagellin monomer from strain JF1 was also digested with 3,000 μg of trypsin per ml under the same conditions. The reaction mixture was separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was then probed with rabbit antisera raised against GP1 flagellins or rabbit antisera raised against JF1 flagellins, as previously described (10).

Amino acid sequencing and total amino acid composition. Bands selected for sequencing were transferred to a PVDF membrane (Bio-Rad) as previously described (10) and sequenced by a pulsed-liquid phase sequencer (Applied Biosystems). Amino acid analysis of flagellin bands transferred onto PVDF was performed by using precolumn derivatization.

RESULTS

The unusual sensitivity of *M. hungatei* flagellar filaments to Triton X-100 (9, 29) prompted an investigation of their stability to a range of detergents, to extremes of pH, to high temperatures, and to chaotropic agents. Two methods were used to determine filament stability: electron microscopy and a method using centrifugation followed by SDS-PAGE (9). There was no difference in the stability of flagellar filaments obtained from strains GP1 and JF1.

Stability of flagellar filaments. The flagellar filaments of *M. hungatei* were unstable in the presence of most of the detergents used (Table 1). Exposure to Triton X-100, Tween 20, Brij 58, CTAB, and Zwittergent 3-14 all resulted in dissociation of the filaments. The filaments were partially dissociated by exposure to Tween 80 (Fig. 1b) and *N*-octylglucoside. Only the zwitterionic detergent CHAPS and the anionic detergent sodium deoxycholate (Fig. 1c) did not significantly disrupt the flagellar filaments.

The marked instability of the flagellar filaments to detergents did not extend to temperature. The flagellar filaments of *M. hungatei* were stable at 40, 60, and 80°C (Fig. 2 and 3a). At 90°C, the filaments were partially dissociated (Fig. 2 and 3b). Electron microscopy of a filament preparation heated to 100°C for 10 min did not reveal any intact filaments (Fig. 3c). However, a weak band appeared on SDS-PAGE (Fig. 2).

Intact flagellar filaments are often resistant to proteases (21). In this case, intact *M. hungatei* flagellar filaments were not significantly digested by trypsin, pronase, or chymotrypsin under the conditions used, as determined by direct electron microscopic examination (Table 1). In our observation, treatment with proteases reduced any background debris usually present in isolated flagellar filaments.

In addition to the treatments described above, *M. hungatei* flagellar filaments were tested for stability to a range of pHs (Fig. 4), to chaotropic agents, and to EDTA treatment. The complete results from studies of the physical stability of *M. hungatei* flagella are summarized in Table 1. The results of the physical stability studies of *M. hungatei* flagella suggested a protocol for visualizing flagellar basal structures from *M. hungatei.*

Visualization of the flagellar basal structure. Previous attempts to visualize basal bodies from *M. hungatei* were largely unsuccessful because of the use of Triton X-100, which dissociates the filament, although an intact flagellum had been reported in two studies (5, 19). On the basis of the detergent sensitivity results, both deoxycholate and CHAPS were surveyed for use in visualizing basal bodies. The effects of these two detergents on the diether and tetraether lipids present in the cytoplasmic membrane of *M. hungatei* (32) has not been reported. The visualization of flagellar basal structure was performed by the dithiothreitol spheroplasting procedure (31) or by treatment with $NaHCO_3$ (pH 9.5) (12) followed by dissolution of membrane material with sodium deoxycholate. Both procedures result in separation of the internal cells from the sheath. Isolated flagellar filaments were stable in both the spheroplasting solution and the $NaHCO_3$ solution. In one preparation of spheroplasts produced by the dithiothreitol procedure, a cell was seen separating from the sheath and end plug assembly with attached flagella threaded through the end plug (Fig. 5).

The only reproducible structures that appeared after a number of attempts to visualize *M. hungatei* JF1 basal bodies were simple, small, round knobs at the base of the flagellar filament (Fig. 6). No distinctive ring or hook structures were seen. Longer incubations with detergents or incubations at higher temperatures resulted in no visible structures at all.

Amino acid composition. While structural information about the flagellar filaments is important for an understanding of flagellar structure and function, it must be complemented by information at the molecular level. With this in mind, we have examined the primary structure of the component flagellins. The amino acid compositions of the 35,000-M_r flagellin from *M. hungatei* JF1 and both the 24,000- and the 25,000-M_r flagellins from *M. hungatei* GP1 were determined (Table 2). Like other flagellins (both archaeal and bacterial), the three *M.*

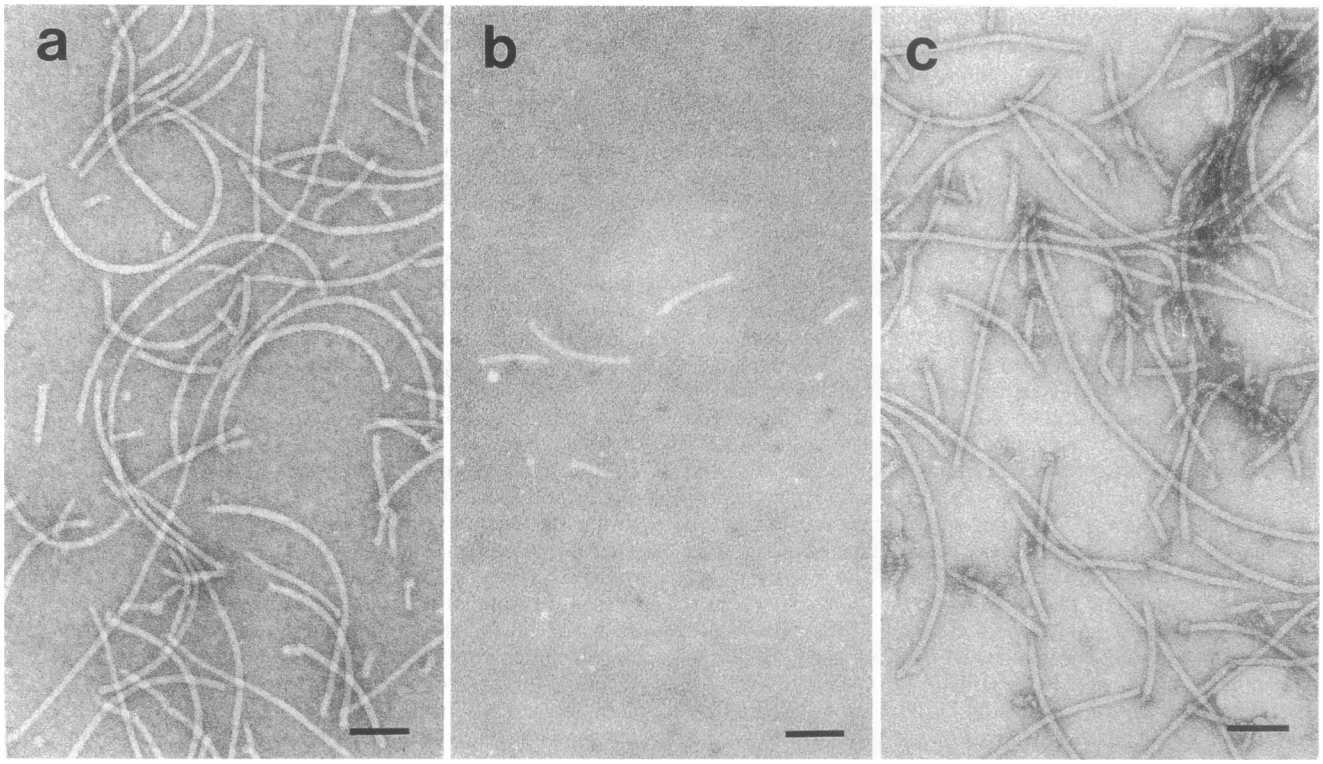

FIG. 1. Electron micrographs of negatively stained preparations (1% uranyl acetate) of isolated *M. hungatei* flagellar filaments before and after treatment with Tween 80 (a and b, respectively) and after treatment with sodium deoxycholate (c). Bars = 100 nm.

hungatei flagellins have low levels of cysteine and histidine and high levels of serine, threonine, and glycine. There are, however, higher levels of proline, tyrosine, phenylalanine, and arginine in the *M. hungatei* flagellins than are common in bacterial flagellins.

Protease digestion and cyanogen bromide cleavage. The flagellins proved to be quite resistant to trypsin, chymotrypsin, endoproteinase Glu-C, and endoproteinase Lys-C (data not shown). Digestion of flagellin monomer from strain GP1 by 48 h of incubation with 800 μg of trypsin per ml was needed to obtain peptide fragments, while digestion for 48 h with 3,000 μg of trypsin per ml was needed to obtain significant digestion of strain JF1 flagellins (data not shown). These fragments could not be used for amino acid sequencing because of the contamination of trypsin and trypsin self-digestion peptides.

Cleavage by cyanogen bromide was used to generate peptide fragments for sequencing. The 35,000-M_r flagellin from *M. hungatei* JF1 was not cleaved by cyanogen bromide (under the same digestion conditions that were successful for the GP1 flagellins) (Fig. 7, lane 2). *M. hungatei* GP1 flagellins (both the 24,000- and the 25,000-M_r flagellins) were cleaved by cyanogen bromide (Fig. 7, lane 4), and three of the resulting peptides (with M_rs of approximately 5,000, 7,000, and 12,000) were sequenced (Fig. 7). Because of the mixed nature of the samples (depolymerized flagella containing both the 24,000- and the 25,000-M_r flagellins), the cyanogen bromide fragments could be from either one or both of the flagellins. Clearly, both flagellins are cleaved by cyanogen bromide. A Western blot (immunoblot) of the GP1 flagellin cleaved by cyanogen bromide and trypsin (data not shown) indicates that the fragments, while still immunologically cross-reactive, are significantly less so than the intact flagellins.

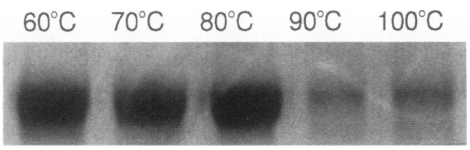

FIG. 2. SDS-PAGE of flagellar filaments after heating (10 min) at the indicated temperatures and recovery by centrifugation. Only the relevant part of the gel is shown.

DISCUSSION

We have examined the effects of a variety of physical and chemical treatments on *M. hungatei* flagellar filaments. In the case of exposure to extremes of pH, chaotropes, and proteases, the stability of *M. hungatei* flagellar filaments is similar to that of bacterial flagellar filaments (27). However, temperature and some detergents appeared to affect *M. hungatei* flagellar filaments very differently from bacterial flagellar filaments.

There are no previous reports of archaeal flagellar filaments being stable at high temperatures, although the flagellar filaments of motile hyperthermophilic archaea must be intact at their growth temperatures (by definition, above 80°C). The stability of *M. hungatei* flagellar filaments at 80°C is in sharp contrast to that of the flagellar filaments of *S. typhimurium* (which are known to be dissociated at 50°C) (27). Dissociation at pH 2 is a common feature of bacterial flagellar filaments (27) and a property also exhibited by the filaments of *M. hungatei*. Such acid dissociation is often a starting point for obtaining reassociated flagellar filaments and has been used to purify flagellins (6, 20, 27). It may be possible to use pH 2.0 dissociation-reassociation of *M. hungatei* flagellar filaments to

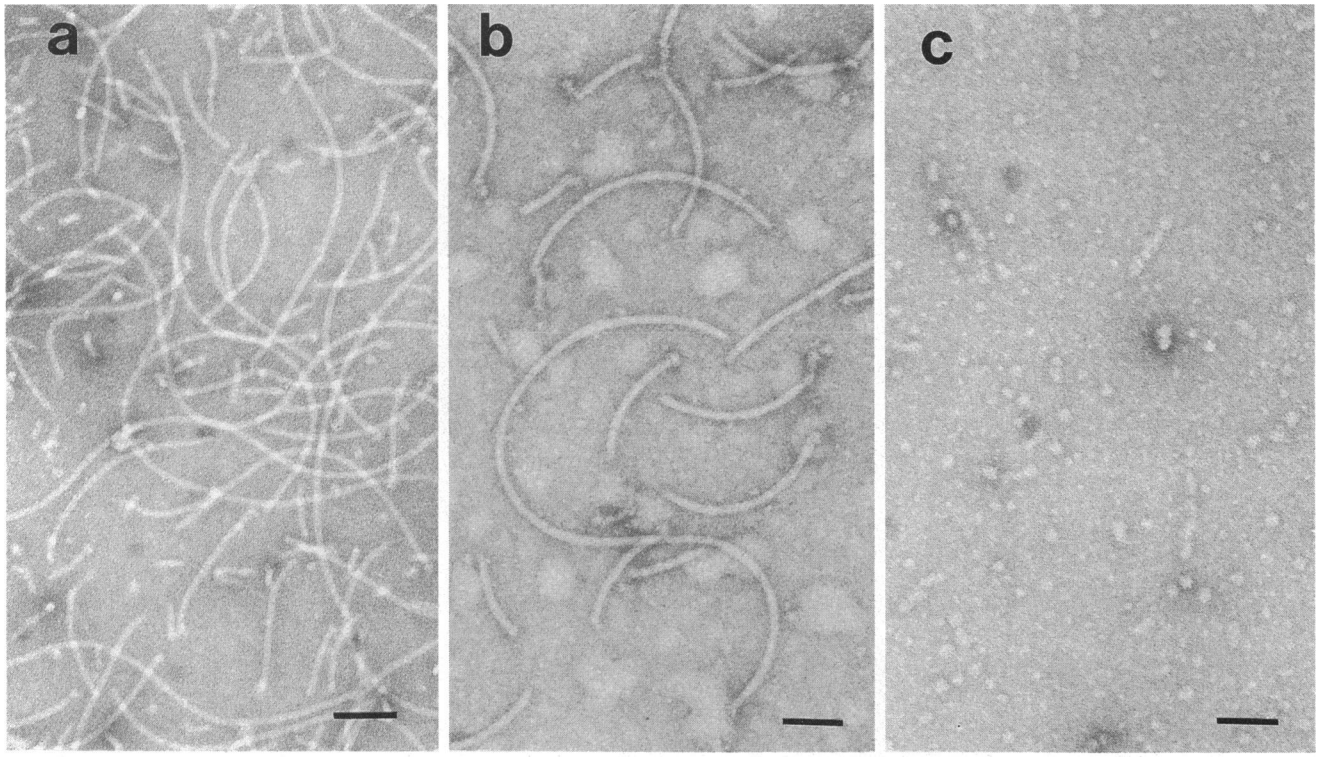

FIG. 3. Electron micrographs of negatively stained (1% uranyl acetate) preparations of *M. hungatei* flagellar filaments after treatment for 10 min at 80°C (a), 90°C (b), and 100°C (c). Bars = 100 nm.

examine the ability of 24,000- or 25,000-M_r flagellins to reform filaments alone, although that is beyond the scope of this work.

We have previously shown that flagellation in *M. hungatei* is affected by the Ca^{2+} concentration in the growth medium (10). Furthermore, it has been shown that divalent cations are required in *Rhizobium meliloti* for flagellar filament stability (26). For these reasons, we investigated the stability of *M. hungatei* flagellar filaments in various concentrations of the Ca^{2+} chelator EDTA. Since the *M. hungatei* filaments were stable in all the concentrations of EDTA examined, it appears that the loss of flagellation of *M. hungatei* cells in low-Ca^{2+} growth conditions is, unlike the case for *Romeliloti*, not due to flagellar filament instability.

The dissociation of flagellar filaments by nonionic detergents such as the Triton and Tween series is, to our knowledge, unknown in bacteria, and indeed, Triton X-100 is often used in the isolation of flagella (6, 7). Sensitivity of flagellar filaments to dissociation by Triton X-100 has been observed in a number of archaea (9). The ability of a detergent to dissociate *M. hungatei* flagellar filaments is not based on critical micellar concentration (CMC) or on charge but may be related to the micellar size of the detergent, as both CHAPS and deoxycholate form small micelles (14). When the structures of the detergents that do not dissociate the filaments are examined, a bulky steroid ring structure is the nonpolar moiety in both deoxycholate and CHAPS.

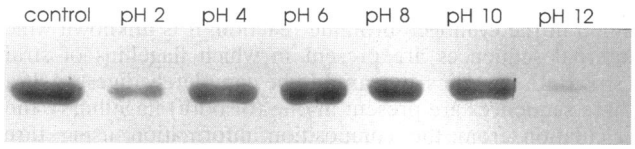

FIG. 4. SDS-PAGE of flagellar filaments after treatment (1 h at 37°C) at the indicated pHs and recovery by centrifugation. Only the relevant part of the gel is shown.

The finding that deoxycholate and CHAPS did not dissociate the filaments allowed their use in visualizing the basal body structure of *M. hungatei*. The basal body structures of two other methanogens, *M. voltae* and *Methanococcus vannielii*, have been reported to be simple knob-like structures (19), while that of *Methanococcus thermolithotrophicus* has been reported to be a two-ring structure (similar to that of gram-positive bacteria) (5). *M. voltae*, *M. vannielii*, and *M. thermolithotrophicus* have a simple cell envelope structure consisting of an S-layer surrounding the cytoplasmic membrane (16).

From our study, the basal structure of *M. hungatei* is also simple. No discernible ring or hook structures are apparent, although the latter may be obscured by attached membranous material. Three other studies have reported *M. hungatei* flagellar basal structure; in two, Triton X-100 was used in the process (5, 19), while in the third study no detergents were used (3, 34). In this study as well as several others (5, 9, 29), Triton X-100 has been shown to dissociate *M. hungatei* flagellar filaments. Furthermore, each of the previous studies presented a different structure. The basal structure observed in this study most closely resembles that found by Southam as reported by Wilson and Beveridge (34).

The flagella of *M. hungatei* must span the length of the end plug assembly, which may be as large as 80 to 100 nm (29). In Fig. 5, the flagella are still anchored to the cytoplasmic membrane but have been threaded through the end plug (or at least the component of the end plug responsible for the accumulation of negative stain). Clearly, if any structure exists

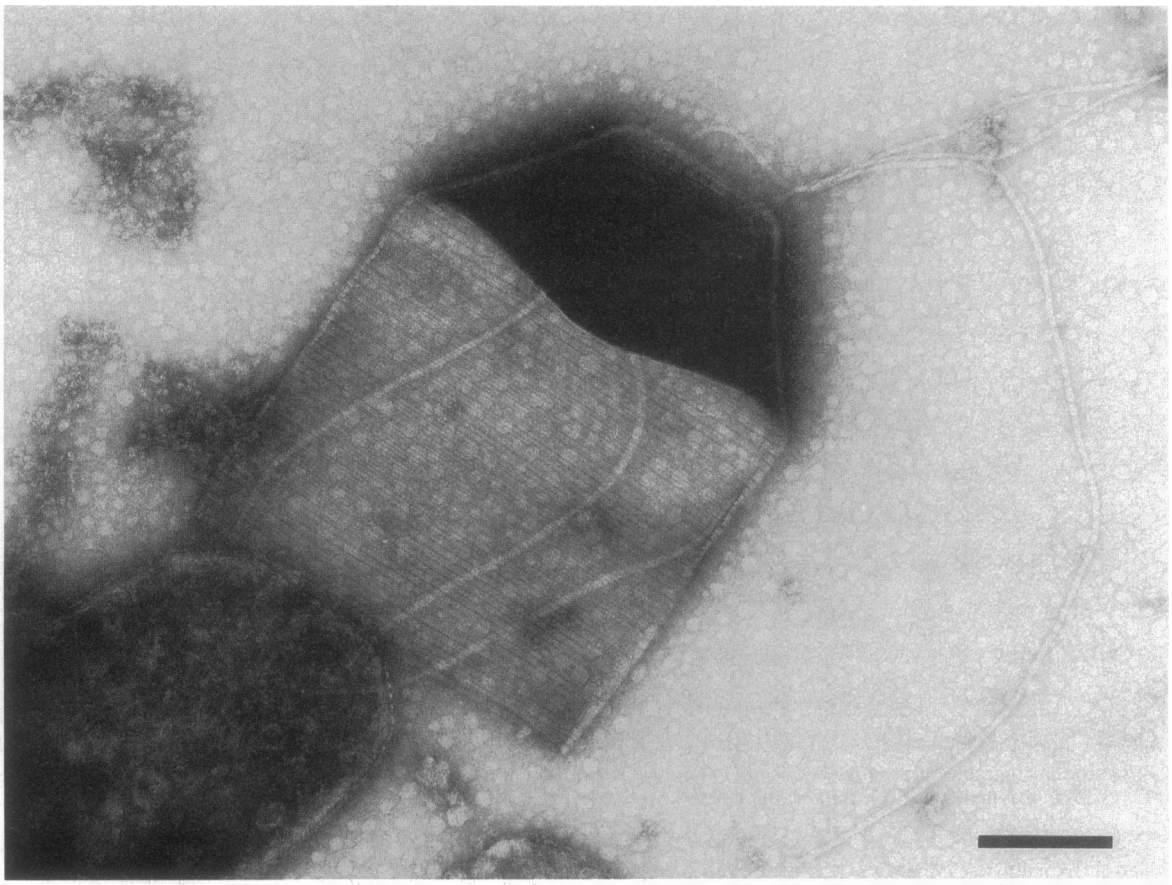

FIG. 5. Negatively stained preparation (1% uranyl acetate) of an *M. hungatei* cell in the process of spheroplasting. Note the flagella, still attached to the spheroplast and threaded through the end of the sheath. Bar = 150 nm.

at the flagellum-end plug junction, it is not an integral part of the flagellum itself.

It may seem odd that an organism with such a complex envelope ultrastructure has a simple flagellar basal structure. However, there are no reported additional structures or modifications of flagellar basal bodies in bacteria that possess S-layers. Although complex, the ultrastructure of *M. hungatei* has been described as an assortment of S-layers (12, 28). In fact, at the poles (where the cell is flagellated), without the end plug the cell envelope of *M. hungatei* is analogous to that of *M. voltae*.

The basal structures observed in gram-positive organisms lack the L and P rings seen in gram-negative bacteria. These rings are thought to act as a bushing in gram-negative bacteria, a job performed by the thick peptidoglycan layer in gram-positive bacteria. The end plug structure probably accomplishes the same function in *M. hungatei* (29). The important components in terms of physiological function (e.g., the motor and switch proteins) of the basal structure in bacteria are not revealed by the techniques employed here but require more elegant and sophisticated techniques for visualization (7, 20).

Archaeal flagella are different from bacterial flagella, not only at the ultrastructural level, but also at the molecular level. Archaeal flagella are composed of multiple flagellins that in primary sequence are not homologous to bacterial flagellins but do have extensive homology at the N terminus among themselves. Since archaeal flagellins are often glycosylated, it is possible that multiple flagellins could arise from different posttranslational modifications of the same gene product rather than multiple gene products. However, in both of the archaea whose flagellin genes have been cloned, multigene families have been identified (i.e., *M. voltae* [18] and *H. halobium* [13]), although the number of genes does not correlate with the number of flagellin bands observed upon SDS-PAGE analysis of purified flagellar filaments.

In *M. hungatei*, the amino acid compositions of the three flagellins suggest that the flagellins are in fact related but different gene products. *M. hungatei* flagellins have a higher proportion of proline residues (mol%, 3 to 5) than is common in bacterial flagellins (average mol% of all bacterial flagellins available, 0.81 [15, 34]). The *M. hungatei* JF1 35,000-M_r flagellin also contains cysteine (mol%, 2.76), which is extremely uncommon in bacterial flagellins (average mol%, 0.02 [15, 34]). The deduced amino acid compositions of flagellin genes of *M. voltae* and *H. halobium* have higher proportions of proline (mol%, 1.5 to 3) than bacterial flagellins, and the *flaA1* gene of *M. voltae* codes for 0.91 mol% cysteine (two residues).

Since the 24,000- and the 25,000-M_r flagellins were combined in the cyanogen bromide reaction, it is unknown which internal sequences are present in which flagellins of strain GP1. Both flagellins appeared to be completely digested. If all three sequences are present in one (or both) flagellin(s), then calculation from the composition information using three methionines (based on the number of fragments generated by the cyanogen bromide cleavage) indicates that the proteins would be over 400 residues long and would have an apparent

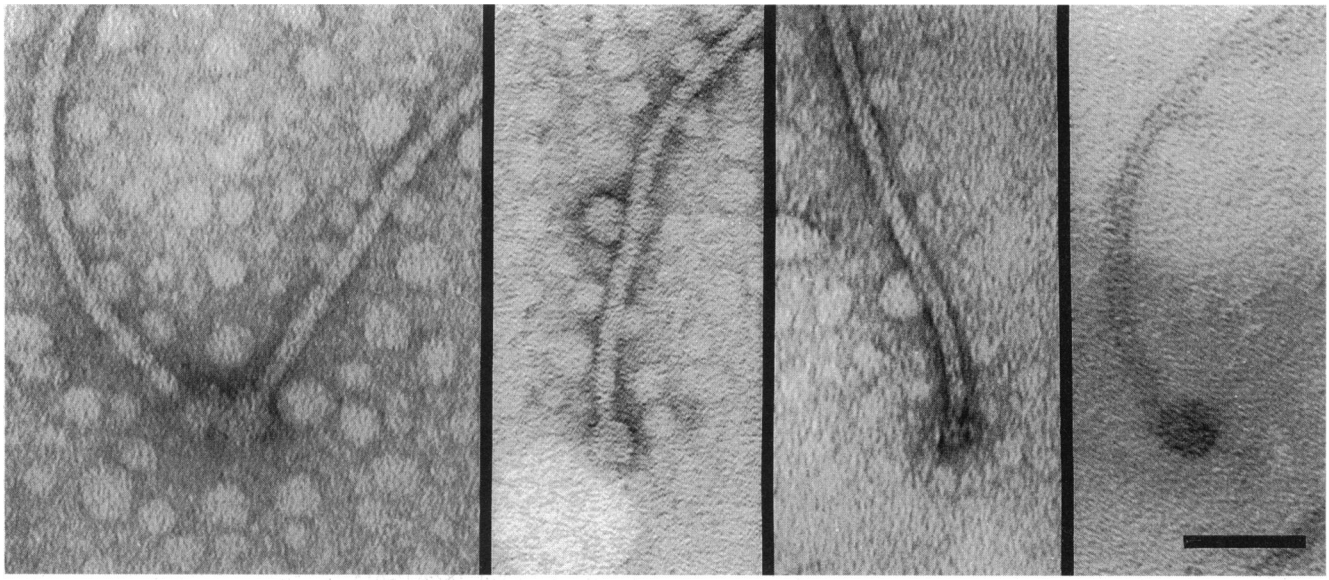

FIG. 6. Several *M. hungatei* flagella with attached basal structures prepared by treatment of envelope preparations with sodium deoxycholate (negatively stained with 1% uranyl acetate). Note the absence of defined hook or ring structures. Bar = 75 nm.

molecular weight of more than 44,000, which is clearly not the case. Thus, either the composition of methionine is not accurate (it is not uncommon to underrepresent methionine under the conditions employed) or all three sequences are not present in the same flagellin. However, the three *M. hungatei* flagellins are identical for at least the first 20 amino acids at the N terminus (8, 29).

No sequences homologous to the three internal sequences were found by using BLAST electronic mail searches (2). However, the sequence from the smallest fragment (approximate M_r = 5,000) is high in proline, and *M. voltae* flagellins have regions rich in proline near the C terminus (18). The 7,000-M_r sequence and a minor contaminating secondary sequence (not shown) from the 12,000-M_r fragment are the same. The first eight residues of the 7,000-M_r fragment are identical to the last eight residues (following a methionine) of the N-terminal sequence for the 25,000-M_r flagellin (Fig. 7). Thus, with reasonable assurance, we know the first 48 residues of at least one of the *M. hungatei* GP1 flagellins.

The serendipitous sequencing of overlapping fragments will make it easier to generate an oligonucleotide probe from the amino acid sequence, one of the primary goals for obtaining internal sequence information. To date, no genes from *M. hungatei* have been cloned and sequenced. We have been unsuccessful in isolating the gene(s) for *M. hungatei* flagellins

TABLE 2. Amino acid compositions of flagellins from *M. hungatei* GP1 and JF1

Amino acid	Mol% of flagellin		
	JF1 35,000 M_r	GP1 25,000 M_r	GP1 24,000 M_r
B	8.01	9.07	7.19
Z	12.97	12.21	9.27
S	11.10	7.80	10.87
G	11.92	15.94	16.55
H	0.00	0.00	0.00
R	5.47	0.92	1.70
T	7.42	12.80	11.22
A	5.47	6.00	8.58
P	5.05	3.13	3.37
Y	3.45	3.84	3.66
V	5.47	6.78	6.49
M	1.88	0.71	0.62
C	2.76	0.00	0.00
I	3.30	5.19	3.30
L	8.41	7.08	8.15
F	3.10	3.30	2.67
K	3.99	5.92	6.65
W	ND[a]	ND	ND

[a] ND, not determined by the method employed.

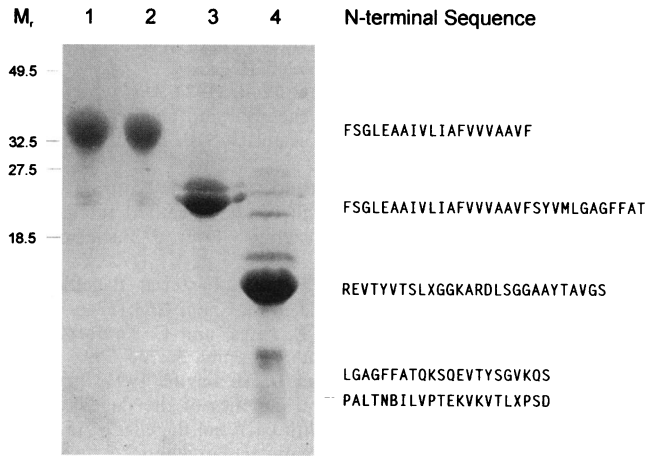

FIG. 7. Cyanogen bromide cleavage and sequencing of the resulting fragments for *M. hungatei* flagellins. Lanes 1 and 2, control and cyanogen bromide-treated JF1 flagellins, respectively (there is no observable cleavage of JF1 flagellins); lanes 3 and 4, control and cyanogen bromide-treated GP1 flagellins, respectively (24,000 and 25,000 M_r). The amino acid sequences for three of the cyanogen bromide-generated fragments (with approximate M_rs of 5,000, 7,000, and 12,000) and the N-terminal sequences for the three flagellins themselves (which are identical for at least the first 20 residues) are shown. M_rs are indicated in thousands.

by using a mixed oligonucleotide probe generated from the N-terminal amino acid sequence, the procedure used successfully for isolating the flagellins of *M. voltae* (18). Internal sequence data should allow use of PCR techniques to generate a partial flagellin sequence which would be more successful in Southern hybridizations.

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