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Isolation of Flagella from the Archaebacterium *Methanococcus voltae* by Phase Separation with Triton X-114

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The flagella of *Methanococcus voltae* were isolated by using three procedures. Initially, cells were sheared to release the filaments, which were purified by differential centrifugation and banding in KBr gradients. Flagella were also prepared by solubilization of cells with 1% (vol/vol) Triton X-100 and purified as described above. Both of these techniques resulted in variable recovery and poor yield of flagellar filaments. Purification of intact flagella (filament, hook, and basal body) was achieved by using phase transition separation with Triton X-114. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified flagella revealed two major proteins, with molecular weights of 33,000 and 31,000. This result indicates the likely presence of two flagellins. The filament had a diameter of 13 nm. The basal structure consisted of a small knob, while a slight thickening of the filament immediately adjacent to this area was the only evidence of a hook region. Flagella from three other *Methanococcus* species were isolated by this technique and found to have the same ultrastructure as flagella from *M. voltae*. Isolation of flagella from three eubacteria and another methanogen (*Methanospirillum hungatei* [*M. hungatii*]) by the phase separation technique indicated that the detergent treatment did not affect the structure of basal bodies. Intact ring structures and well-differentiated hook regions were apparent in each of these flagellar preparations.

The methanogenic archaebacterium *Methanococcus voltae* is a motile marine coccus with tufts of flagella and a type three archaebacterial cell envelope, i.e., an envelope in which the sole component lying external to the cytoplasmic membrane is a regularly structured protein layer (17, 37). In *M. voltae*, this protein layer consists of component subunits (M_W, 75,000) arranged in a hexagonal array with a center-to-center spacing of 10 nm (18). The insertion of a flagellum into this unusual type of envelope, characteristic of many archaebacteria, is expected to be different from that observed in the multilayered envelopes of eubacteria.

Little work has been reported on the characterization of the flagella of methanogenic archaebacteria. Recent work on a member of the extremely halophilic branch of archaebacteria, *Halobacterium halobium*, demonstrated that the flagellar filament consisted of three different sulfated glycoproteins (1, 39). In the case of the methanogens, neither the biochemistry nor the morphology of flagella has been well characterized. A fundamental question is whether methanogen flagella are structurally or biochemically similar to those found in eubacteria or halophiles. Both flagella and fimbriae are present on methanogens (11) and have been demonstrated in *M. voltae* (18).

Eubacterial flagella have been well characterized (3, 13, 22, 27, 28) and shown to consist of three morphologically distinct regions (filament, hook, and basal body). The flagellar filament is responsible for propelling the cell and, in most cases, consists of an assembly of one type of flagellin subunit, although there are exceptions, for example, in *Caulobacter crescentus* (12), *Bdellovibrio bacteriovorus* (34), and *Treponema phagedenis* (21). The hook acts as a universal joint or flexible coupling between the basal body and the filament. The basal body has the dual function of both anchoring the filament and hook to the cell and providing the rotational motion of the flagellum. This complex structure consists of a central axis with several stacked rings (usually four in gram-negative bacteria and two in gram-positive bacteria). Flagellar rotation is driven by the free energy stored across the cytoplasmic membrane via an electochemical proton gradient (24) or, in some marine and alkalophilic bacteria, by a sodium motive force (29). In some organisms, there may also be a number of membrane-associated ring structures (7).

M. voltae represents an excellent system for the study of methanogen flagella since the organism is well characterized, grows well in defined medium (38), and is the sole methanogen in which a genetic transfer system has been developed (4). In addition, this organism is osmotically sensitive, and therefore, the production of spheroplasts, a step common to other flagellum isolation procedures (9, 10), is not required.

In the current study, three methods were used to isolate flagella from this organism: shearing, solubilization of intact cells with Triton X-100, and phase transition separation of solubilized envelope proteins with Triton X-114. The technique of phase transition separation with Triton X-114 was suitable for the isolation of intact flagella (hook, filament, and basal structure) from various methanogens, as well as from three eubacteria. This method may have application for other bacterial systems.

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MATERIALS AND METHODS

Bacteria and growth conditions. *M. voltae* was obtained from L. Hook (Ohio State University, Columbus); *M. vannielii*, *M. deltae*, and *M. maripaludis* were obtained from W. B. Whitman (University of Georgia, Athens), and *Methanospirillum hungatei* (*M. hungatii*) JF1 was obtained from G. D. Sprott (National Research Council, Ottawa, Canada).

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Methanogens were maintained at 37°C in Balch medium III (2; *M. voltae*, *M. deltae*, and *M. maripaludis*), modified Balch medium III (containing 4 g of NaCl per liter; *M. vannielii*), or JMA media (14; *Methanospirillum hungatei*). For flagellum isolation, cells were grown at 37°C in 1-liter bottles modified to accept serum stoppers (Pegasus Industrial Specialties, Scarborough, Ontario, Canada). Cultures were pressurized daily with CO_2-H_2 (1:4 [vol/vol]) and harvested in early stationary phase.

The eubacteria utilized were *Escherichia coli* K30 (from C. Whitfield, University of Guelph, Guelph, Ontario, Canada) and *Pseudomonas aeruginosa* (Queen's University departmental strain; from D. Agnew), and both were maintained on yeast extract slants at 4°C. For isolation of flagella, these cultures were grown in 1 liter of LB broth at 37°C with agitation at 150 rpm. *Aquaspirillum serpens* VHA was maintained on yeast extract-peptone-sodium acetate medium and grown in 100 ml of minimal medium as previously described (19). All cells were harvested by centrifugation at 6,000 × *g* for 10 min at 4°C.

Preparation of flagella. (i) Shearing. *M. voltae* cells (from 2 liters of culture) were suspended into approximately 200 ml of 0.1 M Tris buffer (pH 7.5) containing 2.0% (wt/vol) NaCl and sheared at the high speed setting of a Waring blender (Waring Products Co., Winsted, Conn.) for 90 s (23). The sheared suspension was centrifuged (6,000 × *g* at 4°C) to remove the whole cells, and the supernatant, containing the flagella, was centrifuged at 80,000 × *g* for 90 min. The pellet containing sheared flagella was suspended in buffer overnight at 4°C, loaded onto a KBr gradient (0.5 g of KBr per ml [25]), and centrifuged for 24 h at 260,000 × *g*. The resulting single diffuse band (which usually occurred in the bottom third of the centrifuge tube) was recovered and dialyzed against distilled water.

(ii) Solubilization of intact cells. *M. voltae* cells (from 1 liter) were gently suspended in 200 ml of 0.1 M Tris buffer (pH 7.5) containing 2% NaCl. Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) was slowly added to a final concentration of 1% (vol/vol). Addition of the detergent resulted in the immediate lysis of the cells and a marked increase in the viscosity of the solution which was reduced by the addition of DNase (Boehringer Mannheim Canada, Laval, Quebec, Canada) and $MgCl_2$. The solution was centrifuged at 80,000 × *g* for 90 min, and the pellet containing flagella was suspended overnight in 10 ml of 0.05 M Tris buffer (pH 7.5). Flagella were subsequently purified by banding in a KBr gradient as described above.

(iii) Phase separation. For the phase transition separation of flagella with Triton X-114 (Sigma), the starting material was the envelope fraction from each of the above bacterial strains. All four *Methanococcus* species were lysed by suspension of the harvested cells in distilled water with gentle mixing and DNase treatment to reduce solution viscosity. Envelopes were recovered by centrifugation (22,000 × *g* for 30 min at 4°C). Spheroplasts of *E. coli* and *P. aeruginosa* were prepared in the presence of lysozyme and EDTA by the method of Suzuki et al. (33). Spheroplasts of *A. serpens* were prepared as reported by Koval and Murray (19). Spheroplasts were lysed by suspension in distilled water containing DNase, and the envelopes were recovered by centrifugation. For *Methanospirillum hungatei*, spheroplasts were prepared by the alkaline-dithiothreitol method (31). Cell lysis and envelope recovery were as described above.

The phase transition separation procedure (Fig. 1) was adapted from the method of Bordier (5). Envelopes were suspended in cold 10 mM Tris hydrochloride (pH 7.5) containing 150 mM NaCl (Tris-buffered saline) and treated with 1% (vol/vol) Triton X-114 for 30 min at 4°C with occasional mixing. For eubacteria, 5 mM EDTA can be included with the detergent. The samples were then incubated at 37°C to induce phase separation and centrifuged at 300 × *g* for 3 min. The aqueous phase was removed, and the oily droplet at the bottom of the tube (detergent phase) was discarded. The combined aqueous phase (approximately 17 ml) was concentrated to a 1.0-ml volume with an ultrafiltration apparatus (model 8010; Amicon Corp., Lexington, Mass.) with a PM 30 filter (M_w cutoff, 30,000; Amicon).

Column chromatography. Triton X-100 (1% [vol/vol] final concentration) was added to the aqueous phase of Triton X-114 extraction to solubilize adherent membrane material associated with the flagella. The sample was loaded on a column (30 by 10 cm) packed with Sepharose CL-4B (Pharmacia, Uppsala, Sweden) and fractionated with 10 mM Tris hydrochloride buffer (pH 7.5) containing 1% Triton X-100. Fractions of approximately 0.3 ml were collected, concentrated by ultrafiltration, and examined by minigel sodium

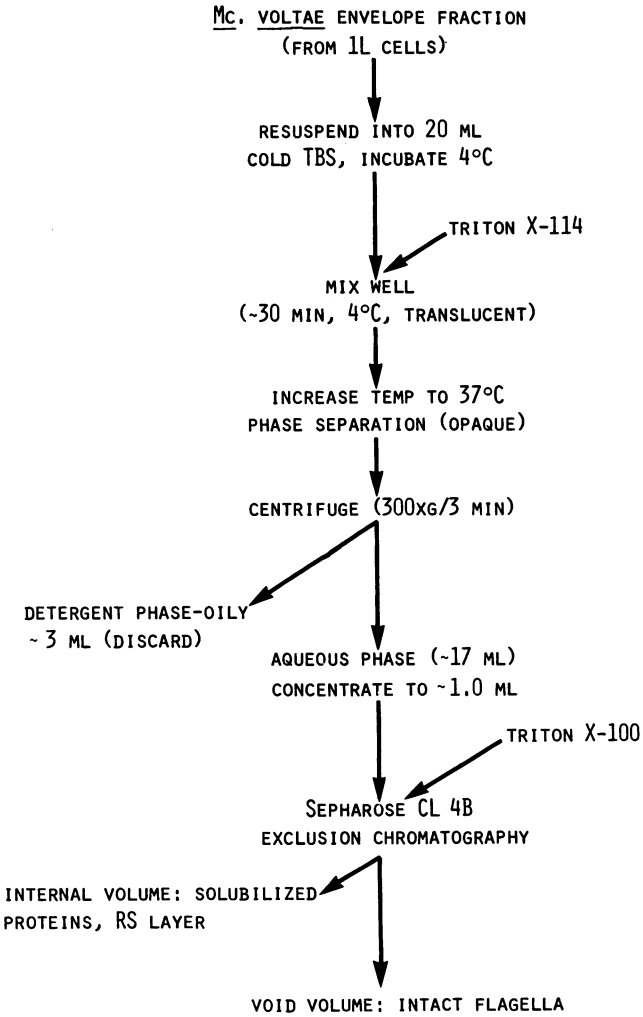

FIG. 1. Flow diagram showing the purification of flagella from *M. voltae* by phase transition separation with Triton X-114. Abbreviations: L, liter; TBS, Tris-buffered saline; TEMP, temperature; RS, wall protein.

dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electron microscopy.

SDS-PAGE. SDS-PAGE was by the method of Laemmli (20), as described previously (18). For rapid analysis of samples, a minigel apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) was routinely used.

Protein determination. Protein determinations were carried out by using the Pierce BCA protein assay reagent kit and albumin standard (Pierce Chemical Co., Rockford, Ill.).

Electron microscopy. Samples were negatively stained and supported on carbon-Formvar-coated copper grids. A 400-mesh grid was inverted over 1 drop of the flagellum preparations in detergent. After 1 min, the grid was washed on 6 drops of water to remove the detergent and stained with 1% uranyl acetate (pH 4.4) containing bacitracin (50 μg/ml) as a wetting agent. Specimens were examined with a Philips EM 300 electron microscope operating at 60 kV.

RESULTS

As a preliminary step in the investigation of the flagella of *M. voltae*, flagellar filaments were isolated by mechanical shearing of cells with a Waring blender. Since this organism is osmotically sensitive, shearing was carried out in buffer containing 2% (wt/vol) NaCl. A crude flagellar preparation was obtained by differential centrifugation, and SDS-PAGE of this material showed the presence of five major bands and a variety of minor components (Fig. 2). SDS-PAGE examination of material from the KBr band indicated the presence of two major protein bands, with molecular masses of 33 and 31 kilodaltons (kDa) (Fig. 2). Flagellar filaments of various lengths and a diameter of 13 nm were seen by negative-staining electron microscopy (Fig. 3).

Treatment of *M. voltae* cells or envelopes with Triton X-100 is effective for solubilizing the regularly structured wall layer (18). It was thus reasoned that whole-cell solubilization might be useful for isolating intact flagella. Cell suspensions were treated with Triton X-100 (1% [vol/vol], final concentration) and subjected to differential centrifugation, and the pellet after centrifugation at 80,000 × *g* was recovered. The resulting pellet had a waxy appearance and was difficult to suspend. SDS-PAGE of the suspended pellet again indicated an enrichment of the 33- and 31-kDa proteins with minor amounts of contaminating proteins (results not shown). No basal bodies on the flagellar filaments were detected by electron microscopy. Both shearing and solubilization of cells with Triton X-100 resulted in variable recovery and poor yields of flagellar filaments. The flagella of this organism may be quite delicate, such that the combined effects of centrifugation and suspension disrupt flagellar structure.

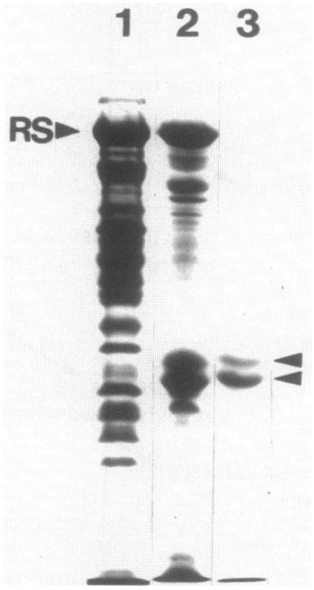

FIG. 2. SDS-PAGE of *M. voltae* proteins in whole cells (lane 1), sheared crude flagellum preparation (lane 2), and KBr gradient-purified flagella (lane 3). The wall protein (RS protein) is shown. Arrows indicate the flagellins.

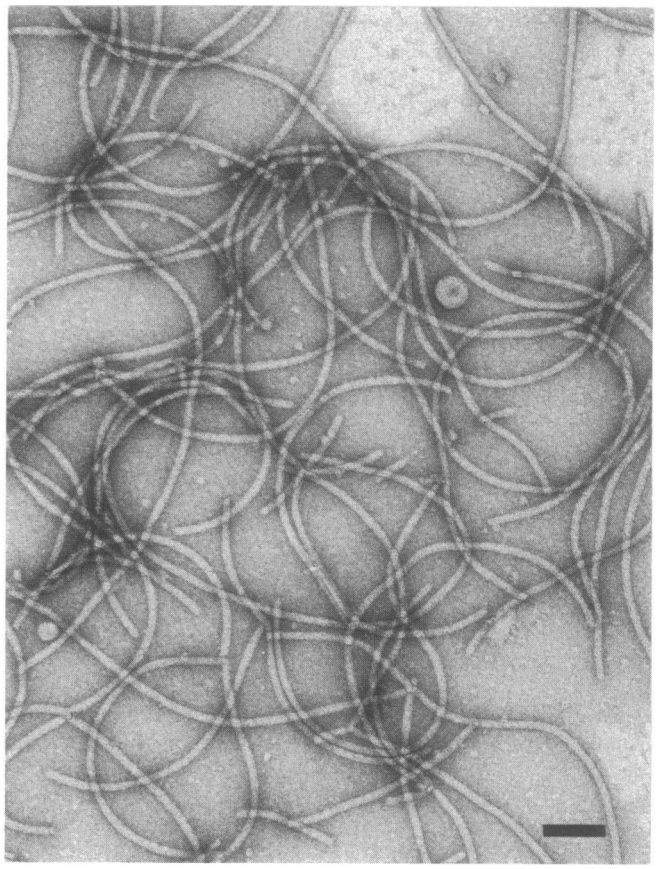

FIG. 3. Negatively stained (1% uranyl acetate) preparations of *M. voltae* sheared flagellar filaments from a KBr gradient. Bar equals 100 nm.

The technique of phase transition separation with Triton X-114 is useful for separating membrane-bound hydrophobic and hydrophilic proteins (5). It was reasoned that the envelope of *M. voltae* may contain very few hydrophilic proteins other than the wall protein and flagellins. Hence, it was thought that this technique could be useful for separating flagella from the majority of hydrophobic proteins present in cell envelopes. Cell envelopes, rather than whole cells, were used as the starting material to reduce contamination with soluble cytosol proteins. Treatment of cell envelopes with Triton X-114 and subsequent separation of the aqueous and detergent phases of centrifugation resulted in an enrichment of the 33- and 31-kDa flagellins, as well as a limited number of other bands (including the wall protein) in the aqueous phase (Fig. 4). Examination by electron microscopy of the

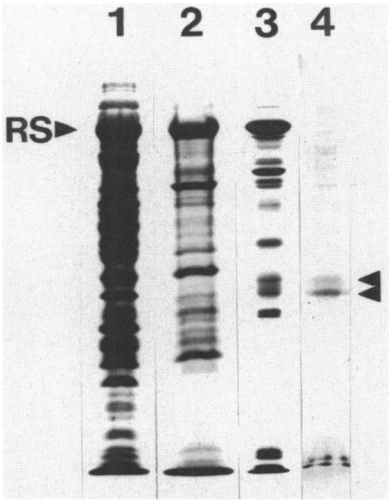

FIG. 4. SDS-PAGE showing purification of intact flagella from *M. voltae* by the Triton X-114 phase separation technique. Lanes: 1, whole cells; 2, cell envelopes; 3, Triton X-114-extracted envelopes (aqueous phase); 4, column-purified intact flagella. Arrows indicate flagellins. The wall protein (RS) is shown.

concentrated crude Triton X-114 aqueous extract from *M. voltae* envelopes revealed some intact flagella. Many of the flagella were still associated with envelope material, as indicated by both electron microscopy and SDS-PAGE (presence of the 75,000-kDa wall protein).

Fractionation of the aqueous extract through a Sepharose CL-4B exclusion column did not greatly enhance the purification of flagellar proteins. This result was likely due to the presence of adherent envelope material, as indicated by the presence of the wall protein (results not shown). Treatment of the aqueous extract with 1% (vol/vol) Triton X-100 and passage of the sample through the exclusion column in a buffer containing 1% (vol/vol) Triton X-100 resulted in the effective separation of intact flagella from envelope proteins, as shown by both SDS-PAGE (Fig. 5) and electron microscopy (Fig. 6). The yield of flagella by this method was 1.6 mg/g of dry cell weight. No basal body containing rings or a hook structure was apparent on any of the flagellar filaments of *M. voltae*. Instead, many of the flagella had a small knob at the end and a slight thickening of the filament in the region corresponding to the hook (Fig. 6). Column-purified flagella did not differ in appearance from those found in the crude aqueous extract. Intact *M. voltae* cells, osmotically protected in 2% NaCl, were also extracted directly with Triton X-114 to determine the effect of sodium on basal body structure. Examination of this material by electron microscopy revealed that these flagella were not structurally different from those isolated in the absence of NaCl (data not shown). This result suggests that there is no requirement for sodium for the maintenance of flagellar structure. Intact flagella were also isolated from *M. vannielii*, *M. deltae*, and *M. maripaludis* by using the phase transition technique. The ultrastructure of *M. vannielii* flagella is shown in Fig. 7. The flagellar structure in all *Methanococcus* species examined was identical to that found in *M. voltae*, i.e., a knoblike basal structure with a poorly differentiated hook.

The lack of a typical basal structure and a well-defined hook region suggested that the detergent treatments may have disrupted these structures. Consequently, the flagella from a number of different organisms were isolated by using the phase separation technique. None of the flagella from these organisms was purified to the same degree as those of *M. voltae*. Triton X-114, like Triton X-100, did not disrupt the gram-negative outer membrane unless EDTA was present. In the absence of EDTA, some free intact flagella were observed. However, many flagella were still attached to the outer membrane, and this complex partitioned into the upper aqueous phase. More free flagella were observed in the Triton X-114-EDTA aqueous extract. Electron microscopic examination of the aqueous phase after Triton X-114 extraction of *A. serpens*, *E. coli*, *P. aeruginosa*, and *Methanospirillum hungatei* JF1 envelopes revealed the presence of flagella (Fig. 7). In all of these organisms, the ring structure of the basal body was well preserved and the hook region can clearly be seen as a differentially staining area adjacent to the basal body. In *Methanospirillum hungatei*, the basal structure consisted of a single pair of rings followed by a knoblike appendage. Further treatment of *Methanospirillum hungatei* crude flagellum extracts with Triton X-100 (1% [vol/vol]) had no effect on the basal body structure. The ring structure of the two eubacteria indicated clear differences in the sizes of the corresponding rings, whereas the lengths of the hook regions were similar. Clearly, the use of Triton

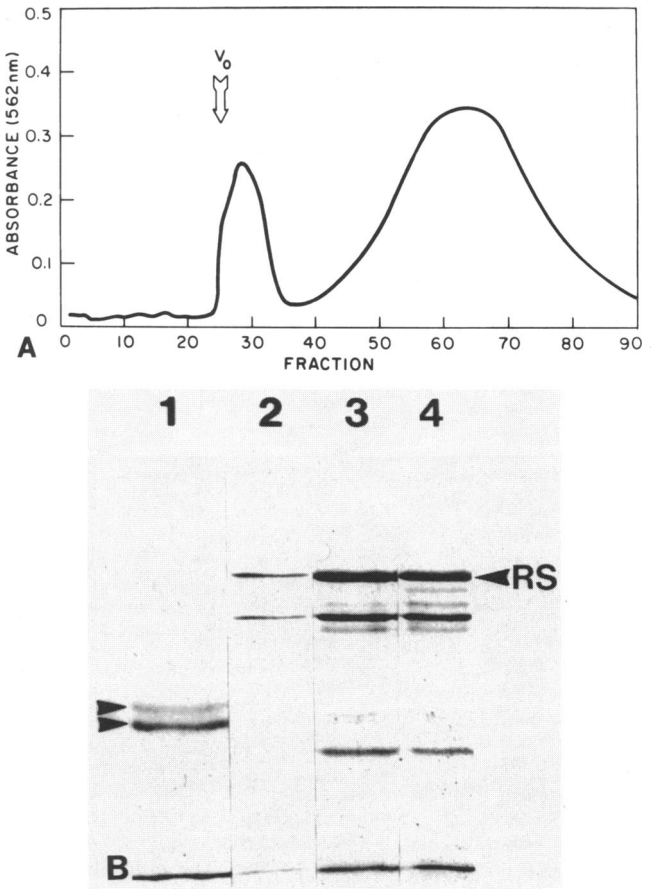

FIG. 5. (A) Elution profile of Triton X-114-extracted *M. voltae* envelopes on Sepharose CL-4B gel developed in 1% (vol/vol) Triton X-100. Fractions were assayed for protein by using the Pierce BCA reagent kit, and A_{562}s were determined. One unit of optical density equals 0.8 mg of protein. V_o, Void volume. (B) SDS-PAGE minigel of selected fractions from column chromatography. Lane 1, Fraction 29; lane 2, fraction 45; lane 3, fraction 55; lane 4, fraction 65. Intact flagella were observed by electron microscopy in the first peak, which corresponded to the void volume. Arrows indicate the flagellins. The wall protein (RS) is shown.

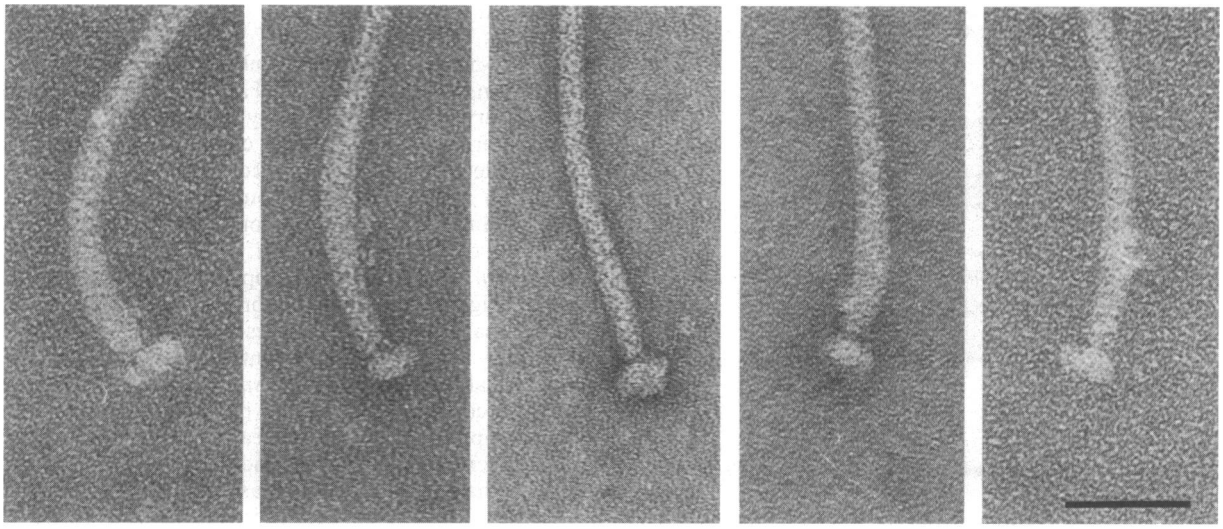

FIG. 6. Several intact flagella from *M. voltae* prepared by Triton X-114 extraction of envelopes and negatively stained with 1% uranyl acetate. Bar equals 50 nm.

X-114 did not affect flagellum basal structure in any of these organisms.

DISCUSSION

Isolation of flagella by mechanical shearing or solubilization of *M. voltae* cells with Triton X-100 resulted in flagellar filaments of various lengths and few intact flagella. While this result was not unexpected for the sheared cell preparations, it was surprising for the Triton X-100 preparation, as this was considered a gentle method of recovering flagella. The inability to recover intact flagella could have been the result of flagellar sensitivity towards Triton X-100 and/or the stress associated with centrifugation, suspension of pellets, and banding common to both isolation methods. The use of Triton X-100 is common in standard flagellum isolation techniques for eubacteria and does not appear to affect flagellar morphology (9). The various lengths of filament which resulted from both shearing and whole-cell solubilization with Triton X-100 suggested that the flagella of *M. voltae* are quite delicate. In addition, both procedures were not very reproducible and resulted in poor yields of flagella.

In conjunction with our work on the cell envelope of *M. voltae* (18), we decided to try a temperature-induced phase separation in Triton X-114 to fractionate envelope proteins. Triton X-114 is a nonionic detergent of the Triton series, which has fewer hydrophilic oxyethylene residues in the molecule. This property results in a cloud point of 20°C for Triton X-114 and the separation of detergent and aqueous

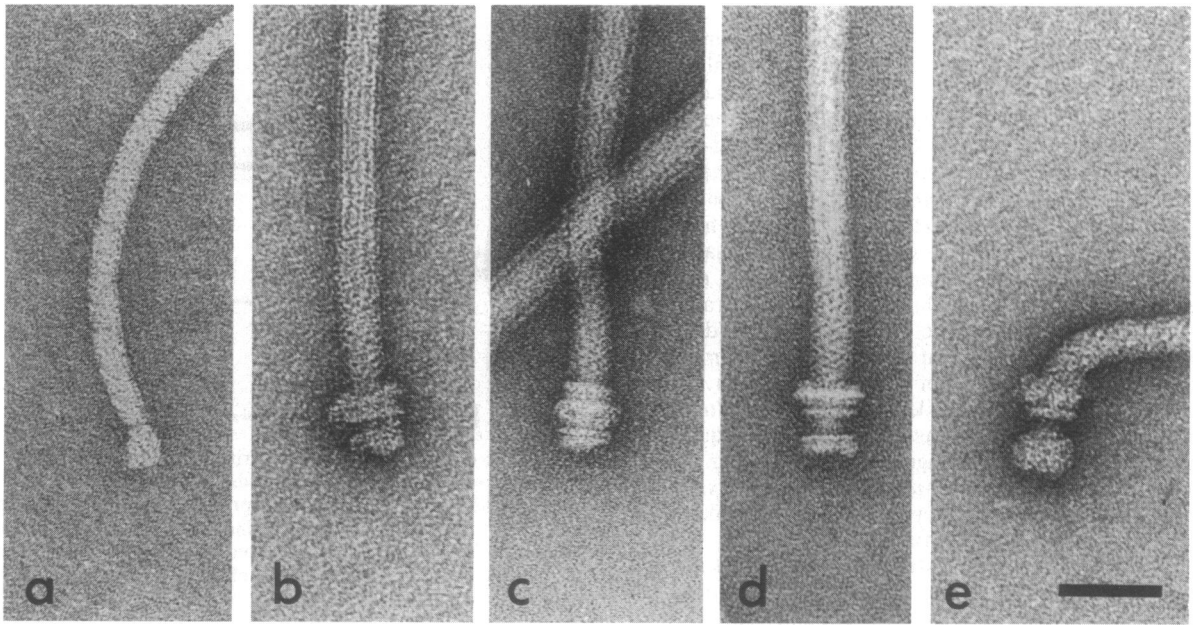

FIG. 7. Intact flagella prepared by Triton X-114 extraction of envelopes and negatively stained with 1% uranyl acetate. (a) *M. vannielii*, (b) *Methanospirillum hungatei*, (c) *E. coli*, (d) *P. aeruginosa*, (e) *A. serpens*. Bar equals 50 nm.

phases above this temperature. We anticipated that the regularly structured wall protein would separate into the upper aqueous phase, while the hydrophobic integral membrane proteins would partition into the lower detergent-rich phase. This result was achieved, but in addition, we found that intact flagella also partitioned into the upper phase because of the hydrophilic nature of the flagellar filament. Fimbriae are more hydrophobic than flagella and were not observed in the aqueous phase. This technique has a distinct advantage for use with archaebacteria possessing this simple envelope structure, since only one membrane is present to dissociate. It should also be particularly useful for gram-positive organisms, although none were tried in this study.

The flagella present in the concentrated crude aqueous extract were long and often still associated with residual envelope material. Treatment of this extract with 1% (vol/vol) Triton X-100 solubilized the contaminating envelope material and allowed the recovery of purified intact flagella in the void volume of the Sepharose CL-4B column.

Analysis of flagella from *M. voltae* indicated that the flagellar filaments were composed of two proteins (i.e., flagellins) with molecular masses of 33 and 31 kDa. The molecular arrangement of the flagellins is not known. In the majority of characterized eubacterial strains, the flagellar filament consists of an assembly of a single flagellin monomer (36). However, the presence of two or more flagellins per filament is not without precedent. Within the eubacteria, both *C. crescentus* (12) and *B. bacteriovorus* (34), for example, possess two or more flagellins, with each flagellin arranged in a distinct region on each filament. Within the archaebacteria, *Halobacterium* flagella contain three different but related flagellins (39), although their organization within the filaments has not yet been reported.

When intact flagella were examined by electron microscopy, an unusual basal structure with a knoblike appearance, but lacking the typical rings of eubacterial flagella, was observed. The structure of basal bodies varies from organism to organism, but in most cases, the basal body consists of a rod and a symmetrical stack of rings (22). However, the relative abundance of this simple basal structure in preparations of purified flagella suggests that these specimens represented the intact flagella of *M. voltae*. A basal structure similar to that of *M. voltae* has been reported for the flagellum of *Bacillus licheniformis* (35).

To determine whether detergent treatment affected the isolation of intact basal bodies, Triton X-114 extractions were carried out on three eubacteria with well-characterized flagellar basal structures. Intact basal bodies (Fig. 7) were readily apparent in crude extracts derived from both *P. aeruginosa* and *E. coli*. Some intact basal bodies were seen in crude extracts of *A. serpens* flagella, but the yield was not improved over that reported by Coulton and Murray (8). Flagella isolated from *Methanospirillum hungatei*, another methanogen with an envelope structure quite distinct and more complex than that found in *Methanococcus* species (30, 31), had basal structures consisting of two rings (as found in gram-positive eubacteria) with small knoblike appendages at the end. It is interesting to note that isolated sheath fragments of *Methanospirillum hungatei* also partitioned into the aqueous phase (data not shown). With these four organisms, the hook region was also clearly seen in negative stains as a differentially staining area adjacent to the basal body. Thus, phase transition separation with Triton X-114 did not disrupt the basal structure in either the eubacteria or *Methanospirillum hungatei*.

An additional factor which may have affected the isolation of intact flagella is salt concentration. *M. voltae* is a moderate halophile and requires 0.4 M (2% [wt/vol]) NaCl for optimal growth (38). Although sodium ions do not appear to play a role in the stability of the regularly structured wall protein (18), there could be a dependence for sodium for the maintenance of basal body integrity. Phase transition separation in the presence of 2% (wt/vol) NaCl yielded crude flagellum preparations containing flagella which did not differ in appearance from those isolated in the absence of a high salt concentration. Clearly, sodium concentration does not play a role in basal body integrity.

It appears that all *Methanococcus* species may possess flagella with similar ultrastructures. When crude flagellum extracts from *M. maripaludis* (16), *M. vannielii* (32), and *M. deltae* (6) were examined by electron microscopy, all exhibited the same ultrastructure as flagella from *M. voltae*. *M. deltae* was originally characterized as nonmotile, but we found it to be flagellated. The flagella of *M. jannaschii* are inserted as two bundles near one pole of the cell (15), but the ultrastructure of the proximal end of the flagellum has not been described. The absence of rings (normally associated with different layers of the complex eubacterial envelope) may be a consequence of the much simpler wall structure of *Methanococcus* species, which consists solely of a regularly structured protein layer external to the cytoplasmic membrane. The anchoring of the flagellum to this kind of envelope may be unusual and may require a cytoplasmic structure as suggested for *Halobacterium* species (1). Polar membrane-like structures have been observed in *M. voltae* (18) and may be involved in such anchoring. It may be that methanogens which share the type three archaebacterial wall also share this novel type of basal structure. If this is the case, flagellated members of other genera with the type three wall, such as the genus *Methanogenium* (26), would be expected to show the same type of basal structure. This work is currently being carried out.

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