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A Biochemical and Cytological Analysis of the Complex Periplasmic Flagella from *Spirochaeta aurantia*

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The periplasmic flagella of *Spirochaeta aurantia* were isolated and were found to be ultrastructurally and biochemically complex. Generally, flagellar filaments were 18 to 20 nm in diameter and appeared to consist of an 11 to 13-nm-wide inner region and an outer layer. The hook-basal body region consisted of two closely apposed disks connected to a hook by a rod. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified flagella together with a Western blot analysis of a motility mutant that produces hooks and basal bodies but not flagellar filaments revealed that the filaments were composed of three major polypeptides of 37,500, 34,000, and 31,500 apparent molecular weight (37.5K, 34K, and 31.5K polypeptides) and three minor polypeptides of 36,000, 33,000, and 32,000 apparent molecular weight (36K, 33K, and 32K polypeptides). Purified hook-basal body preparations were greatly enriched in three polypeptides in the range of 62,000 to 66,000 apparent molecular weight. Immunogold labeling experiments with a monoclonal antibody specific for the 37.5K flagellin and one that reacts with an epitope common to the 36K, 34K, 33K, 32K, and 31.5K flagellins revealed that the 37.5K major polypeptide was a component of the outer layer, whereas one or more of the other polypeptides constituted the core.

The spirochetes represent an ancient and phylogenetically distinct group of bacteria (32) possessing a characteristic morphology and a unique type of motility. All spirochetes have flagella that are contained entirely within the periplasmic space, where they wrap around a helical protoplasmic cylinder comprising the peptidoglycan-cytoplasmic membrane complex and the cytoplasmic region (23). One end of each flagellum is inserted near one pole of the cylinder, whereas the other end is free. The number of flagella per cell ranges from 2 to more than 100, depending on the species, with generally equal numbers inserted at each pole (11, 23). These periplasmic flagella are required for motility (10, 28, 31) and, like other bacterial flagella, are thought to propel cells by means of rotation driven by a proton motive force (5, 18).

Morphologically, the periplasmic flagella resemble other bacterial flagella in that they consist of a filament and a hook-basal body (HBB) region. However, the flagellar filaments of a number of spirochetes appear to be relatively complex, consisting of an inner core and an outer layer, which, in some cases, may be covered by a striated sheath (23, 24). This complexity extends itself to biochemical composition; the flagella of many (7, 11, 13, 28, 30, 33) but not all (4) spirochetes are thought to consist of several different polypeptide subunits, although there is often some controversy regarding the number of different flagellar polypeptides for a given species (7, 11, 13, 28). The reasons for this structural and biochemical complexity are not yet clear but may be related to the unusual location of the flagella and to the way they function to propel cells.

Spirochaeta aurantia, a free-living spirochete isolated from freshwater sediments (9), has two periplasmic flagella. Motile cells exhibit runs in relatively straight lines punctuated by reversals of swimming direction and by flexing (16, 21, 22). A model proposed to account for these behaviors defines runs as occurring when the flagellar motor at one end of the cell is rotating clockwise and the motor at the other cell end is rotating counterclockwise; a reversal occurs when both motors switch synchronously, whereas a flex is generated when the flagellar motors are both rotating clockwise or both rotating counterclockwise (16, 21). Furthermore, the swimming pattern of *S. aurantia* is modified by the addition of attractants: runs increase, flexing decreases, and reversals are suppressed (16). These changes in behavior imply a coordinating clockwise-counterclockwise mechanism and a motor-switch-synchronizing device for generation of reversals. Although an electrogenic component has been implicated in *S. aurantia* chemotaxis (19, 20), the nature of the sensory signal has not yet been determined.

S. aurantia is the model for spirochete chemotaxis (16, 19–22, 25) and the only spirochete for which any information on the bioenergetics of motility (18) is available, yet the periplasmic flagella from this species have not been biochemically characterized. As part of ongoing studies of the components of the chemosensory pathway of this organism, we describe here the purification of *S. aurantia* flagella and the ultrastructural and biochemical characterization of the filament and of the HBB region.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used were *S. aurantia* M1 (9) and a nonflagellated mutant, NP-7 (31). For large-scale cultures, 5 liters of GTY broth (22) in a 6-liter Erlenmeyer flask were inoculated with 100 ml of a culture in the late-logarithmic phase. Incubation was at 30°C on a magnetic stirring plate operating at a low-speed setting. After 24 h, the cells were harvested by centrifugation at 4°C. The yield was approximately 1 g (wet weight) of cells per liter, and cell pellets were stored at −70°C.

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Isolation and purification of flagella. Intact flagella were isolated by a modification of the method of Aizawa et al. (1). Frozen cells (7 to 10 g) were suspended to a final volume of 100 ml in an ice-cold sucrose solution (0.5 M sucrose, 0.1 M Tris hydrochloride [pH 8] at 5°C) by gentle stirring at 4°C. Five ml of lysozyme (Sigma Chemical Co., St. Louis, Mo.) (2 mg/ml in 10 mM Tris hydrochloride [pH 8] at 5°C) and 10 ml of 0.1 M EDTA (pH 8.0) were added to the cell suspension, and the mixture was gently stirred for 1 h at 4°C. The cell suspension was then warmed to room temperature, and DNase I (Sigma) was added to a final concentration of 50 μg/ml. The cells were lysed by the dropwise addition of 20 ml of 15% (vol/vol) Triton X-100 in deionized water. At this point, the suspension clarified considerably and became very viscous. Then 10 ml of 0.1 M $MgSO_4$ was added, and the suspension was stirred at room temperature until the viscosity decreased, which occurred within the first minute. Cells and cell debris were removed by low-speed centrifugation (3,000 × *g*, 10 min). The supernatant fluid containing the flagella was then subjected to high-speed centrifugation (197,000 × *g*, 10°C, in a Beckman SW41 Ti rotor for 1 h), and the resulting pellets were suspended in TET buffer (10 mM Tris hydrochloride, 5 mM EDTA, 0.1% Triton X-100 [pH 8.0]) (1) by gentle stirring at 4°C for 1 h. An equal volume of 4% (vol/vol) Triton X-100 (in deionized water) was added, and stirring was continued for another hour. Insoluble debris were removed by low-speed centrifugation (3,000 × *g*, 10 min), and the supernatant fluid was subjected to high-speed centrifugation as above. The resulting pellets were suspended in 22 ml of TET buffer to which 10 g of solid KBr was added. The mixture was then centrifuged in a Beckman SW41 Ti rotor at 178,000 × *g* at 15°C for 36 h. The flagella formed bands in the center of the tube and were collected with a Pasteur pipette, dialyzed against TET buffer, collected by high-speed centrifugation, and suspended in 10 mM potassium phosphate buffer (pH 7.2).

Isolation of HBBs. HBBs were isolated by a modification of the procedure of Joseph and Canale-Parola (24). Flagella purified from 30 g of cells were collected by high-speed centrifugation for 2.5 h and then suspended in 5 ml of deionized water. After the pH was adjusted to 2.25 by the addition of 0.2 N HCl, the suspension was incubated at room temperature for 40 min and then subjected to high-speed centrifugation for 3 h. The supernatant fluid, which contained disaggregated filament components, was poured off, and the walls of the tube were wiped to remove traces of flagellins. The invisible pellet containing the hooks and basal bodies was suspended in 100 μl of 10 mM potassium phosphate buffer (pH 7.2).

Gel electrophoresis, Western blotting, and concanavalin A staining. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out by the procedure established by Laemmli (27) as described elsewhere (26). The resolving gels contained either 10% or 12.5% acrylamide as indicated. Staining of gels was either with Coomassie blue (26) or with silver (38), as indicated.

Western blotting (immunoblotting) (36) of polypeptides in SDS-polyacrylamide gels and immunoenzymatic development were performed by the procedure of Tortorello and Dunny (35). When concanavalin A was used as a probe, the procedure used was a modification of that described by Clegg (12). The glycosylated protein chicken ovalbumin (Sigma) and the nonglycosylated protein bovine serum albumin (Sigma) were used as positive and negative controls, respectively. After blotting, the filter was incubated overnight in 0.15 M NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% Triton X-100 in 50 mM Tris hydrochloride (pH 7.4) (35). The filter was then incubated for 1 h in TS buffer (0.1 M Tris hydrochloride [pH 7.5] at 25°C, 0.15 M NaCl, 50 mM $CaCl_2$, 50 mM $MnCl_2$) containing 0.5 mg of concanavalin A (Sigma) per ml and then washed four times (15 min each time) in TST buffer (TS buffer containing 0.1% Triton X-100). The filter was then incubated for 1 h in TST buffer containing 50 μg of horseradish peroxidase (Sigma) per ml. After two 10-min washes in TST, the filter was developed with 4-chloro-1-naphthol (Sigma).

Protein concentrations were determined by the Bradford method (8) with reagents obtained from Bio-Rad Laboratories, Richmond, Calif.

Electron microscopy and immunogold labeling. Intact flagella and HBBs were negatively stained as described below. Copper grids (300 mesh) supporting carbon-coated Formvar films were floated film side down on small drops of the material to be examined for 5 min. The grids were then transferred to drops of stain for 40 s. Stains were 3% ammonium molybdate (pH 7.6), 2% potassium phosphotungstate (pH 7.0), or 1% uranyl acetate (pH 4.3), as indicated.

For immunogold labeling, all incubations and washes were carried out by floating grids on drops of reagents. Intact flagella (20 μg/ml in phosphate-buffered saline) were adsorbed onto grids for 30 min. The excess fluid was blotted with a tissue, and the grids were incubated for 45 min on 30-μl drops of either undiluted hybridoma supernatant fluid or rabbit antiserum directed against the *Treponema phagedenis* 33,000-dalton flagellar polypeptide (33K peptide) diluted 1:100 in PBT (phosphate-buffered saline containing 0.1% bovine serum albumin and 0.05% Tween 20). The grids were then washed twice by incubation for 10 min on 200-μl drops of PBT. This was followed by a 45-min incubation on a 30-μl drop of secondary label which was goat anti-mouse immunoglobulin G (1:10 in PBT) in the case of flagella treated with monoclonal antibody (MAb) .1H11G7, goat anti-rabbit immunoglobulin G (1:50 in PBT) for flagella treated with anti-*T. phagedenis* 33K polypeptide, and protein A (1:50 in phosphate-buffered saline containing 1% bovine serum albumin) for flagella treated with MAbs .3E9F6 and .2A6B4. All secondary labels were conjugated to 10-nm gold particles (Janssen Life Sciences Products, Beerse, Belgium). The grids were then washed twice as described above, washed once for 10 min on a 100-μl drop of phosphate-buffered saline, rinsed briefly in deionized water, and stained with 3% ammonium molybdate (pH 7.6) for 40 s.

All grids were examined with a Phillips 301 transmission electron microscope operating at 80 kV.

Preparation of antibodies. Antiserum to purified flagella was raised in a New Zealand White rabbit. On day 1, the rabbit was inoculated intramuscularly with 40 μg of purified flagella in Freund complete adjuvant. The rabbit was boosted subcutaneously on days 21 and 125 with 500 μg of purified flagella in PBS and was bled on day 141. The rabbit antiserum to the *T. phagedenis* 33K flagellar polypeptide was a gift from Nyles Charon. Mouse monoclonal antibodies to purified *S. aurantia* flagella were prepared by the Cornell University Biotechnology Institute Monoclonal Antibody Laboratory by a modification of the protocol of Galfre et al. (17).

RESULTS

Purification of periplasmic flagella. The flagella of *S. aurantia*, like those of all other spirochetes, are completely enclosed in the periplasmic space and presumably are at-

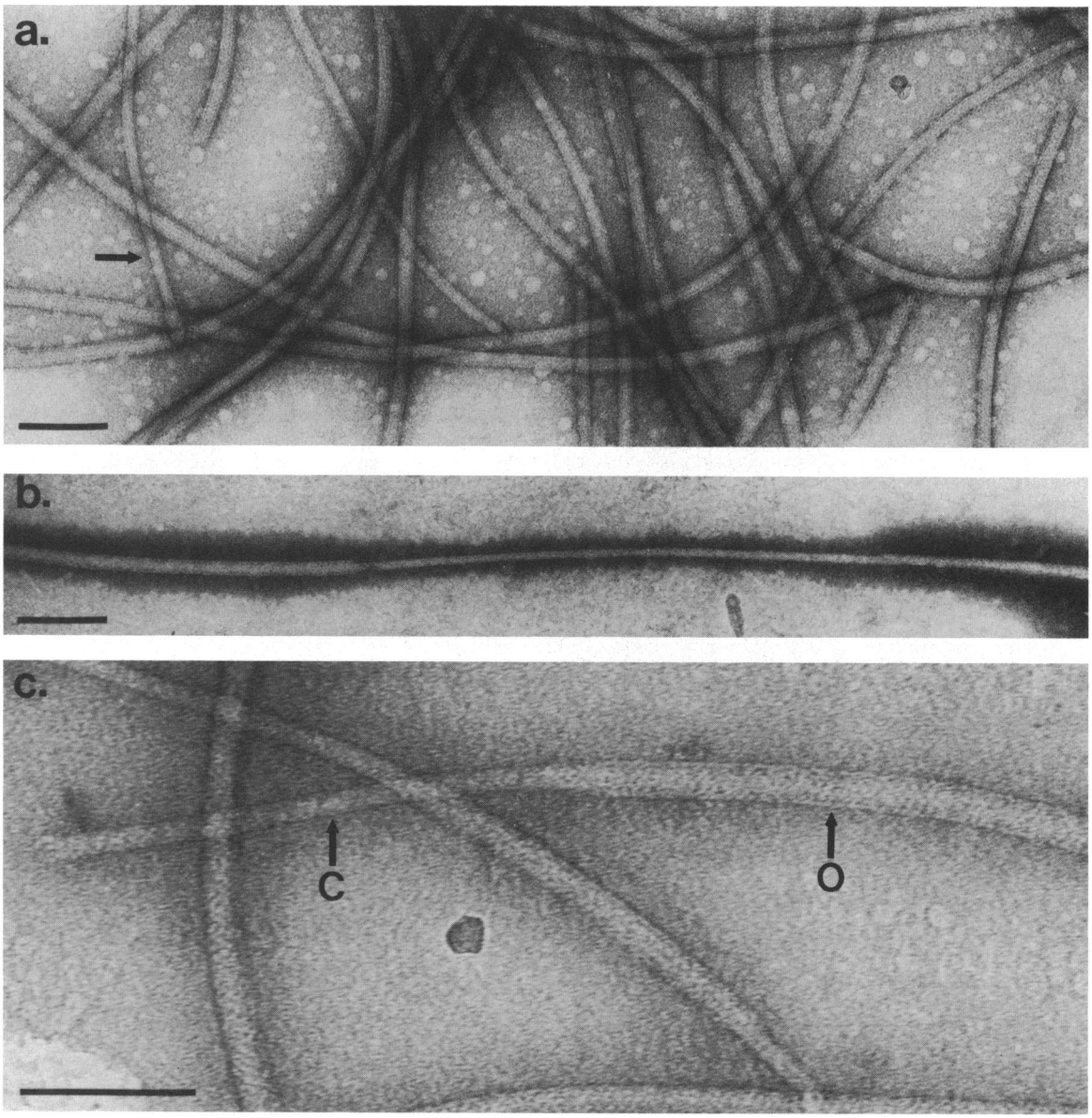

FIG. 1. Electron micrographs of the filamentous portion of *S. aurantia* flagella. The arrow in panel a points to a thin filament. C, Core; O, outer layer. Bars, 100 nm. Stains were (a) 1% uranyl acetate (pH 4.3), (b) 2% potassium phosphotungstate (pH 7.0), and (c) 3% ammonium molybdate (pH 7.6).

tached to the peptidoglycan layer and the cytoplasmic membrane by the rings of the basal body (23). To purify intact flagella for ultrastructural and biochemical analyses, it was therefore necessary to separate them from all envelope components. The purification procedure we employed consisted of gentle lysis of the cells with lysozyme-EDTA, treatment with 2% Triton X-100 (which completely solubilizes the cytoplasmic and outer membranes [26] while leaving the flagella intact), and banding by KBr isopycnic gradient centrifugation. In the KBr gradient, the flagella reproducibly formed two distinct and well-separated bands with densities of 1.29 and 1.30 g/cm^3. An electron microscope examination of each band indicated that it contained flagella, many possessing basal bodies. Furthermore, we could not detect morphological differences between the flagella of the upper or lower KBr band, although differences in the polypeptide composition were apparent (see below).

Ultrastructure of purified *S. aurantia* flagella. Electron microscopy of purified flagella revealed that filaments were composed of an inner core and an outer layer (Fig. 1). When fields of negatively stained flagella were examined, both thick and thin filaments were evident (Fig. 1a). The thick filaments were 19.3 ± 2.8 (standard deviation) nm for the denser flagella, and 18.3 ± 2.8 nm for flagella from the upper KBr band, whereas the thin filaments from the lower KBr band measured 12.1 ± 1.4 nm and those from the upper band measured 12.3 ± 1.1 nm in diameter. The proportion of thin to thick filaments was similar in either case, with thin filaments constituting 14% of the total population for the denser flagella and 17% for the less dense flagella. Electron micrographs of selected filaments at higher magnification show more clearly the outer layer and the inner core (Fig. 1b and c). Since the diameter of the thin filaments (Fig. 1a) corresponded to that of the inner region (Fig. 1b and c), the

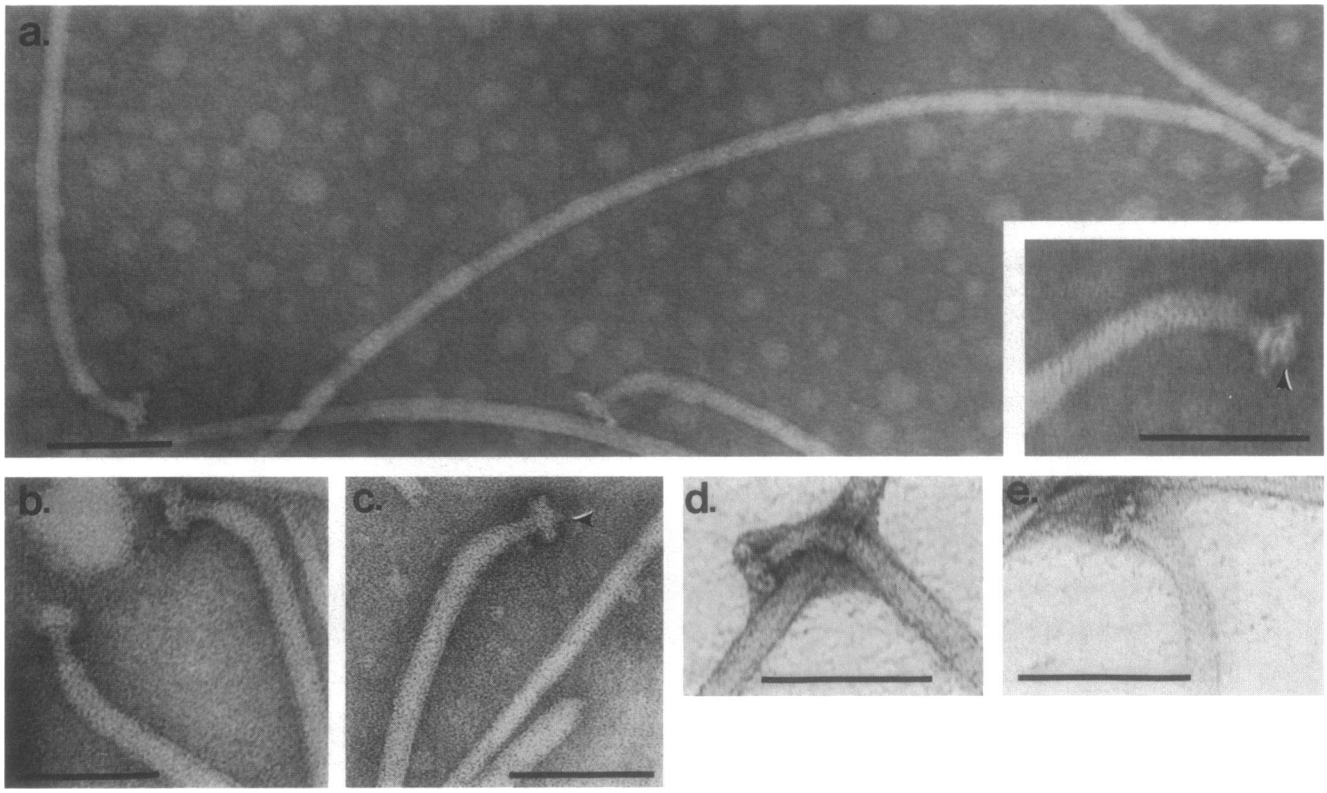

FIG. 2. Electron micrographs of the HBB region of *S. aurantia* flagella. Bars, 100 nm. The stain was 3% ammonium molybdate (pH 7.6). Arrowheads in panels a and c point to buttonlike structures.

thin filaments probably represent those from which the outer layer had been stripped, perhaps during purification. The HBB region consisted of two closely apposed rings, 40 to 45 nm in diameter, connected to a hook 18 to 20 by 45 to 50 nm by a rod 12 nm wide and 18 nm long (Fig. 2). There also appeared to be a buttonlike structure similar to that described for *Caulobacter* HBBs (M. J. B. Stallmeyer, D. De Rosier, S.-I. Aizawa, R. M. Macnab, K. Hahnerberger, and L. Shapiro, Biophys. J. **47**:48a, 1985). The rings exhibited a variety of configurations. In some cases they had a mushroomlike appearance (Fig. 2b) as described by Holt for other spirochetes (23), and in other cases they were either stacked (Fig. 2c) or concave to one another (Fig. 2e). Furthermore, the outer layer of the filament did not appear to extend over the hook (Fig. 2b).

Polypeptide composition of the flagella. To establish the polypeptide composition of the flagella, flagella from the two KBr gradient bands were examined by SDS-polyacrylamide gel electrophoresis. There were major polypeptide bands with apparent molecular weights of 37,500 and 34,000 (the 37.5K and 34K polypeptides), along with minor bands with apparent molecular weights of 36,000 and 33,000 (the 36K and 33K polypeptides) (Fig. 3). However, in the case of the higher density flagella, there was an additional major band of 31,500 daltons (the 31.5K polypeptide) and an additional minor band of 32,000 daltons (the 32K polypeptide) (Fig. 3). Furthermore, the presence and abundance of the 31.5K polypeptide were dependent on the purification conditions. The more harshly the flagella were treated, the less of this polypeptide there was. For example, when the higher-density flagella were treated with 2% deoxycholate and repurified on a KBr gradient, the 31.5K polypeptide was removed (data not shown). These results suggest that the 31.5K polypeptide is loosely associated with the flagella, or that flagella containing the 31.5K polypeptide are less stable than those without it. Also, since the two types of flagella were at least grossly similar, the 31.5K polypeptide was not critical to the filament structure.

The question then arises whether the 31.5K polypeptide is actually a flagellar constituent or whether it is a contaminant

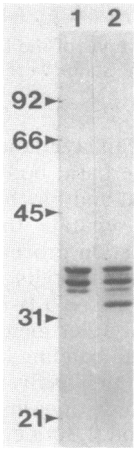

FIG. 3. Coomassie blue-stained 10% SDS–polyacrylamide gel of purified *S. aurantia* periplasmic flagella. Lanes: 1, flagella from the upper band of a KBr gradient; 2, flagella from the lower band of the KBr gradient. The migrations of molecular size standards are indicated to the left in kilodaltons.

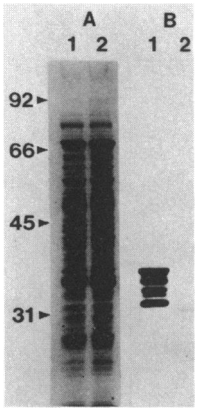

FIG. 4. Western blot analysis of whole cell extracts of *S. aurantia* M1 and the nonmotile mutant NP-7. Lanes: 1, *S. aurantia* M1; 2, *S. aurantia* NP-7. (A) Coomassie blue-stained 10% SDS–polyacrylamide gel. (B) Nitrocellulose blot probed with a 1:100 dilution of rabbit antiserum raised against purified *S. aurantia* flagella.

associating with the flagella during the purification. The more general question is which, if not all, of the six polypeptide bands (Fig. 3) is a component of the flagella? To answer this question, rabbit antiserum raised against purified flagella was used in a Western blot analysis of whole-cell lysates of the wild-type and of a mutant, NP-7 (31), similar in phenotype to *hag* mutants of *Escherichia coli* (34) in that it lacked flagellar filaments but possessed basal body structures (Fig. 4). Although the wild-type cells contained antigens corresponding in molecular weight to all of the polypeptide bands observed in SDS-polyacrylamide gels of purified flagella (Fig. 3), including the 31.5K polypeptide band (the lowest-molecular-weight band in Fig. 4B, lane 1), there was no reaction with the mutant. These results support the conclusion that all of the major and minor flagellar polypeptides (Fig. 3) are components of *S. aurantia* flagella. Furthermore, they must be associated with the flagellar filament, since the mutant cells possess HBBs but not filaments.

In an effort to establish the polypeptide composition of the HBB region, we took advantage of the fact that, when incubated at low pH, *S. aurantia* flagellar filaments dissociated into their component subunits, whereas the HBBs remained intact and could be concentrated by high-speed centrifugation. As indicated by electron microscopy, such HBB preparations consisted primarily of detached hooks, although intact HBBs were also present (Fig. 5). The hooks had a V-shaped indentation at the distal end, and intact HBBs tended to aggregate by their basal bodies (Fig. 5b). Both of these features are also characteristic of HBBs purified from *Salmonella typhimurium* (1).

These purified hooks and the acid-soluble material were analyzed by SDS-polyacrylamide gel electrophoresis. All of the polypeptide bands seen in intact flagella were also present in the acid-soluble material (Fig. 6). This further confirms the conclusion drawn from the Western blot analysis that these polypeptides are associated with the filamentous portion of the flagellum. Three polypeptide bands in the apparent molecular weight range of 62,000 to 66,000 were greatly enriched in the HBB preparation (Fig. 6). These bands were consistently seen in HBB preparations and were visible in preparations of intact flagella when gels were overloaded. An additional 28K band was also present in some HBB preparations but was not consistently observed and therefore may be a degradation product of HBBs. Furthermore, traces of the 37.5K and 31.5K bands were visible. Since the HBB complex constitutes roughly 1% of the mass of an intact flagellum (1) and because our preparation consisted primarily of detached hooks, we feel that the 62K to 66K bands, because they are the most abundant, probably represent hook polypeptides. Although in *E. coli* and *S. typhimurium* (1) the hook is composed of only one polypeptide, it is possible that *S. aurantia* hooks, like its filaments,are composed of more than one polypeptide.

Immunogold localization of flagellar antigens. To establish a correspondence between the structural components of the flagellum and the polypeptides detected by SDS-polyacrylamide gel electrophoresis, mouse MAbs were raised against purified flagella and used in decoration experiments. MAbs were obtained which reacted with only the 37.5K polypeptide (MAb .3E9F6), primarily with the 31.5K polypeptide (MAb .2A6B4), and with all of the polypeptides except for the 37.5K polypeptide (MAb .1H11G7) (Fig. 7). We also included in our analysis a polyclonal antiserum directed against the *T. phagedenis* 33K flagellar antigen, which has been reported to react with whole-cell lysates of *S. aurantia* (29) and also reacts with purified *S. aurantia* flagella. The pattern of reactivity observed with this antiserum was identical to the one obtained with MAb .1H11G7 (Fig. 7). The results of decoration experiments indicated that MAb .1H11G7, as well as the antiserum to the *T. phagedenis* 33K flagellar antigen, reacted with the broken ends of filaments, with regions of the filament where the outer layer appeared to have been stripped off, with thin, frayed fragments of filaments, and with debris (Fig. 8a through d), suggesting that they were recognizing internal filament epitopes. In contrast, the MAb directed against the 37.5K polypeptide appeared to decorate the outside of filaments (Fig. 8e). Although it was not possible always to see the outer layer clearly, the decorated areas generally measured 18 to 20 nm, and thus we feel that this MAb is recognizing an epitope that is part of the outer layer. The patterns of decoration obtained with MAb .1H11G7 and with MAb .3E9F6 were observed with flagella from both the upper and the lower KBr gradient bands. On the other hand, MAb .2A6B4, which reacted specifically with the 31.5K antigen, decorated neither flagella possessing the 31.5K polypeptide (the lower KBr gradient band) nor those lacking it (the upper KBr band), suggesting that the epitope it was recognizing was not accessible to the antibody.

Are filament polypeptides glycosylated? The Western blot results showed that all of the polypeptides with the exception of the 37.5K polypeptide appear to share a common epitope as detected by MAb .1H11G7. These polypeptides were also recognized by antiserum directed against the *T. phagedenis* 33K flagellin. It has been suggested that this cross-reactivity may stem from a common epitope arising from a posttranslational modification such as glycosylation (29). In addition, the pattern of major and minor polypeptides on SDS-polyacrylamide gels of purified *S. aurantia* flagella appears remarkably similar to the pattern observed when the flagella of *Halobacterium halobium* are analyzed (2). In the case of *H. halobium*, this has been demonstrated to result from the glycosylation of three related flagellins (37). To test the hypothesis that *S. aurantia* flagella may contain glycosylated polypeptides, purified flagella were electroblotted onto nitrocellulose and probed with concanavalin A, a lectin that recognizes glucose, mannose, and *N*-acetylglucosamine residues. Mainly the minor 33K polypeptide and, to a lesser extent, the 34K polypeptide reacted

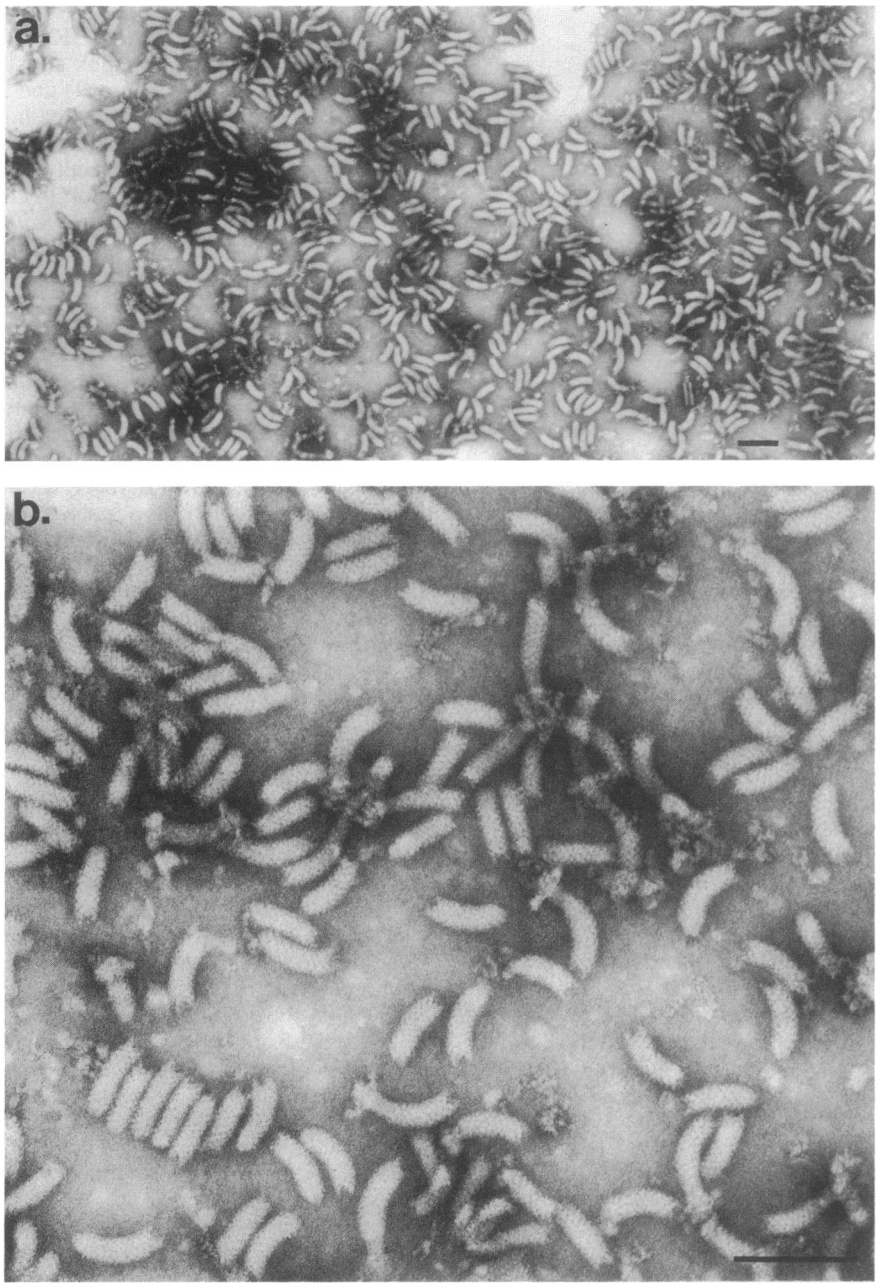

FIG. 5. Electron micrographs of *S. aurantia* HBBs after acid dissociation of the filament. Bars, 100 nm. The stain used was 3% ammonium molybdate (pH 7.6).

with concanavalin A (Fig. 9). This, together with the antigenic cross-reactivity, suggests that at least two of the three minor polypeptides in flagellar filaments may be glycosylated forms of more abundant flagellar polypeptides. Other types of assays for specific types of glycosylation (for example, attempts to cleave glycosyl groups with specific enzymes such as glycopeptidase F and endoglycosidase F) have not been successful (data not shown).

DISCUSSION

The periplasmic flagella of *S. aurantia* were purified and were found to consist of a filament composed of an inner core and an outer layer (Fig. 1) and an HBB region composed of a hook connected to two rings by a rod (Fig. 2). These rings presumably correspond to the M ring and the S ring of flagellated bacteria such as *E. coli* and *S. typhimurium* (1, 14, 15). Unlike *E. coli* and *S. typhimurium* basal bodies, which have four rings, the basal bodies of gram-positive bacteria such as *Bacillus subtilis* (14) which have no outer membrane possess only two rings. Since spirochete flagella do not protrude through the outer membrane, it is not surprising that we observed only two rings associated with *S. aurantia* HBBs. It should be pointed out, however, that the basal bodies of some spirochetes, such as *Leptospira* (23), have been observed to possess four rings, the function

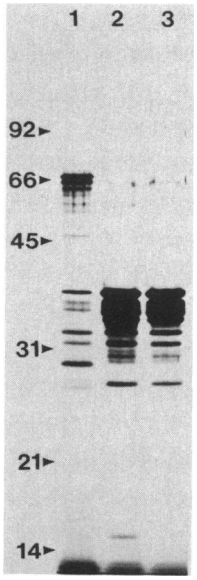

FIG. 6. Silver-stained 12.5% SDS–polyacrylamide gel of acid-treated *S. aurantia* flagella. Lanes: 1, HBBs; 2, material soluble at low pH; 3, intact flagella (control). The migrations of molecular size standards are indicated to the left in kilodaltons.

of which does not appear immediately obvious. As opposed to the external flagella of *E. coli* (14), *S. typhimurium* (1), and *Bacillus subtilis* (14), multilayered filaments appear to be a characteristic shared by many of the spirochetes. They have been observed in species as diverse and as phylogenetically distant as *Leptospira* species (30), *Treponema pallidum* (13), and certain strictly anaerobic members of the genus *Spirochaeta* (24). Although the reasons for such complexity are not yet clear, they may relate to the periplasmic location of the flagella. For example, the flagella of *S. aurantia* wrap around the peptidoglycan, overlapping in the middle of the cell, and when purified have a wavelike form. One would expect that, as compared with the wirelike flagellar filaments of *E. coli* (6), these flagella must be relatively plastic to conform to the shape of the protoplasmic cylinder when the flagella are rotating clockwise or counterclockwise.

Given the structural complexity of the *S. aurantia* flagella, and because purified flagella of other spirochetes have also been reported to consist of several polypeptides (7, 13, 28, 30, 33), it was not surprising to find multiple polypeptides associated with the filament. The results of a Western blot analysis of a mutant lacking a filament but having a basal body structure, together with the results of dissociation experiments, indicated that the filamentous portion of *S. aurantia* flagella was composed of three abundant polypeptides (37.5K, 34K, and 31.5K) and three minor polypeptides (36K, 33K, and 32K) (Fig. 3, 4, and 6). The Western analysis including the filament mutant (Fig. 4) provided tangible evidence that all six of the polypeptides were components of flagella rather than cellular contaminants that bound to flagella during the purification. Because the presence and abundance of the 31.5K major polypeptide was dependent on the purification conditions and because flagella having the 31.5K polypeptide were indistinguishable from ones lacking it, it appears either that this polypeptide was loosely associated with the filament perhaps on the surface and not critical to the structure or that filaments containing this polypeptide were less stable than those without it. Furthermore, it appears that purification procedures can affect the polypeptide composition of *S. aurantia* flagella. This points to one explanation for discrepancies in the literature regarding the number of polypeptides constituting flagella from other spirochetes (7, 11, 13, 28).

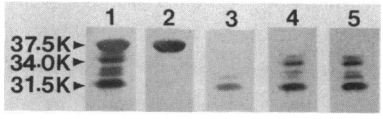

FIG. 7. Western blot analysis of whole cell extracts of *S. aurantia* separated on a 10% SDS–polyacrylamide gel and probed with polyclonal antiserum and MAbs to purified flagella, and with polyclonal antiserum to the *T. phagedenis* 33K flagellin. Lanes: 1, rabbit antiserum to purified *S. aurantia* flagella; 2, MAb .3E9F6; 3, MAb .2A6B4; 4, MAb .1H11G7; 5, rabbit antiserum to the *T. phagedenis* 33K flagellin.

HBB preparations were found to be greatly enriched in three polypeptides in the 60K to 66K range (Fig. 6). Because our HBB preparations consisted primarily of detached hooks, as opposed to intact HBBs, we feel that these polypeptides probably are components of the hook. Although in *E. coli* and *S. typhimurium* (1) the hook is essentially composed of only one polypeptide, it is possible that *S. aurantia* hooks, like its filaments, are composed of more than one polypeptide. Further experiments, including the purification of large quantities of HBBs from the filamentless mutant NP-7, should help to define further the other components of the *S. aurantia* HBB complex.

The results of decoration experiments indicated that the 37.5K polypeptide was associated with the surface of flagellar filaments. The MAb used, .3E9F6, however, did not appear to associate with all flagellar filaments (Fig. 8). The reasons for this heterogeneity are unclear. Because MAb .1H11G7, which is specific for the other five flagellar polypeptides (36K to 31.5K), labeled the broken ends of flagella as well as areas where the outer layer had been stripped, we suspect that it reacted with the core of the flagellar filament. Because this MAb recognized five polypeptides, it is possible that only one or as many as all five of these polypeptides are core constituents. The MAb specific for the 31.5K and the 32K polypeptides did not decorate purified flagella. Further experiments, including the preparation and use of polyclonal antiserum as well as other MAbs to the 31.5K polypeptide in decoration experiments of intact and thin-sectioned flagella will be necessary to determine the location of this filament constituent.

Our results bear a striking similarity to those obtained recently with the flagella of *Treponema pallidum* (3, 13). Cockayne et al. (13) found that *T. pallidum* flagella were ultrastructurally complex, consisting of an inner core and an outer layer, which they called a sheath. Furthermore, the *T. pallidum* flagella were composed of more than one polypeptide. Immunogold labeling experiments somewhat similar to those described here indicated that the core was composed of a major 33.5K polypeptide and a minor 34K polypeptide, whereas the sheath appeared to consist of a 37.5K polypeptide and perhaps a 31.5K polypeptide. The core component was antigenically related to the 33K flagellin of *T. phagedenis*, which is immunologically cross-reactive with what appears to be the core of the *S. aurantia* filament. Because our monoclonal antibodies .1H11G7 and .2A6B4 cross-react with some of the flagellins of *T. phagedenis* and *Treponema hyodysentariae* and with polypeptides in whole cell lysates of *Spirochaeta halophila* (B. Brahamsha and E. P. Green-

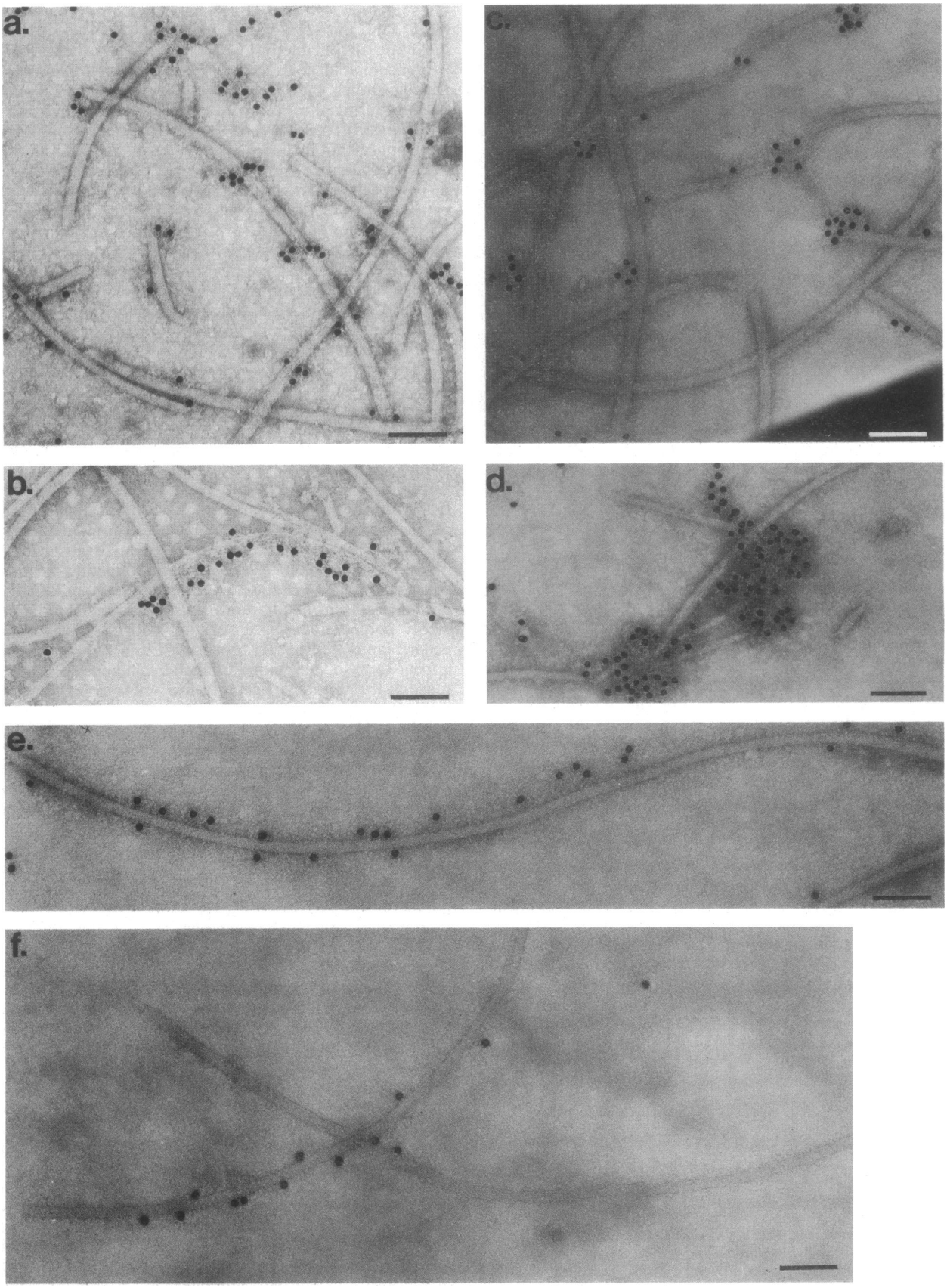

FIG. 8. Electron micrographs of immunogold-labeled *S. aurantia* flagella. (a and b) MAb .1H11G7; (c and d) anti-*T. phagedenis* 33K flagellin; (e and f) MAb .3E9F6.

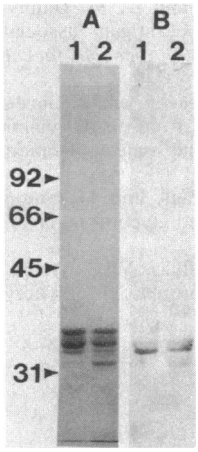

FIG. 9. Concanavalin A blot of purified *S. aurantia* flagella. Lanes: 1, flagella from the upper band of a KBr gradient; 2, flagella from the lower band of the gradient. (A) Coomassie blue stain. (B) Concanavalin A-treated nitrocellulose filter. Each lane contained 10 μg (total) of protein.

berg, unpublished results), it is possible that the flagella of many diverse spirochetes may share a related core and may differ in their outer layers.

The concanavalin A blotting results (Fig. 9) suggest that some of the minor flagellar polypeptides of *S. aurantia* may be glycosylated. Further studies, including the molecular cloning of the *S. aurantia* flagellins, should reveal the molecular basis of the antigenic similarity existing not only among the flagellins of *S. aurantia* but also among those of distantly related spirochetes.

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