Electron Microscopic Observations of Structures Associated with the Flagella of *Spirillum volutans*

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Electron microscopy of thin-sectioned *Spirillum volutans* (ATCC 19554) showed that at the insertion site of the flagellum there was a cylindrical structure with a diameter of ca. 36 nm which extended ca. 19 nm into the cytoplasm. This structure, termed a cytoplasmic flagellar base, enclosed a central rod which was continuous with the hook. There was a continuation of the flagellar base into the peptidoglycan layer, enclosing ringlike structures and the central rod. The flagellar hook and proximal part of the flagellar filament contained a central channel which was large enough to accommodate the flagellin subunit. The flagella of fixed cells may project perpendicularly from the outer membrane in a position corresponding to a trailing, swimming orientation or may bend almost parallel to the membrane in a leading orientation. Maximum bending occurred in the hook region, which may be the structure responsible for executing changes in swimming direction.

Recent research has concentrated on studying the flagellar bases in isolated flagella, since there is convincing evidence that bacterial flagella are inert helical structures rotated from their flagellar bases (2, 3, 20).

The structure of the flagellar base is reviewed by Smith and Koffler (22), Silverman and Simon (21), and Macnab and Aizawa (13). Silverman and Simon (21) noted that occasional electron micrographs show cytoplasmic structures associated with the base of the flagellum in *Escherichia coli* and *Salmonella* spp. They concluded that these are labile structures or are intermittently associated with the flagellar base and noted that "if such structures exist, techniques for fixing and stabilizing them will have to be developed before they can be isolated."

It is important that cytoplasmic structures associated with the insertion sites of flagella should be documented, since unequivocal demonstration of their presence will encourage the study of their biochemical properties and may lead to a better understanding of the way in which bacterial flagella move. *Spirillum volutans* was chosen for a study of the structure of cytoplasmic elements associated with the base of flagella because it is lophotrichous, with large numbers of flagella located in a small area. Thus, a section through the cell pole will contain several flagella. It is more difficult to study the structure of flagellar bases in peritrichous bacteria like *E. coli*, where flagella are sparse.

The study of flagellar bases in thin sections requires preparation of sections which are dark grey (20 nm thick, according to Sakai [17]), or thinner. The intracytoplasmic flagellar bases described in this study were ca. 19 by 36 nm; sections with gold interference colors are 96 to 102 nm thick (17), and the structures of interest are obscured by overlying material. It should be realized that for geometric reasons a dark-grey section does not include the structures of interest below every flagellum or fragment of a flagellum which appears in the plane of the section.

The structures described occurred consistently in all the numerous micrographs examined in this study, taking into account variations in appearance due to the plane of the section.

**MATERIALS AND METHODS**

*Spirillum volutans* (ATCC 19554) was grown, at 30°C in ATCC medium 234 and cinematographed as described by Swan (24). In some experiments cells were vortexed for 1 min or sonicated for 40 s in a Unisonics ultrasonic bath before fixation for transmission electron microscopy. Fixed cells were vacuum filtered onto a Millipore filter (pore size, 0.45 μm) for transmission electron microscopy and onto a Nucleopore filter (pore size, 0.4 μm) for scanning electron microscopy. No agar was included in the medium of the cells grown for scanning electron microscopy.

The most satisfactory preservation was obtained by fixing cells for 30 to 45 min in a fixative containing 0.8% (vol/vol) glutaraldehyde, 0.6% (wt/vol) Formalin, and 0.02% (wt/vol) 2,4,6-trinitrophenol in 0.01 M cacodylate buffer (pH 6.9). The cells were washed in 0.05 M cacodylate buffer and postfixed in 1% osmium tetroxide in the same buffer. Further processing for transmission electron microscopy was by standard procedures, including a tannic acid treatment, as detailed by Swan (23), except that some preparations were also block stained with uranyl acetate as described by Swan et al. (25). Sections were examined with a Jeol 100C electron microscope. For scanning electron microscopy, cells attached to Nucleopore filters were dehydrated after fixation, critical point dried, gold coated, and examined with a Jeol 35C. The magnification of the transmission electron microscope was verified by means of a carbon grating, and that of the enlarger was verified by means of a graticule.

Wherever possible, mean values are given for measured structures, followed by the standard deviation (SD) and number of measurements (N). The number of measurements of one type of structure in a single micrograph is limited to two. The data contain measurements of more micrographs than are reproduced here.

**RESULTS**

*Spirillum volutans* is a gram-negative bacterium with the cell body in the form of a left-handed helix (Fig. 1A). Bundles of flagella were located at the cell poles and occurred in a trailing configuration (Fig. 1B) or leading configuration (Fig. 2A) in both live and fixed cells, although
the arrangement of flagellar filaments in the bundles was clearly distorted in the critical-point-dried material. The light microscopic appearance of a swimming cell is shown in Fig. 1B (inset).

Directly below the flagella in thin sections of *Spirillum volutans* were cytoplasmic structures. A cylindrically shaped flagellar base extended to a depth of 19 nm (SD, ±2.9; N = 7) into the cytoplasm below the plasma membrane (Fig. 2B, 3B, 3C, and 4D). When sectioned thus, perpendicular to its plane of attachment to the plasma membrane, its diameter was 36 nm (SD, ±3.4; N = 7). This plane of section will be referred to as longitudinal. The cytoplasmic flagellar base had approximately the same diameter in sections parallel to the overlying plasma membrane (Fig. 4A and B). This plane of section will be referred to as transverse. The cylindrical cytoplasmic flagellar base had a wall 5.8 nm thick (SD, ±1.1;
FIG. 2. (A) Polar bundle of flagella (f) oriented in a leading position. Bar, 0.4 μm. (B) Transmission electron micrograph of Spirillum volutans. The peptidoglycan layer between the outer membrane and the plasma membrane is interrupted at the insertion sites of the hooks. At the arrowed flagellar insertion site the following features are seen. The peptidoglycan layer is interrupted by what appears to be a continuation of the hook. Below the plasma membrane and parallel to it is a bilaminar ringlike structure. Projecting at right angles from the edge of the ringlike structure is a trilaminar structure, indicated by the three arrows. This is interpreted as a longitudinal section through a cylindrical structure, the cytoplasmic flagellar base, which encloses a central rod continuous with the flagellar hook. Less easily seen because of the dense material around it is a faint continuation of the wall of the cytoplasmic flagellar base through the peptidoglycan layer. f, Flagellar filament; h, hook; o, obliquely cut cytoplasmic flagellar base; s, sheathlike material around the flagellar filament; arrows, trilaminar structure, interpreted as the cytoplasmic flagellar base cut longitudinally and enclosing the flagellar rod (above the central arrow). Bar, 0.05 μm.

$N = 6$) which resembled a membrane, except for the appearance in it of a subunit structure (Fig. 4A) and its thinness (the plasma membrane is 6.7 nm thick; SD, ±0.9; $N = 6$). The plasma membrane and the wall of the flagellar base were significantly different in thickness by the paired $t$ test. In longitudinal sections of the flagellar base, the flagella in the section which appeared to lack flagellar bases were those which in their basal regions were slightly out of the plane of the section (e.g., the flagella labeled g in Fig. 3C).

In some longitudinal sections (Fig. 2B, 3A, 3B, and 3C) and in all transverse sections (Fig. 4A and B) of the cytoplasmic flagellar base, there was a central structure termed the rod. This had a diameter of 12.1 nm (SD, ±0.7; $N = 8$).

In some longitudinal sections there appeared to be a faint continuation of the wall of the cytoplasmic flagellar base into the peptidoglycan layer of the cell wall, terminating at the outer membrane (Fig. 2B and 3A). This part of the flagellar
base was less easily seen than that lying in the cytoplasm because of the dense staining of the surrounding peptidoglycan layer.

In favorably oriented and heavily stained sections, two ringlike electron-lucent structures lay immediately below the outer membrane beneath the flagellum (Fig. 4C and D). These were 24 nm in diameter (SD, ±2.8 nm; N = 3), and their overall thickness, measured from outer edge to outer edge, was 12 nm (SD, ±0.5; N = 3). In lightly stained sections the peptidoglycan layer appeared interrupted by the insertion of the hook, as described by Swan (24) and seen in Fig. 2B, 3A, and 3C.

A bilaminar ringlike structure 6.5 nm in thickness (SD, ±0.6 nm; N = 2) and extending ca. 31 nm (SD, ±5.8 nm; N
FIG. 4. (A) The cylindrical wall of the cytoplasmic flagellar base, cut in cross section (arrow), encloses a central rod which is not a uniformly electron-dense structure. h, Hook; p, oblique section through a polar membrane; s, sheathlike material around the flagellar filament. Bar, 0.05 μm. (B) Several cytoplasmic flagellar bases (b) are cut in transverse section and contain a central rod. Bar, 0.1 μm. (C and D) Heavily stained sections in which ringlike structures (r) appear as electron-lucent areas between the outer and plasma membranes at the base of the flagellar hook. In (C) the distal part of the hook is bent, and the flagellum lies at an angle of 40° to the cell surface. Bar, 0.05 μm. (E and F) Transverse sections of the flagellar filament with enveloping sheathlike material, which gives the flagella an overall diameter of 23 nm. Within the flagellar filaments, the outer walls of which are merged with the sheath, is a central channel containing some electron-dense material, possibly the inner wall of the flagellum. This is seen only in transversely sectioned flagella (t). Bar, 0.05 μm.

= 2) against the cytoplasmic side of the plasma membrane was associated with the insertion sites of flagella in thin sections of favorably oriented flagellar bases (Fig. 2B). A comparison of the dimensions of basal structures associated with the flagella in Spirillum volutans with some published values for other species is given in Table 1.

In thin longitudinal sections the hook region was 12.0 nm in diameter (SD, ±1.3 nm; N = 6), whereas the flagellar filament was 11.4 nm in diameter (SD, ±0.7; N = 4). For comparison, the diameter of the flagellar filament was 15.6 nm (SD, ±1.4 nm; N = 6) after negative staining with uranyl acetate. Negatively stained material is subject to drying artifacts (15), whereas embedded and sectioned material usually undergoes shrinkage of ca. 5% (11).
The proximal part of the flagellar filament for 0.2 μm (SD, ±0.06 μm; N = 4) distal to the hook was surrounded by sheath-like material (Fig. 2B), which was easily removed by vortexing (Fig. 3A). In transverse sections this material gave the flagellum an overall diameter of 22.8 nm (SD, ±1.2 nm; N = 4) (Fig. 4E and F). It enclosed a relatively electron-lucent region 12.1 nm in diameter (SD, ±0.5 nm; N = 4), which probably represented the space required for the flagellar filament; within this there was electron-dense material, possibly representing staining of the inner wall of the flagellum (Fig. 4E and F). A channel was present in transverse sections of the hook, where it was 6.5 nm in diameter (SD, ±0.5; N = 5) (Fig. 5A), and in the flagellar filament, where it was ca. 5.5 nm in diameter (Fig. 5B).

In thin sections the hook region usually lay at 90° to the plasma membrane, but it was sometimes bent so that the flagella lay at an angle as low as 18° to the plasma membrane (data not shown). Scanning electron micrographs of Spirillum volutans fixed by the same method contained flagellar bundles in a trailing or leading configuration (Fig. 1B and 2A).

**DISCUSSION**

Below the insertion sites of flagella in Spirillum volutans were cytoplasmic flagellar bases, cylindrical structures containing a continuation of the flagellar rod. A comparison of the dimensions of structures associated with the base of the flagellum in Spirillum volutans with some reported values for other species is given in Table 1. A diagrammatic representation of the flagellar base and its relationship to other structures associated with the proximal part of the flagellum in Spirillum volutans is shown in Fig. 6, based on transmission electron microscopy of thin-sectioned material.

Several studies by thin-sectioning electron microscopy suggest that there are cytoplasmic structures associated with the base of bacterial flagella. Murray and Birch-Andersen (14) found that the flagella originate from knobs just inside the plasma membrane and pass through the membranes individually in Spirillum serpens. Van Iterson et al. (27) observed in cells of Proteus mirabilis with relatively electron-lucent cytoplasm that the flagella emerge from rounded structures ca. 25 to 45 nm wide. Tauschel and Drews (26) found in Rhodopseudomonas palustris that basal bodies have the same internal fine structure in transverse and longitudinal sections and interpreted them as spherical bodies, with the flagellum emerging from the center of the basal body.

In a study of Selenomonas ruminantium by thin sectioning, Chalcroft et al. (4) found that each flagellum terminates in a bullet-shaped base (50 by 70 nm) which extends through the thickness of the cell wall and also through the bounding membrane of the flagellar organelle; the flagellar organelle has structural features in common with the polar membrane described by Murray and Birch-Andersen (14) in Spirillum serpens.

In Spirillum volutans there appeared to be a continuation of the wall of the cytoplasmic flagellar base to the outer membrane in thin sections, forming a basal complex 44 nm long. DePamphilis and Adler (6) observed an apparent connection between the L and P rings near their periphery in isolated, negatively stained flagella. DePamphilis and Adler (6) introduced the terminology L, F, S, and M rings for the two pairs of disks or rings at the proximal end of the isolated flagellum in gram-negative cells. They concluded (7) that the cytoplasmic membrane is attached to the proximal ring (termed the M ring), since only the S ring is visible in negatively stained Bacillus subtilis cells. In Ectothiorhodospira mobilis (16) only three rings are visible. Johnson et al. (10) found five rings in naturally released hook structures of Caulobacter crescentus.

It was noted by Coulton and Murray (5) that it is not certain that the locations of all the elements of the basal complex in the DePamphilis and Adler model are correct. Coulton and Murray found it difficult to determine the anatomical associations of the F, S, and M disks. Their measurements indicate that the basal complex requires some 3 to 4 nm more space than the envelope profile would provide. From a combined freeze-fracture, thin-sectioning, and negative-staining study of Aquaspirillum serpens, they proposed that the S ring lies in the plasma membrane and the M ring lies below it in the cytoplasm. In addition, Coulton and Murray found a series of fibrils extending from the innermost M ring, as seen by negative staining. By using thin sections, they described cytoplasmic bulbous protuberances below the insertion sites of flagella in Spirillum volutans and A. serpens.

In a thin-sectioning study of E. coli, Bayer (1) described a ring-like structure adjacent to the plasma membrane at the insertion site of the flagellum and suggested that this represents an area of modified plasma membrane. The present study supports this view; the plasma membrane at the insertion site of the flagellum had an appearance suggestive of two membrane leaflets fused together. It is possible that this ring-like structure, or modified plasma membrane, is visualized as the M ring in negatively stained, isolated flagella as predicted from measurement of cell envelope dimensions (5). In the present study, in addition, two ring-like structures were seen occasionally beneath flagella and immediately below the outer membrane. Their location suggested that they could be either the L and F or the P and S rings seen in isolated flagella. Rings have not been visualized previously in intact thin-sectioned cells, but they are demonstrated after treatment with detergent, rhamnolipid haemolysin, EDTA, and lysozyme to dissolve surrounding components (8).
In *Spirillum volutans* the flagellar filament was surrounded by a sheathlike structure for ca. 0.2 μm distal to the hook; easily removed by vortexing the cells, this sheathlike structure has not been reported in *Spirillum volutans* previously. In transverse sections this material gave the flagellum an overall diameter of 22.8 nm. Its composition is unknown, but it is unlikely to be a fixation precipitate since it was regular in occurrence and location in gently processed cells (i.e., in cells attached to Millipore filters after fixation), although cellular debris was associated frequently with the sheathlike structure.

In *Spirillum volutans* the hook region was little thicker than the flagellar filament. In thin sections the hook region and the flagellar filament usually lay at 90° to the plasma membrane, but the hook was sometimes bent so that the flagellar filament lay at an angle as low as 18°. This could be the result of changes occurring after fixation, but it is possible that the position assumed by a moving flagellum at the time of exposure to the fixative was preserved, as occurs in cilia (18). Cells of *Spirillum volutans* fixed in the same way as for embedded material contained flagellar bundles in a leading or trailing position when examined by scanning electron microscopy. Bending of the hook region may be the mechanism by which *Spirillum volutans* changes flagellar orientation from a trailing to a leading configuration.

A central channel lay within the hook and flagellar filament. In the flagellum of *Spirillum volutans* the channel was ca. 5 nm in diameter. It is possible that only the proximal part of the flagellum has been viewed in this study and that the distal part of the flagellar channel may taper further, but no data is available concerning this possibility. Measurements of the channel in this study were slightly larger than those reported in negatively stained flagella of other bacteria. This could be due to species or preparative differences or may be due to a distal narrowing of the flagellar channel. Macnab (12) calculated that in *Proteus* flagella there is a 4-nm-diameter central channel and a limiting subunit width of 5 nm. Shirakihara and Wakabayashi (19) found a low-density region in the central part of mutant *Salmonella typhi-*murium flagella which were negatively stained; however,
they regarded the evidence for a central hollow core as inconclusive since it was not resolved at sufficiently high resolution. In reconstructions of the flagellar hook in *Caulobacter* and *Salmonella* spp., Wagenknecht et al. (28) found central channels of ca. 2.5 nm and 1.5 nm, respectively. They noted that the space is insufficient to accommodate the free passage of subunits, for which 2.5 nm would be required.

It is established that filament assembly occurs at the distal end of the flagellum; this topic was reviewed by Macnab (12). Flagellin monomer travels down a central channel and is assembled at the distal end of the flagellar filament. Recently, Ikeda et al. (9) reported a short-flagella mutant of *Salmonella typhimurium* which secretes flagellin into the culture medium due to the poor polymerization, which prevents flagellin from being trapped at the distal end of the filament.

The observations reported here on sectioned material indicated that the central channel of the hook and proximal part of the flagellar filament contained sufficient space to accommodate the flagellin monomer in *Spirillum volutans*.

The observations reported here support previous studies which describe the presence of cytoplasmic flagellar bases associated with the insertion sites of bacterial flagella. Rings immediately below the outer membrane were visualized in thin sections of intact cells. As the wall of the cytoplasmic flagellar base was continuous with a similar structure in the peptidoglycan layer, the rings and central rod were enclosed in a cylindrical basal complex which had a total length from top to bottom of 44 nm and a diameter of 36 nm.

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**LITERATURE CITED**


