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Relationship between Cell Wall, Cytoplasmic Membrane, and Bacterial Motility[1]

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High-resolution electron microscopy of polarly flagellated bacteria revealed that their flagella originate at a circular, differentiated portion of the cytoplasmic membrane approximately 25 nm in diameter. The flagella also have discs attaching them to the cell wall. These attachment discs are extremely resistant to lytic damage and are firmly bound to the flagella. The cytoplasm beneath the flagellum contains a granulated basal body about 60 nm in diameter, and a specialized polar membrane. The existence of membrane-bound basal bodies is shown to be an artifact arising from adherence of cell wall and cytoplasmic membrane fragments to flagella in lysed preparations. Based on structures observed, a mechanism to explain bacterial flagellar movement is proposed. Flagella are considered to be anchored to the cell wall and activated by displacement of underlying cytoplasmic membrane to which they are also firmly attached. An explanation for the membrane displacement is given.

Weibull (30) and Lederberg (13) showed that when the cell wall of motile bacteria was either enzymatically removed, or its synthesis and polymerization disturbed by antibiotics, the bacteria became nonmotile, but still retained their flagella. We reported that penicillin converted *Salmonella typhimurium* into osmotically labile spheroplasts whose motility persisted until remaining intact cell wall fragments disappeared (26). We concluded that an association between cell wall and flagella was a necessary prerequisite for translational motility. Later (27) it was demonstrated that not only motility, but the development of functional flagella, was dependent on the integrity of the bacterial cell wall and cytoplasmic membrane.

Structural aspects of flagellar attachment in bacteria have received wide attention in recent years (8), and ever since Weibull's (30) work on *Bacillus megaterium* KM, it has been generally accepted that bacterial flagella penetrate the cell wall and terminate within the cytoplasm. Many reports have appeared (2, 10, 11, 17, 24, 28) on membrane-bound basal granules (sometimes termed blepharoplasts) in shadowed and negatively stained preparations of autolysing bacteria. Murray and Birch-Anderson (15) found that the flagella of *Spirillum serpens* originated just inside the cytoplasmic membrane and that there was a specialized polar membrane in this region. The area immediately beneath the flagella was noted to be conspicuously ribosome-free. Remsen et al. (18) have observed a similarly structured second membrane in *Ectothiorhodospira mobilis*. They designated it as a "basal plate" from which the flagella originated. Glauert, Kerridge, and Horne (9) reported that in *Vibrio metchnikovii* the flagellum is attached to a basal disc which terminates "just inside the plasma membrane." Richie, Keeler, and Bryner (20) reinvestigated both negatively stained and thin sections of *Vibrio fetus* and confirmed the paucity of ribosomal structures, and the presence of a basal disc, and a cone-shaped basal granule which was not membrane-bound.

The mode of flagellar attachment in peritrichously flagellated bacteria also has been investigated. Van Iterson, Hoeniger, and van Zanten (29) made a comprehensive study of *Proteus mirabilis* swarmers and found the flagella to be anchored, often by means of a hook, in rounded structures approximately 50 nm wide. Abram and co-workers (1-3) demonstrated that in *P. vulgaris* and several *Bacillus* species, the flagella have a hooked basal portion, and that spherical bodies 39 to 43 nm in diameter are part of, or lie immediately adjacent to, the cytoplasmic membrane.

In view of these various reports concerning basal bodies and possible sites of origin of bacterial flagella, and the fact that a rigid cell wall is essential in functional motility, a comprehensive

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study, using primarily polarly flagellated bacteria, was undertaken to obtain, if possible, definitive information about the site of origin of bacterial flagella, and the relationship existing between them and the cell wall and cytoplasmic membrane.

MATERIALS AND METHODS

Organisms. The bacteria used were *V. metchnikovii* ATCC 7708, *S. serpens* ATCC 11330, *Pseudomonas aeruginosa* (University of Maryland Culture Collection #401), *B. licheniformis* (ATCC 9945A), and *Serratia marcescens* ATCC 274. All organisms were cultivated in Trypticase Soy broth (BBL). To reveal internal structures not visible in intact bacteria, they were subjected to controlled lysis by methods described below.

Autolysis. Autolysis was achieved by washing 5-ml amounts of 18-hr broth cultures and suspending them in 5 ml of sterile, distilled water at room temperature (ca. 20 C) or at 4 C for periods up to 4 weeks. The degree of lysis was generally found proportional to the time allowed.

Alkaline lysis. With the exception of *P. aeruginosa*, all organisms lysed when 5-ml amounts of 18-hr broth cultures were centrifuged and suspended in 5 ml of 0.2 M carbonate-bicarbonate buffer at *p*H 9.4 (23). After 4 hr, most bacteria showed varying amounts of membrane separation and cytoplasmic clearing.

Ultrasonic treatment. When separation of flagella from cell soma was desired, bacteria autolysing in distilled water at 4 C for 2 weeks were subjected to 5- or 10-sec treatments (20 kc) in a Mullard Ultrasonic Disintegrator (Measuring & Scientific Equipment Ltd., London, England). This resulted in a tearing away of flagella with membrane fragments still attached to their terminal portions.

Plasmolysis. Plasmolysis was achieved by centrifuging 5-ml amounts of 18-hr broth cultures and suspending for 30 min in 5 ml of 1.7 M KNO_3 in 0.05 M phosphate buffer at *p*H 7.0.

Negative staining. The usual method for electron microscopic examination of whole and lysing bacteria was a modification of that developed by Brenner and Horne (4). One drop (3 to 5 μliters) of the bacterial suspensions prepared by the methods listed above was placed on a 200-mesh, Athene-type, copper grid covered with a Formvar film. This was followed by a drop of 0.8% (w/v) aqueous potassium phosphotungstate at *p*H 7.0. The excess was immediately removed by touching filter paper to the droplet edge. The preparations were allowed to dry for 1 min or longer, after which they were examined in the electron microscope.

Thin sectioning. After centrifugation, the bacterial pellet was prefixed in 5% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (*p*H 6.2) for 1 hr. The pellet was then washed in five changes of 0.1 M cacodylate buffer (*p*H 6.2), postfixed in 2% (w/v) sodium barbital buffered osmium tetroxide (*p*H 6.2) and polymerized in Vestopal W by the method of Ryter and Kellenberger (21). Sections 50 to 60 nm in thickness were cut on an LKB Ultrotome (Model 4801A) equipped with a DuPont diamond knife E. I. du Pont de Nemours & Co., Inc., Wilmington, Del. and collected on Formvar-coated 200-mesh copper grids. The sections were poststained for 1 min with lead citrate (19).

Electron microscopy. An RCA EMU-3F electron microscope (Radio Corporation of America, New York, N.Y.) equipped with a 250-μm condenser and 50-μm platinum objective apertures was used. Electron micrographs were made using 50-kv accelerating voltage with the gun bias control set to give the lowest possible beam current at plate magnifications of $\times$ 5,000 to 46,000. Photography was done using 2 by 10 inch Kodak Electron Image Plates.

RESULTS

Each flagellum of negatively stained autolysing and alkali-lysed *V. metchnikovii* and *P. aeruginosa* had a single disc and a double disc at its base (Fig. 1a–d). The double disc is located distal and the single disc proximal to the cytoplasmic membrane. The double discs are approximately 4 nm apart, and the space between the distal double disc and the proximal single disc is about 15 nm. The average disc diameter is 25 nm.

Examination of the distal disc revealed a series of projections on it about 6 nm apart (Fig. 1b). Flagella completely free of cellular material were never seen with both discs on them. The discs also were not seen in thin sections (Fig. 2a). Thin sections of *V. metchnikovii* showed a flagellum penetrating the cell wall and terminating at the cytoplasmic membrane. The wall and cytoplasmic membrane at the point of flagellar insertion appeared flat, unlike the generally wavy morphology of the outer cell wall layer, suggesting identity of these flat areas and the discs seen on flagella of lysing cells. Furthermore, the diameter of these flat areas and the gap between them was identical to the measurement of the discs of the negatively stained flagella of lysing cells. No membrane-bound basal bodies were seen in the thin sections. Favorable sections showed a ribosome-poor area beneath the flagellum (Fig. 2a,b).

The flagella originate outside the cytoplasm and are attached at the cytoplasmic membrane to the proximal disc, which may be a specialized and differentiated area of the cytoplasmic membrane (Fig. 1a–d). This attachment appeared extremely firm, since very few detached flagella were seen, and since the cytoplasmic membrane was observed to pull outward with the flagellum in lysing bacteria (Fig. 1a–c). The diameter of the discs and the gap between them remained constant, despite the disintegration of the cytoplasmic membrane and cell wall. The distal discs originate at the cell wall (Fig. 1c) and are not as firmly bound to it

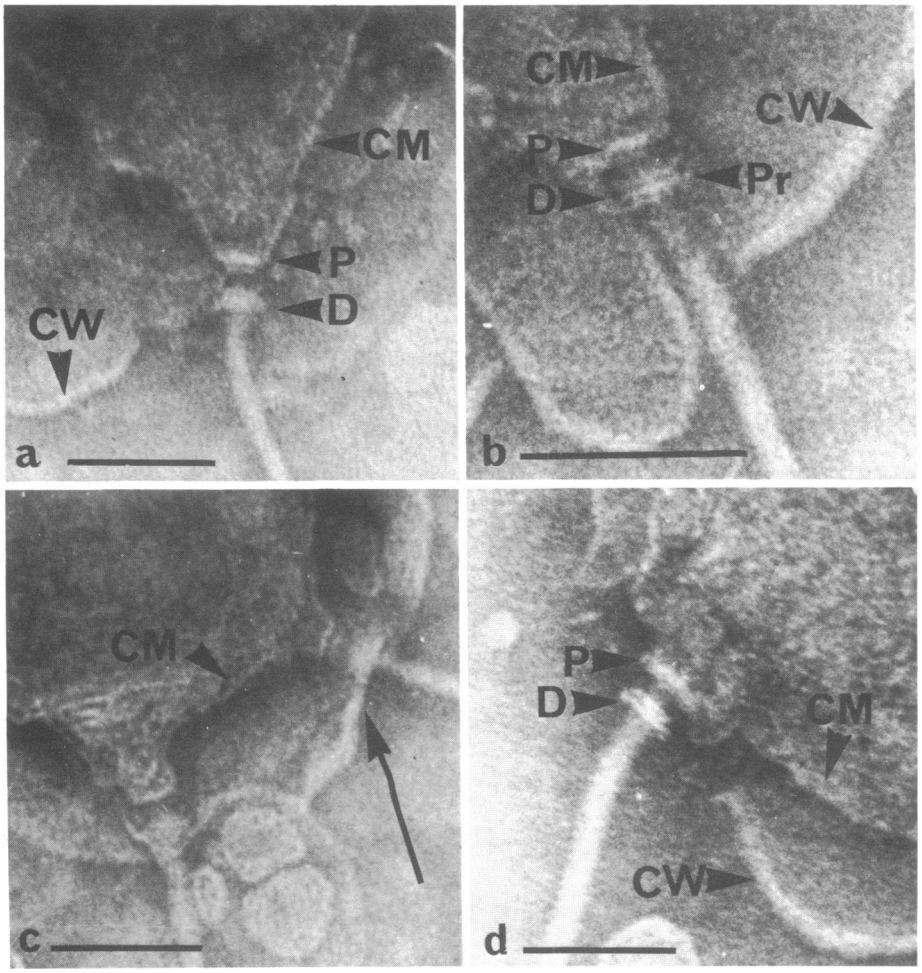

FIG. 1. *Negatively stained autolysing V. metchnikovii showing structures seen at the base of the flagellum. The proximal disc (P) appears to be continuous with the cytoplasmic membrane (CM); (a, b, d) the cell wall is separated from the distal disc (D), and the arrow (c) indicates where the distal disc is seen continuous with the cell wall; (b) and (d) show the distal disc as a composite of two thinner discs; an enlarged side view (b) shows a series of projections (Pr) at the periphery of the distal discs. The bar denotes 100 nm in all photographs.*

(Fig. 1a–b) as is the proximal disc to the cytoplasmic membrane. When lysis proceeds to cell dissolution, the disc may be seen with five or six protrusions on it (Fig. 3a–b). Only remnants of the distal discs are seen at this stage, indicating that even they eventually disintegrate as lysis proceeds.

Autolysed *V. metchnikovii* (Fig. 4a) and plasmolyzed *S. marcescens* (Fig. 4b) both show fibrous structures (56 nm in length) in a radial arrangement originating at the base of the flagellum. It has not been possible to determine, using negative stains, whether these fibers are part of the cytoplasmic membrane, above it, or below it. A second structure was noted in lysed *V. metchnikovii*, which appeared as a membrane extending from the base of the flagellum into the cytoplasm (Fig. 4c).

Thin sections and negative stains of plasmolysed *P. aeruginosa* and negative stains of sonically disrupted autolysing *V. metchnikovii* revealed the presence of spherical membrane-bound structures at the bases of flagella. Figure 5a–c is a series of thin sections of plasmolysing *P. aeruginosa*. In Fig. 5a, the flagellum is seen originating from an untreated organism. Figure 5b shows plasmolysis with the separating of the membrane at the poles, except at the site of the flagellum where the attachment is firmer. As plasmolysis proceeds, a part of the membrane remains at-

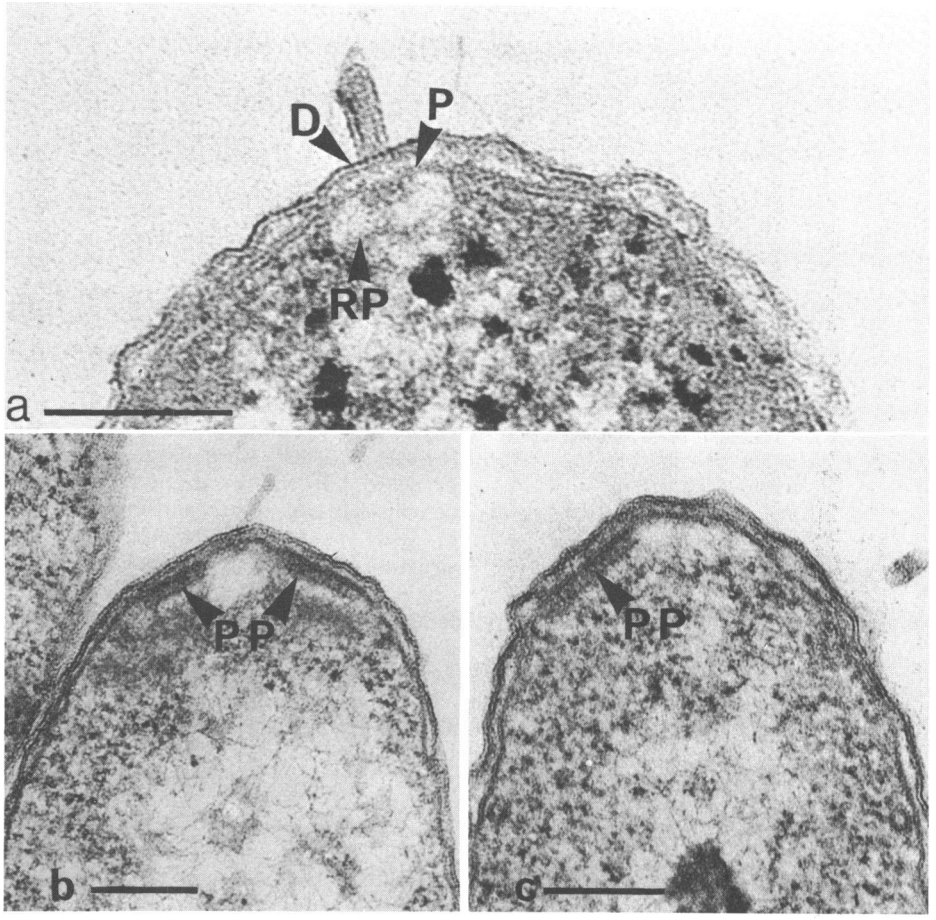

FIG. 2. *Thin sections of V. metchnikovii through the area of flagellar attachment (a). The distal (D) and proximal (P) discs on the flagellum appear as "flat" areas of the cell wall and cytoplasmic membrane, whereas the cell wall in other areas appears wavy; the cytoplasm shows a ribosome-poor area (RP) beneath the proximal disc; (b) and (c) show the presence is a polar plate (PP).*

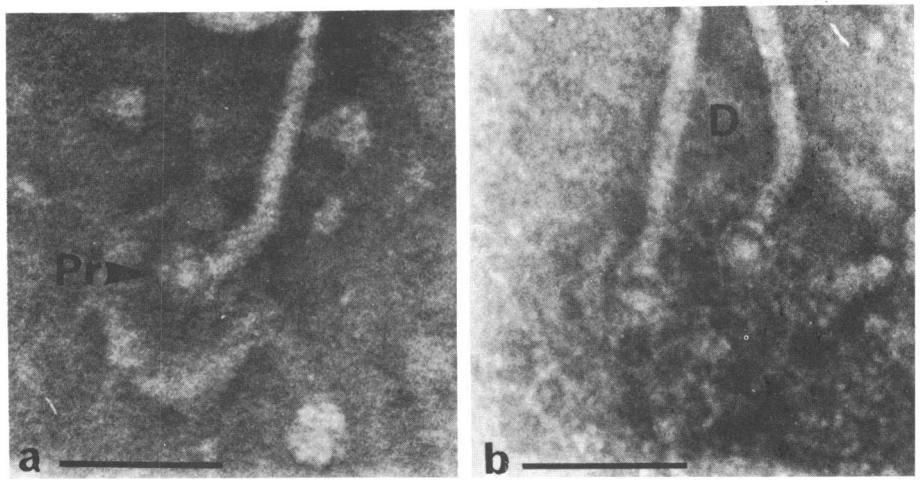

FIG. 3. *Negative stains of alkali-lysed V. metchnikovii. Flagella with under side of flagellar discs; (a) five peripheral projections (Pr) may be seen on the disc; (b) the distal disc is no longer seen except for occasional remnants (D).*

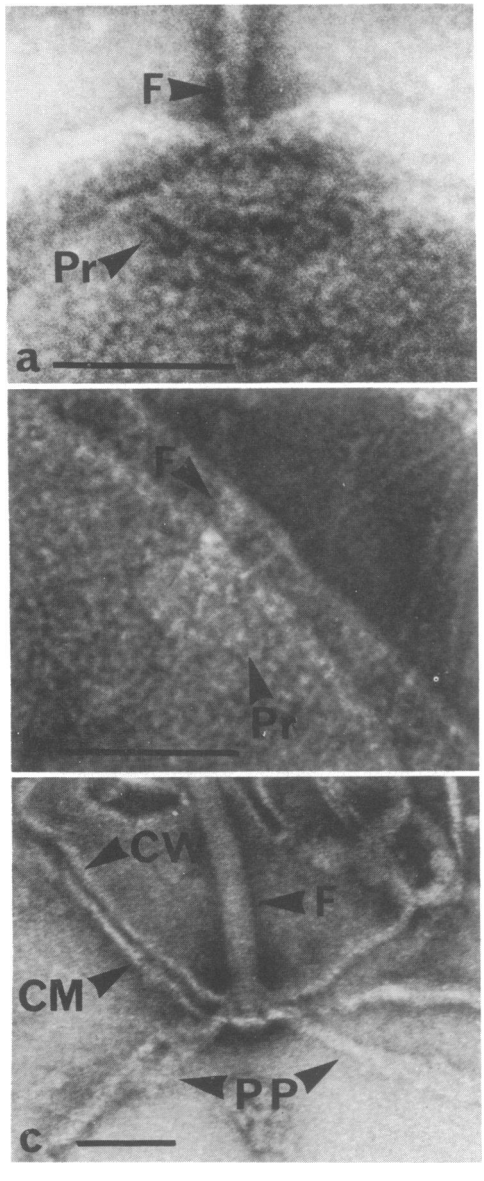

FIG. 4. *Autolysing V. metchnikovii (a) and plasmolysed S. marcescens (b) showing a circular area of fiber-like projections (Pr) originating at the flagellum (F). Autolysed V. metchnikovii (c) with the cell wall (CW), cytoplasmic membrane (CM) and what may be polar plate (PP) fragments attached to a sheathed flagellum (F).*

tached to the flagellum and closes up, thus appearing as a "basal bulb" (Fig. 5c). Figure 5d shows the same results in a negatively stained preparation.

The basal-bulb structure was also demonstrated in *V. metchnikovii* subjected to prolonged autolysis (Fig. 6). In some cases, a cytoplasmic membrane fragment was seen as a spherical structure adhering to the flagellum in the space between the cell wall and cytoplasmic membrane. Basal bulbs on isolated flagella bound by a double membrane were produced by short bursts of high frequency sound on bacteria autolysing in distilled water for 14 days at 4 C. The double membrane arises during tearing away of the cytoplasmic membrane and cell wall from the autolysed cells, giving the appearance of basal bulbs of varying dimensions bounded by two membranes of varying diameters (Fig. 6b–c).

The only structure found in the cytoplasm at the base of flagella was a cluster of ribosome-like particles, but this arrangement is difficult to demonstrate consistently and it may be seen only in some preparations. Figure 7a shows this structure located in the area where the flagellum originates in a thin section of *V. metchnikovii*. It also was observed in *P. aeruginosa* (Fig. 5a), and in negative stains of autolysing *V. metchnikovii* (Fig. 7b) and *S. serpens* (Fig. 7c). The clusters are approximately 60 nm in diameter and do not appear to be membrane-bound.

DISCUSSION

It appears that the discs on the flagella of *V. metchnikovii*, *S. serpens*, and *P. aeruginosa* originate from, or are parts of, the cytoplasmic membrane and cell wall. This contrasts with previous reports (5, 9, 15, 18) wherein the discs are said to terminate in the cytoplasm. Remsen et al. (18) stated that the basal discs acted as a clamp anchoring the flagella to a specialized polar membrane, or polar plate, located within the cytoplasm. Our results clearly demonstrate that the flagellar basal discs are located outside the cytoplasm. The observed diameter of the basal discs (25 nm) is in agreement with that reported for *Rhodospirillum rubrum* (5), *V. metchnikovii* (9), and *Ectothiorhodospira mobilis* (18).

Doetsch (6, 7) proposed that bacterial flagella might be inert-rigid or semi-rigid helices, driven by a membrane-associated area, thus not invoking the necessity of contractile wave formation by flagella. This region was postulated to enclose flagellar "basal bulbs" and the flagellum could be activated from within the bacterium by some arrangement of contractile ring structures. Two modifications of this proposal may be made in light of the present evidence. The first omits the membrane-bound basal bulb since it appears to be an artifact. The second eliminates any fully rotating parts at the locus of flagellar insertion.

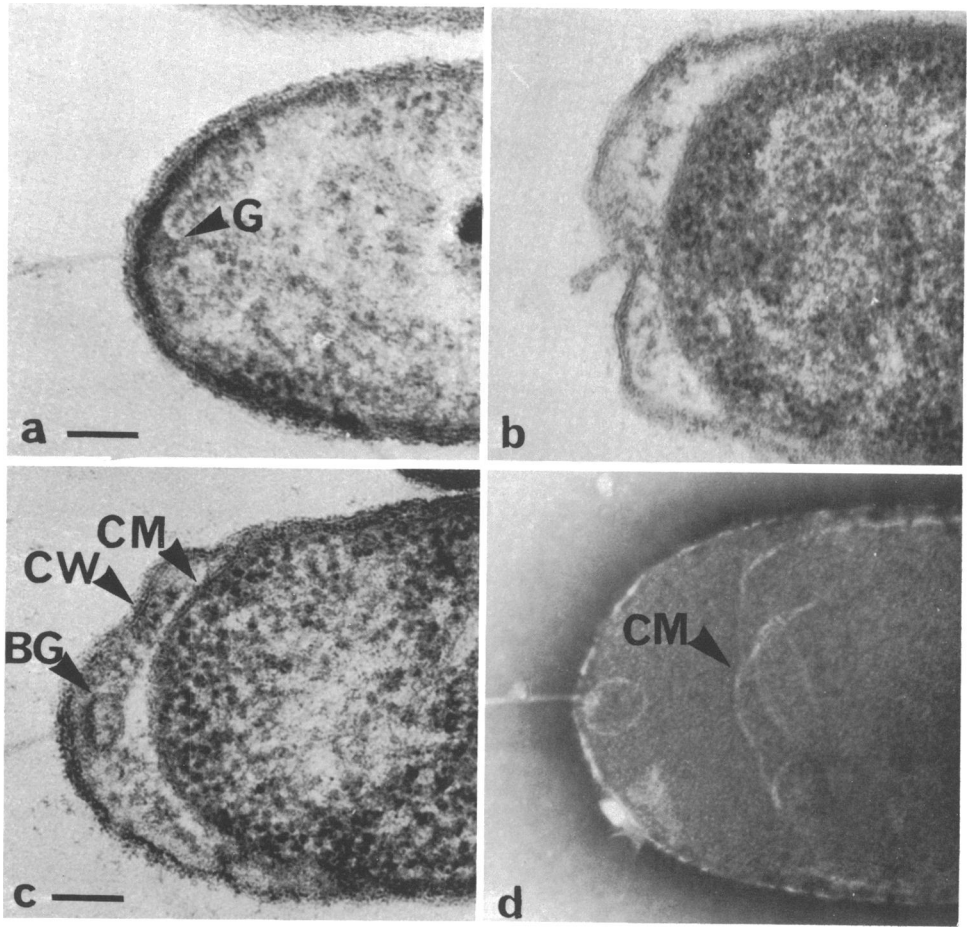

FIG. 5. *Thin sections of plasmolysing P. aeruginosa. (a) The flagellum is attached to the cell wall and cytoplasmic membrane in an untreated cell; (b) plasmolysis results in separation of cell wall and cytoplasmic membrane, except at the locus of flagellar insertion where the attachment seems firmer; (c) round fragment (BG) of membrane remains with flagellum in a plasmolysed cell; note that cytoplasmic membrane (CM) is separated from the cell wall (CW). Similar results may be seen in negatively stained plasmolysed P. aeruginosa (d). Note the cluster of dense, granular material (G) at the base of flagellum (a).*

We now view the mechanism of flagellar motion to be based on a circular wobbling of the disc attached to the cytoplasmic membrane. The discs attaching the flagellum to the cell wall are believed to be an anchoring point. The net result on the flagellum would be the transcription of a cone of revolution by its "hook" portion (Fig. 9b). To produce this cone of revolution, and to evoke circular motion to the remainder of the flagellum, the "hook" portion of the flagellum would have to be rigid. There is evidence supporting this view (2, 3, 14). The remainder of the flagellum would, then, primarily be a propelling device in an efficient form, and its helical structure solely a reflection of the tertiary protein configuration. A mechanical model of this system was found to operate as proposed and it was capable of exerting measurable thrust.

The motility mechanism is, then, dependent on the rigidity of the specialized area of the cell wall seen as a double disc located on the hook portions of flagella (Fig. 1). It is the fulcrum which enables the organism to transfer the activating force via the rigid hook to its flagellum. The cell wall-associated disc has not been seen in flagella of gram-positive bacteria (Fig. 8). This may be due to the structure of the gram-positive cell wall (22) which is mainly composed of the rigid murein sacculus, and teichoic or teichuronic acid polymers. These relatively

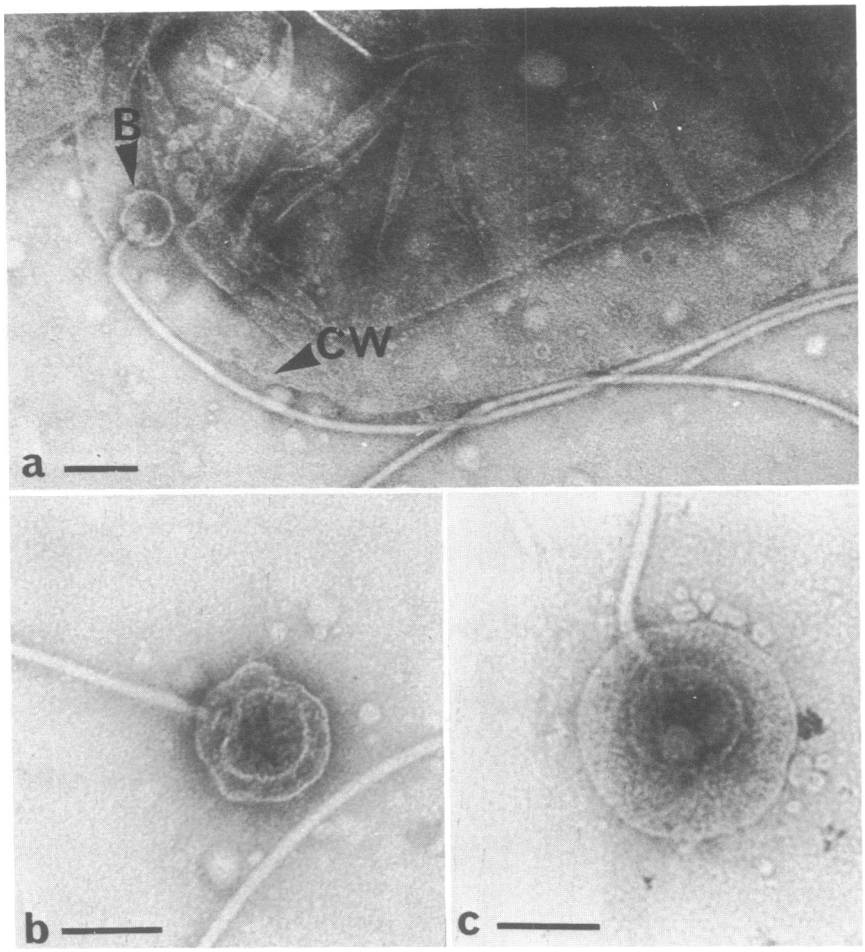

FIG. 6. *Negative stain of autolysing V. metchnikovii showing "basal bulbs." Although still within the cell wall (CW), the "basal bulb" (B) consists of a single spherical fragment of cytoplasmic membrane adhering to the flagellum; (b) and (c) show isolated flagella with fragments of cytoplasmic membrane and cell wall appearing as "basal bulbs" of varying diameters bounded by a double set at membranes.*

thicker cell walls could be sufficiently rigid to provide the flagella with satisfactory anchoring points. Gram-negative bacteria, having a much thinner, rigid, mucopeptide layer, would require a specialized thicker, nonflexible area around the flagellum, which appears as the set of double discs in electron micrographs.

It is further proposed that this view of flagellar attachment and activation explains the cessation of motility when the cell wall is damaged or removed. Penicillin-induced and diaminopimelic acid-deprived spheroplasts become nonmotile and this condition coincides with loss of the rigid mucopeptide layer (26, 27). Motility resumes when spheroplasts synthesize murein sacculus polymers and revert to *rod*-forms. Loss of motility is irreversible in ethylenediaminetetraacetate-lysozyme-formed spheroplasts, presumably due to complete removal of the mucopeptide layer. The same is true for protoplasts of gram-positive bacteria (30). Once the cell wall is depolymerized, there exists no anchor for the flagellar hook. The activating force cannot be effectively transferred to the flagellum, hence, motility ceases.

Two mechanisms may be considered to account for the proposed membrane displacement. One envisages a series of contractile fibers at the base of each flagellum. Such fiber-like structures may be seen in Fig. 4a and 4b. It appears more likely, however, that these short radiating fllaments are extensions of the proximal disc and serve to

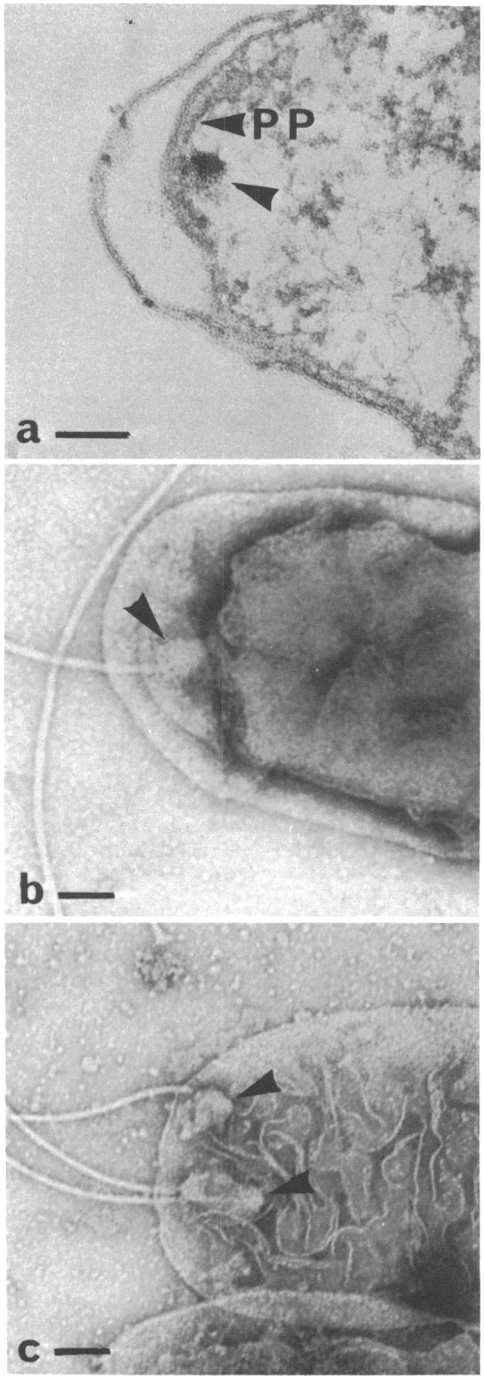

FIG. 7. *Thin section (a) and negative stain (b) of V. metchnikovii and a negative stain of S. serpens (c) showing the presence of a dense structure in the cytoplasm at the base of flagella (arrows). Note the presence of a polar plate (PP) in the thin section (a).*

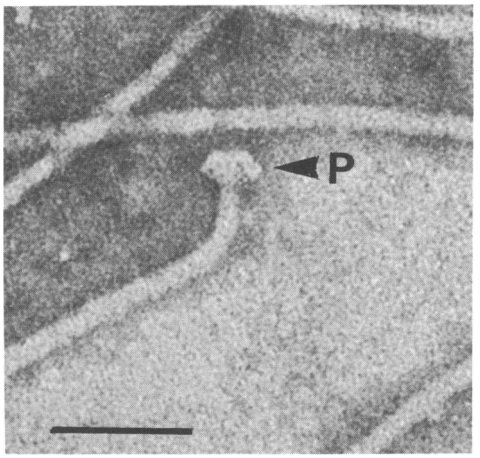

FIG. 8. *Negative stain of isolated B. licheniformis flagella. Only one disc (P) per flagellum is present.*

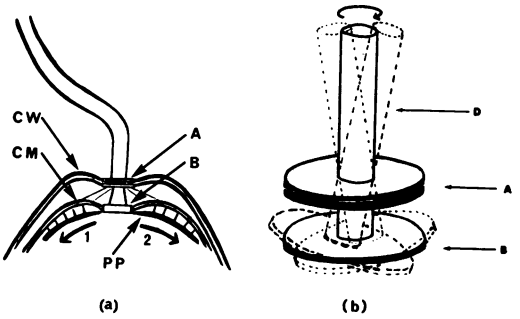

FIG. 9. *Model of proposed flagellar-activating system as deduced from electron microscopic studies of polarly flagellated bacteria (a, b). (A) Distal disc anchoring flagellum to the cell wall; (B) proximal disc firmly attached to the cytoplasmic membrane which is moved (1, 2) by a sequentially contracting and relaxing polar plate (PP). The resulting wobbling motion (b) of the proximal disc (B) creates a cone of revolution (arrow) via the rigid hook portion of the flagellum (D).*

anchor the flagellum tightly to the cytoplasmic membrane. A second possible mechanism would involve a displacement of the cytoplasmic membrane at the flagella-containing poles by an underlying polar membrane, or a polar plate. In Fig. 2, 4c, and 7a, there is seen a polar membrane in the cytoplasm. An alternately contracting and relaxing polar plate is the more likely mechanism, since it would displace the cytoplasmic membrane and all the flagella attached to it. This view eliminates the need to postulate separate movement and coordination mechanisms for each flagellum in lophotrichously flagellated bacteria.

The presence of a polar membrane, also called

polar plate, and its association with flagella has been previously reported (12, 15, 18). Murray and Birch-Andersen (15) postulated that it could supply energy for motility. Keeler et al. (12) suggested that electron transport systems might be located within the membrane. Remsen et al. (18) believed that the flagella of *E. mobilis* were attached to it. These reports and our study do not show the polar plate in thin sections of every organism observed. Some sections show only parts of the plate. This may be due to the fact that the polar plate is not a continuous structure, but a series of discontinuous structures "radiating" from the pole of the bacterium (Fig. 9a), which would make a sequential circular series of contractions more feasible. Such fiber-like structures would in all probability not easily be seen in all thin sections.

The existence of membrane-bound basal granules in polarly flagellated bacteria has been debated since 1953, when Van Iterson (28) reported them in flagellated bacteria. Pease (17) claimed to have resolved the controversy by showing their presence in bacteria in a series of shadowed electron micrographs of *Vibrio* species and *Spirillum* species. As recently as 1965, Newton and Kerridge (16) suggested that these bodies might be artifacts since they had not been reported in thin sections or in negatively stained preparations. Tawara (25) demonstrated a basal granule bounded by a thin membrane in negatively stained *V. cholerae*. Our results show basal bulbs in both negatively stained preparations and thin sections (Fig. 5a–d). We believe that the membrane-bound basal bulbs are artifacts resulting from the persistent adherence of cytoplasmic membrane to the flagellum as the bacterium plasmolyses. Furthermore, basal bulbs of varying dimensions bounded by a double membrane were produced by ultrasonic disruption of autolysing bacteria (Fig. 6a–c). These clearly result from adhering cytoplasmic membrane and cell wall fragments to the flagellar hooks.

The only structure considered to be a real entity at the flagellar terminus was a dense cluster of ribosome-like granules in the cytoplasm at the base of flagella approximately 60 nm in diameter (Fig. 5a, 7a). Negatively stained preparations also showed similar structures when autolysis had not proceeded too far (Fig. 7b–c). This cluster is similar to that reported by Ritchie, Keeler and Bryner (20) as a "cone-shaped basal granule" in *V. fetus*. The nature of this structure is unknown. Furthermore, it cannot be seen in all thin sections. At times, a ribosome-poor area is seen in this region (Fig. 2). It may be that the granules are present during active flagellar synthesis, later dispersing and being replaced by the ribosome-poor area. Whether this is really the case is presently under investigation.

ACKNOWLEDGMENT

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