JOURNAL OF BACTERIOLOGY, Jan. 1971, p. 396-407
Vol. 105, No. 1

Attachment of Flagellar Basal Bodies to the Cell Envelope: Specific Attachment to the Outer, Lipopolysaccharide Membrane and the Cytoplasmic Membrane

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Received for publication 23 October 1970

A procedure is described for the purification of the *Escherichia coli* outer membrane (lipopolysaccharide or L membrane) with flagella still attached. The resulting lipopolysaccharide membrane was in the form of vesicles that had a trilaminar structure in thin section and contained about 55% lipopolysaccharide and 45% protein. T2 or T4 phage preadsorbed to *E. coli* were found attached to the purified lipopolysaccharide membrane. Flagella were bound to the purified lipopolysaccharide membrane specifically at the basal body ring closest to the hook (the L ring). The cytoplasmic membrane in preparations from osmotically lysed *E. coli* spheroplasts or *Bacillus subtilis* protoplasts was specifically attached to flagella at the basal body ring farthest from the hook (the M ring). In the *E. coli* preparation, lipopolysaccharide membrane was also present and was attached to the L ring. From these data and a knowledge of the structure and dimensions of the *E. coli* flagellar basal body and cell envelope, a model for flagellar attachment is deduced.

In an accompanying paper (9), we show that the basal end of *Escherichia coli* flagella consists of a hook attached to a basal body composed of two pairs of rings mounted on a rod. It has been known for some years that the basal end of the bacterial flagellum is attached to the cell envelope (33). The purpose of the present paper is to determine the details of this attachment.

A general structure for the cell envelope of gram-negative bacteria [exemplified by *E. coli* (10) in Fig. 24] consists of three structural components (13): (i) an inner membrane—the cytoplasmic membrane—made of lipid and protein; (ii) an outer membrane—the lipopolysaccharide or L membrane—made of lipopolysaccharide, protein, and phospholipid; and (iii) an intermediate layer—the peptidoglycan layer—made of peptidoglycan, digestible with lysozyme, and possibly some protein. The L membrane and peptidoglycan layer are the major components of the cell wall.

Recent studies have shown that the basal end of bacterial flagella is attached to both the cell wall and the cytoplasmic membrane (1, 2, 7, 12, 15, 25, 29, 31). However, more detailed interpretation of the data is difficult because of the small dimensions and close proximity of the various flagellar and cell envelope components, as well as the problem of identifying these components in the negatively stained and sectioned bacteria or bacterial lysates that were used in the above studies. Therefore, in these studies the structure of the flagellar basal end and its specific relationship with the cell envelope are not clear.

To determine the specific attachments between the basal end of flagella and the cell envelope, we purified the lipopolysaccharide membrane and found that the basal body ring closest to the hook is attached to this membrane. We then found that, in preparations of osmotically lysed spheroplasts, the ring farthest from the hook is attached to the cytoplasmic membrane. From these data and a knowledge of the structures and dimensions of the *E. coli* flagellar basal body (9) and cell envelope (10), a model for flagellar attachment was deduced.

MATERIALS AND METHODS

Electron microscopy. Materials were negatively stained with phosphotungstate, *p*H 7.2, as previously described (9). Phosphotungstate was superior to uranyl acetate, *p*H 4.5, for revealing L membrane. Thin sections through L membrane were prepared as described by dePetris (10). All preparations were viewed in a

Siemens Elmiskop I electron microscope at 80 kv by use of a liquid nitrogen decontamination device and a 50-μm thin metal objective aperture.

Bacteria. *E. coli* K-12 strain AW330 and *Bacillus subtilis* 168, Marburg strain, are motile bacteria described in an accompanying paper (8).

Purification of the outer, L membrane of E. coli. L membrane from *E. coli* was purified by exactly the same procedure that was used for purification of intact flagella (8), except for the modifications cited below. Only these modifications are described in detail. The major modification is that, once spheroplasts have been formed, Mg^{2+} is used instead of ethylenediaminetetraacetic acid (EDTA) to preserve the L membrane.

Spheroplasts were formed from *E. coli* by using lysozyme and EDTA. After the cells were incubated for 1 hr with lysozyme and EDTA, 1 M $MgCl_2$ was added to give a molar ratio of Mg^{2+}/EDTA = 6, and the suspension was incubated at 30 C for 30 min. Lysis was then obtained within one min after addition of Triton X-100. The lysate was incubated with deoxyribonuclease I. In the presence of Mg^{2+}, L membrane is resistant to dissociation by Triton X-100, but the cytoplasmic membrane is solubilized. The lysate is turbid because of the presence of L membrane in the form of vesicles.

The buffer used throughout the remainder of the procedure was 0.1 M tris(hydroxymethyl)aminomethane (Tris) and 10 mM $MgCl_2$, adjusted to *p*H 7.8 with HCl at 26 C (Tris-Mg buffer). Centrifuging the $(NH_4)_2SO_4$-treated lysate resulted in a viscous floating layer and a dense white pellet. The floating layer was collected with a pipette and combined with the pellet. After dialysis of this material, it was placed over sucrose layers and was centrifuged by using an SW-25 rotor in a Spinco model L at 10,000 rev/min for 60 min. The sucrose layers were collected and were dialyzed against Tris-Mg buffer. The dialyzed material was then diluted with Tris-Mg buffer, and Triton X-100 was added. The material was centrifuged for 10 min at 5,000 $\times$ *g* (R_{max}); the pellet was discarded, and the supernatant material was fractionated on a CsCl gradient.

The result was a single white flocculent band, 50 to 55 nm from the meniscus at a density of 1.34 g/ml, which was identified as L membrane with flagella attached. The supernatant liquid from the 10,000 rev/min centrifugation yielded four bands on CsCl, between 1.32 and 1.30 g/ml, which contained various ratios of L membrane and flagella.

The preparation of L membrane with bacteriophage attached was carried out by the following procedure. A suspension of 10^9 *E. coli* cells per ml was prepared in 0.035 M potassium phosphate (*p*H 7.1), 0.08 M NaCl, and 10% (w/w) sucrose. Phage T2 were added (100 phage per bacterium), and the suspension was incubated for 4 min at 37 C. The suspension was then adjusted to contain 0.1 M Tris (*p*H 7.8), 60 μg of lysozyme/ml, and 2 mM EDTA. After incubation for 10 min at 30 C, L membrane was prepared as described above.

Preparation of membranes from spheroplasts and protoplasts. *E. coli* was grown in 10 ml of tryptone broth (8) to an optical density at 590 nm of 1.2; the culture was then centrifuged and washed once by centrifugation in double-distilled water. To form spheroplasts (26), the pellet was dispersed and the cells were suspended for 20 min at 30 C in 2.0 ml of a solution containing 50 μg of lysozyme/ml, 1.5 mM EDTA, 10% sucrose, and 0.1 M Tris (*p*H 7.8 at 26 C). The spheroplasts were then osmotically lysed by a sudden 1:20 dilution into a lysis medium (designed to study adenosine triphosphatase activity) containing: 2 mM $MgCl_2$, 20 mM NaCl, 20 mM KCl, and 50 mM Tris-maleate buffer, *p*H 7.0. After 2 to 5 min, the resulting cytoplasmic membranes and remaining (16, 23) L membranes with their attached flagella were collected by centrifugation at 12,000 $\times$ *g* for 20 min. The pellet was resuspended in 0.25 ml of lysis medium containing 15 μg of deoxyribonuclease I per ml and incubated at room temperature for 10 min.

B. subtilis was grown (8) in 10 ml of Penassay Broth (8), and cytoplasmic membranes were prepared as described for *E. coli*. Protoplasts of *B. subtilis* were not stable in the lysozyme-EDTA-sucrose-Tris medium and lysed before their dilution into lysis medium.

Adenosine triphosphatase assay. Adenosine triphosphatase activity was localized in preparations of membranes from spheroplasts and protoplasts (*see above*) by electron microscopy using the $Pb_3(PO_4)_2$ precipitation technique (18, 32) modified as follows. Samples of freshly prepared membranes were incubated for 30 to 45 min in a reaction mixture (18, 32) containing 2 mM adenosine triphosphate (ATP), 2 mM MgCl, 20 mM KCl, 20 mM NaCl, 1 mM lead acetate, and 40 mM Tris-maleate at *p*H 7.0. Samples were then negatively stained with either 0.5% phosphotungstate or 0.5% ammonium molybdate as described above. A variety of experimental conditions was tried, such as varying the Mg^{2+}/ATP ratio between 1 and 3 and the concentration of ATP and Mg^{2+} between 1 and 5 mM, or using $CaCl_2$ instead of or in addition to $MgCl_2$. The lead acetate concentration was varied between 0.5 and 1.5 mM.

RESULTS

Characterization of purified outer, L membrane. Electron microscopy of negatively stained L membrane preparations (Fig. 1) showed the presence of vesicles and some flagella. These vesicles could not be cytoplasmic membrane since: (i) the vesicles are stable in the presence of Triton X-100 and Mg^{2+}, whereas cytoplasmic membranes are rapidly solubilized by Triton X-100 (5) even in the presence of Mg^{2+} (as indicated by the lysis of spheroplasts by Triton X-100 when Mg^{2+} is present); and (ii) cytoplasmic membranes would have a density of approximately 1.1 to 1.2 g/ml generally found with lipoproteins (20), whereas these vesicles have a density of 1.34 g/ml.

In addition, the vesicles were identified as L membrane based on the following criteria. (i) Purified lipopolysaccharide, the major constituent of L membrane (13, 23), also forms vesicular structures in the presence of Mg^{2+} (DePamphilis, *manuscript in preparation*). (ii) Electron microscopy of thin sections through material from L membrane preparations showed the trilaminar structure (Fig. 2) characteristic of the L membrane in *E. coli* cell walls (10, 13), and

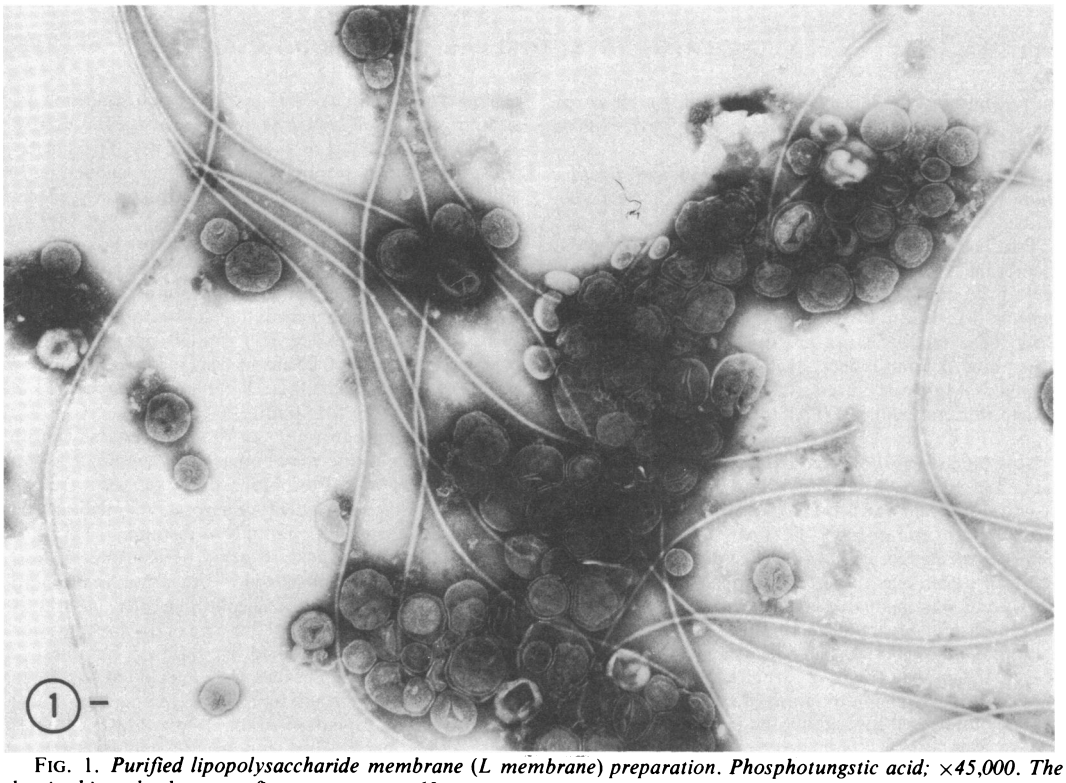


FIG. 1. *Purified lipopolysaccharide membrane (L membrane) preparation. Phosphotungstic acid; ×45,000. The bar in this and subsequent figures represents 60 nm.*

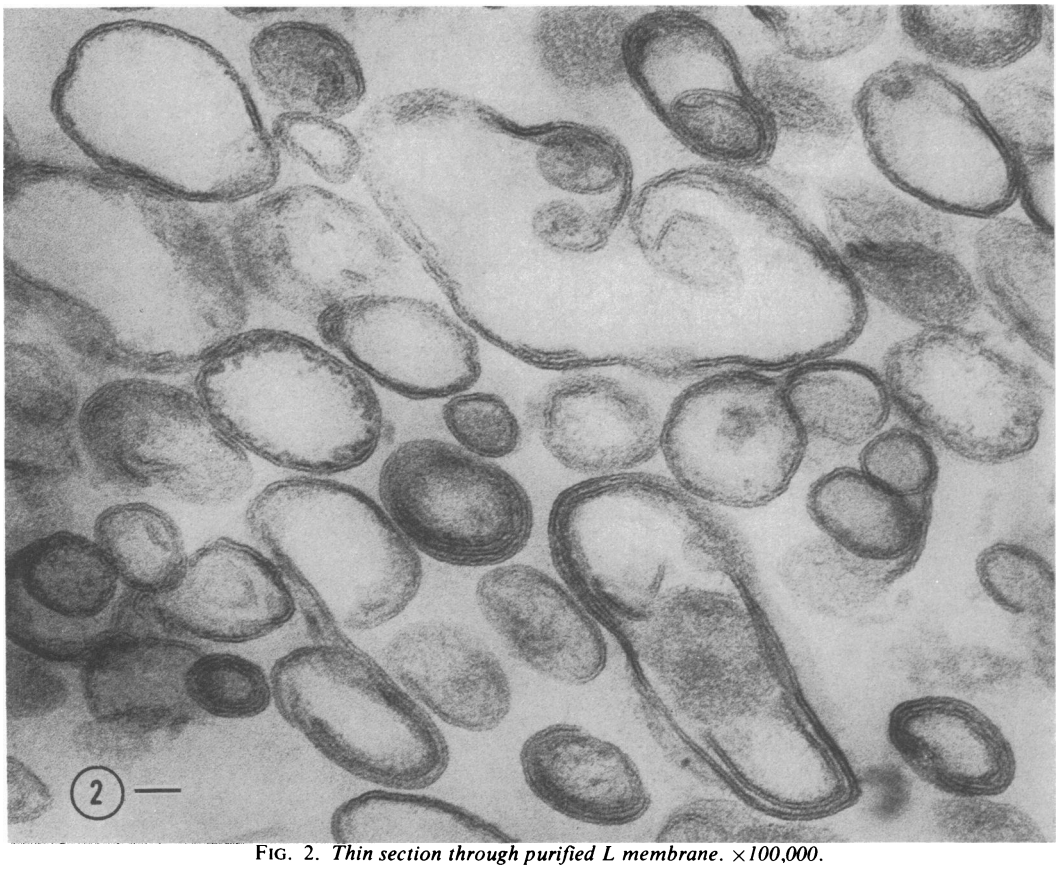


FIG. 2. *Thin section through purified L membrane. ×100,000.*

the coiled appearance and thickness (9 ± 0.8 nm, Fig. 2) reported in L membrane isolated by a different procedure (19). (iii) Chemical analysis (DePamphilis, *manuscript in preparation*) of L membrane preparations and lipopolysaccharide purified from the same *E. coli* strain demonstrated similar molar ratios of heptose, glucose, 7-keto-3-deoxyoctonate, and phosphate. These compounds are characteristic of lipopolysaccharide (23). The L membrane preparations contained about 55% lipopolysaccharide and 45% protein, some of which was flagella and pili. L membrane has also been shown to contain small amounts of phospholipid (17), but we did not assay for it. (iv) After T2 or T4 phage adsorption to intact cells, we were able to isolate L membrane with phage attached to the outside of the vesicles. About 80% of the vesicles had one or more phage attached (Fig. 3). The phage were oriented perpendicular to the surface of the vesicle and had contracted sheaths and empty heads. This identifies the vesicles as originating from the outer layer of the cell envelope which contains the T2 and T4 phage receptor sites (34).

Specific attachment of flagellar basal bodies to purified L membrane. The flagella in the L membrane preparation were attached by their basal bodies (Fig. 4, 5, 7, 8, 9). We have shown (9) that flagellar basal bodies from *E. coli* consist of two pairs of rings mounted on a rod. Inspection of L membrane vesicle-basal body complexes shows that vesicles are specifically attached to the basal body ring closest to the hook, therefore called the L ring (Fig. 4, 5, 7, 8), and the remaining three rings are not involved. Infrequently basal bodies were seen with an extension of their L rings (Fig. 6), which is probably due to attachment of L membrane. Similar variations in ring diameter were not seen with the other three rings.

Some basal bodies were found encapsulated by vesicles with a diameter of 60 to 70 nm (Fig. 7) and looked similar to basal body "structures" frequently reported (1, 14, 15, 24, 27, 28, 31; D. Abram, Bacteriol. Proc., p. 30, 1968 and p.29, 1969; A. E. Ritchie and J. H. Bryner, Bacteriol. Proc., p. 29, 1969). This raised the question of whether such vesicles attached to basal bodies represent a true structure of the basal body or artifacts resulting from vesiculation of L membrane. When T2 had been adsorbed to cells before L membrane purification, phage were found adsorbed to the outside surface of basal body-vesicle complexes (Fig. 8, 9). Therefore, the vesicles attached to flagellar basal bodies found in L membrane preparations originated from vesiculation of the outer membrane of the cell envelope.

Data further confirming the attachment of L rings to L membrane will be presented in a later paper, where it will be shown that dissociated L membrane or purified lipopolysaccharide reassembles into vesicles with concomitant attachment to flagellar basal body L rings.

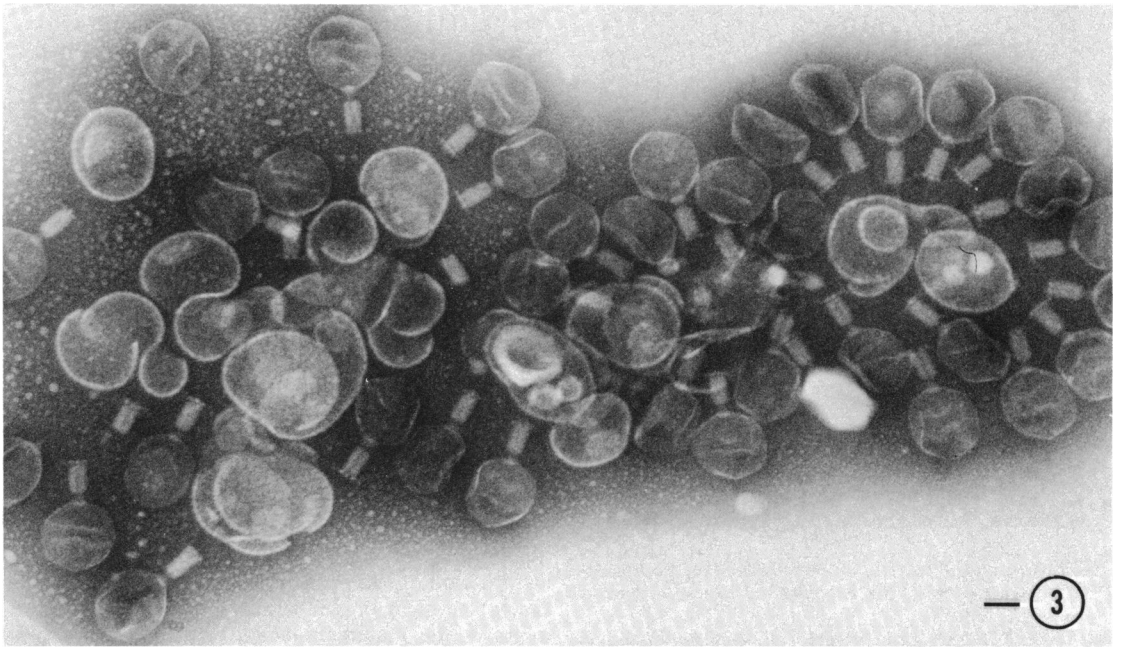
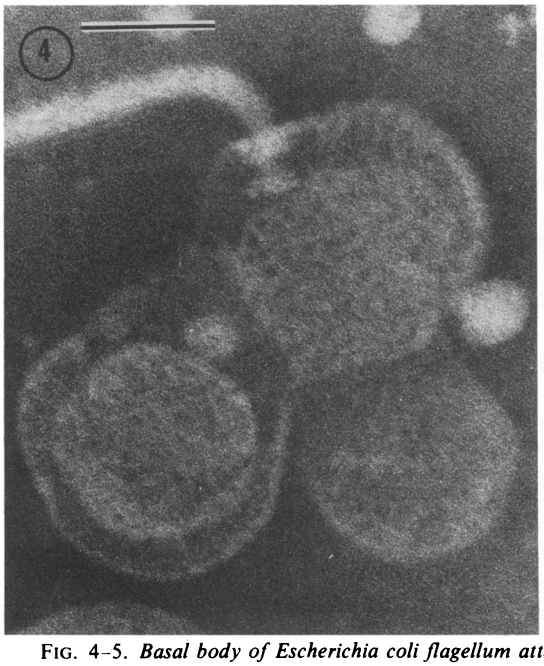


FIG. 3. *T2 phage attached to purified L membrane vesicles. Phosphotungstic acid;* ×*77,000.*



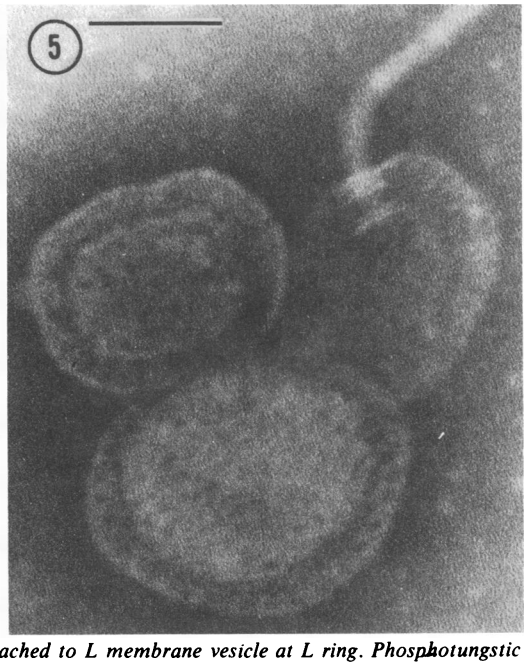
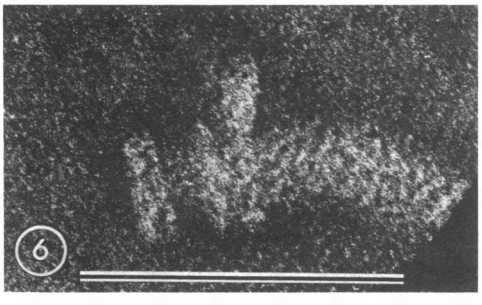


FIG. 4–5. *Basal body of Escherichia coli flagellum attached to L membrane vesicle at L ring. Phosphotungstic acid; ×290,000.*



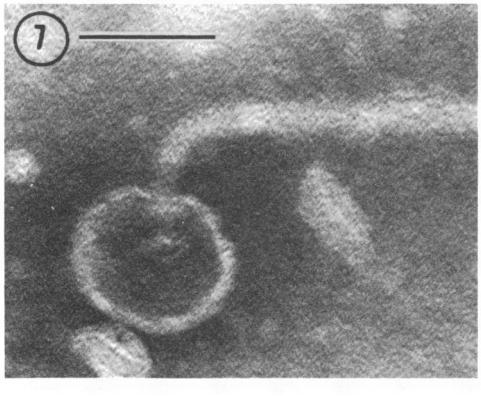


FIG. 6. *Basal body of an intact flagellum from Escherichia coli with L ring extended on one side. Uranyl acetate; ×712,000.*

FIG. 7. *Basal body of intact E. coli flagellum enclosed in a sealed L membrane vesicle. Phosphotungstic acid; ×300,000.*

Specific attachment of flagellar basal bodies to cytoplasmic membrane. To examine the association of flagellar basal bodies with the cytoplasmic membrane, *E. coli* spheroplasts were lysed osmotically. This yields pieces ranging in size from entire spheroplasts (Fig. 10) down to small fragments (Fig. 15, 16). The pieces contain cytoplasmic membrane and often L membrane, and these can be identified and distinguished by the following criteria.

(i) Flagella can be used as a marker for the "outside" surface of the spheroplast fragment. Thus, in Fig. 11 to 13, the flagella show which is the outer, L membrane and which is the inner, cytoplasmic membrane. (ii) The two membranes can often be distinguished by a difference in texture and configuration (Fig. 10, 11, 13). Figure 10 is consistent with the report of Birdsell and Cota-Robles (4) that in thin sections of spheroplasts the L membrane is folded up, exposing the cytoplasmic membrane. (iii) Adenosine triphosphatase, is known to be located in the cytoplasmic membrane (19). By using the $Pb_3(PO_4)_2$ detection method (18, 32) on preparations of osmotically lysed spheroplasts, this activity was localized in the area labeled cytoplasmic membrane. (iv) In the presence of $MgCl_2$, Triton X-100 dissolves the cytoplasmic membrane but not the L membrane. Such treatment of osmotically lysed spheroplasts removes the material labeled cytoplasmic membrane from the basal bodies and exposes the bottom ring.

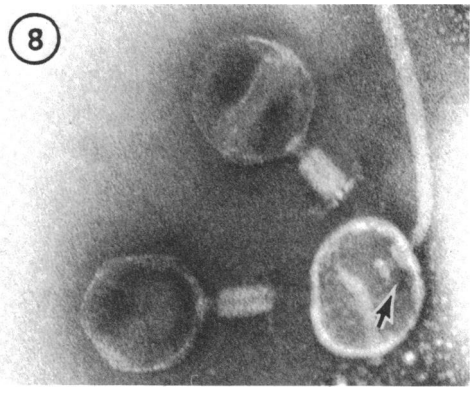


FIG. 8. *Basal body (arrow) of an E. coli flagellum enclosed in a sealed L membrane vesicle with T2 phage attached to the vesicle. Phosphotungstic acid; ×160,000.*

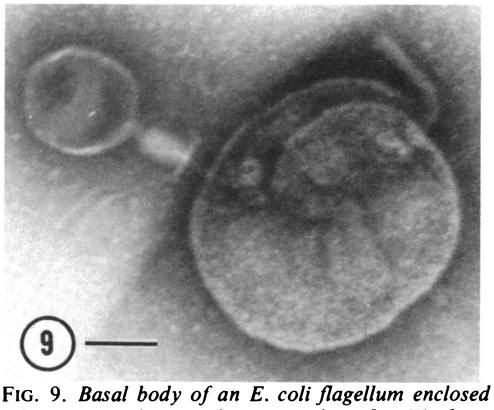


FIG. 9. *Basal body of an E. coli flagellum enclosed in a large, opened L membrane vesicle with a T2 phage attached to the vesicle. Phosphotungstic acid; ×155,000.*

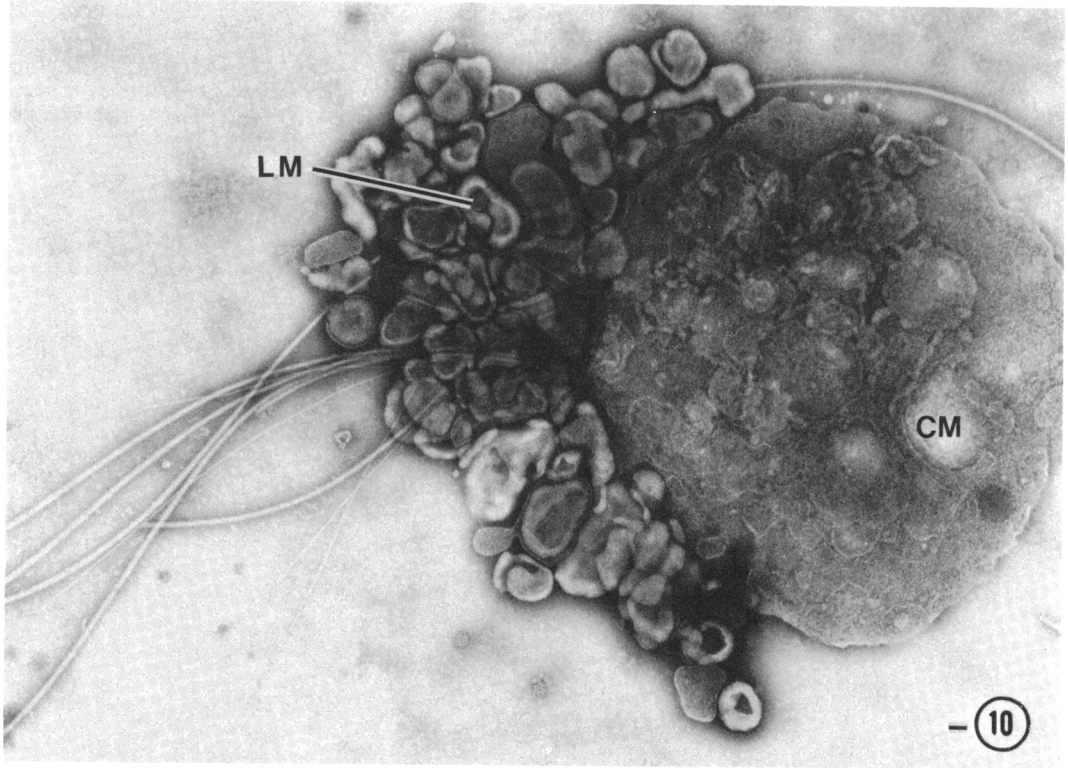


FIG. 10. *Partially damaged Escherichia coli spheroplast found in a preparation of osmotically lysed spheroplasts. L membrane (LM) has folded away from the cytoplasmic membrane (CM) and assumed a vesicular configuration. Phosphotungstic acid, ×37,000.*

In these preparations of osmotically lysed spheroplasts, flagellar basal bodies were bound to the cell envelope. The L membrane was specifically attached to the part of the basal body proximal to the hook, the L ring (Fig. 11–13). This confirms the results described above with purified L membrane. The end of the basal body distal to the hook was attached to the cytoplasmic membrane (Fig. 11–16). In these figures a basal body ring, the S (for "supramembrane") ring, is gener-

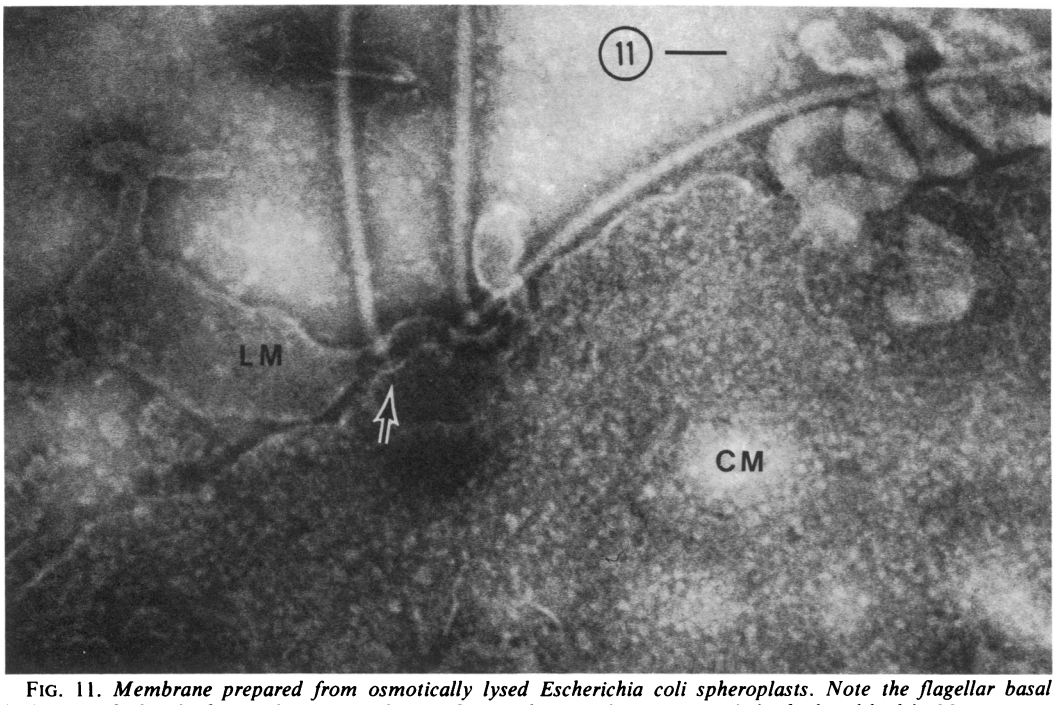


FIG. 11. *Membrane prepared from osmotically lysed Escherichia coli spheroplasts. Note the flagellar basal bodies attached to both cytoplasmic membrane (CM) and L membrane (LM). Only the basal body's M ring is attached to the cytoplasmic membrane (arrow). Phosphotungstic acid; ×133,000.*

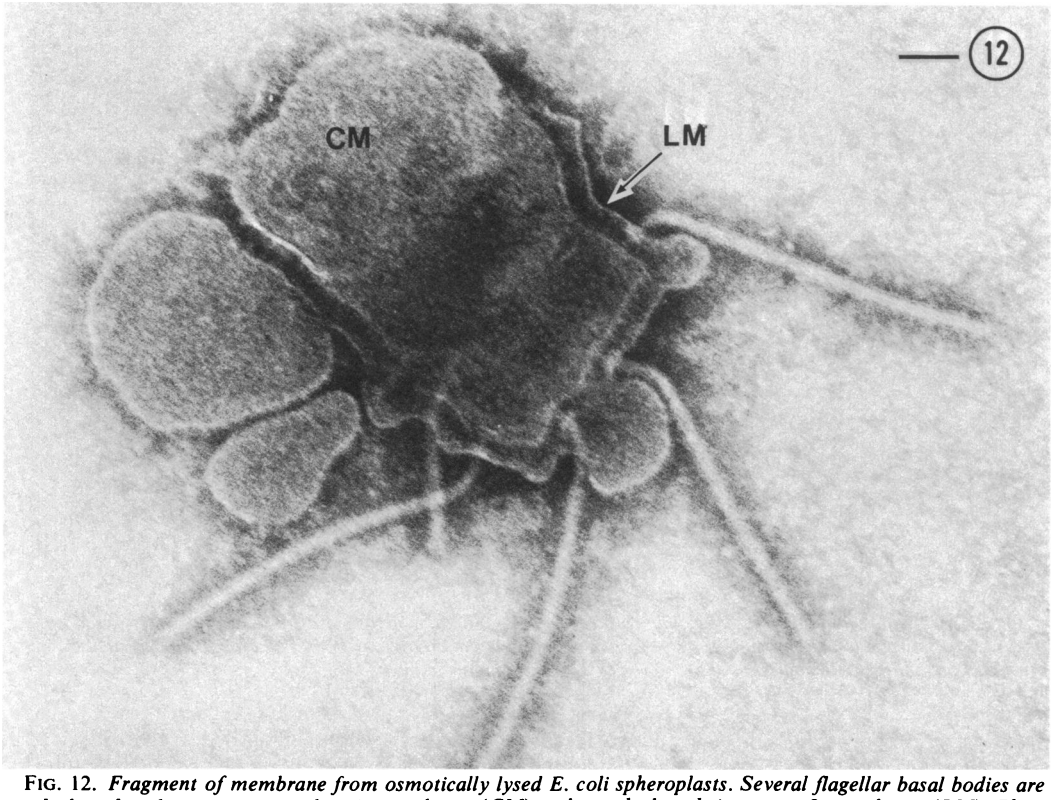


FIG. 12. *Fragment of membrane from osmotically lysed E. coli spheroplasts. Several flagellar basal bodies are attached at their bottoms to cytoplasmic membrane (CM) and attached at their tops to L membrane (LM). Phosphotungstic acid; ×133,000.*

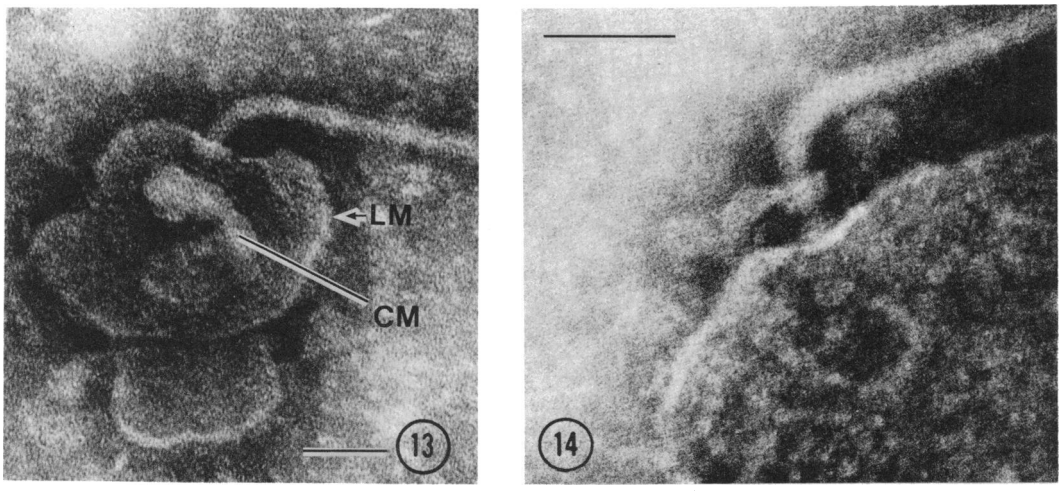


FIG. 13. *Basal body of an Escherichia coli flagellum attached to a fragment of L membrane (LM) and cytoplasmic membrane (CM). The S ring is visible above the cytoplasmic membrane. Phosphotungstic acid; ×185,000.*

FIG. 14. *Basal body of an E. coli flagellum attached to cytoplasmic membrane. The S ring is visible above the membrane. Phosphotungstic acid; ×290,000.*

ally visible just above the cytoplasmic membrane, leaving us with the conclusion that the M (for membrane) ring is the specific site of basal body attachment to the cytoplasmic membrane. We have not found any evidence for a part of the basal body extending past the M ring into the cytoplasm, but the possibility remains that a fragile or unattached component of the flagellar base has been lost.

Some flagellar basal bodies were found with spherical "structures" attached to the bottom (Fig. 18). These "structures" are evidently artifacts resulting from vesiculation of a cytoplasmic membrane fragment. They were never seen when basal bodies were attached to long stretches of cytoplasmic membranes (for example Fig. 11, 12). One specimen was found with eight basal bodies attached to a single membrane sphere (Fig. 17), so it appears that each flagellum does not have a sphere at its base. Figure 19 shows artifacts derived from vesiculation of body cytoplasmic and L membranes.

The attachment of flagella to cytoplasmic membranes prepared from *B. subtilis* protoplasts was also examined. In an accompanying paper (9) we show that flagellar basal bodies from *B. subtilis* are equivalent to *E. coli* basal bodies without the top pair of rings. In Fig. 20 cytoplasmic membrane must be specifically attached to the bottom-most ring (the M ring), since only the S ring is visible. Membrane fragments on flagellar basal bodies were often seen (Fig. 21–23) but spherical "structures" were not found.

We attempted with the electron microscope to locate a possible flagellar adenosine triphosphatase in preparations of membranes from spheroplasts and protoplasts by using the $Pb_3(PO_4)_2$ precipitation technique. Adenosine triphosphatase, i.e., localized areas of $Pb_3(PO_4)_2$, was readily detected in cytoplasmic membranes from *E. coli* and *B. subtilis*, but no adenosine triphosphatase activity was found in flagellar filaments, hooks, or basal bodies. No correlation was found between the sites of membrane adenosine triphosphatase activity and the sites of attachment of flagella. Attempts by others to demonstrate an adenosine triphosphatase in flagellar filaments were also negative (3, 11, 22).

DISCUSSION

Figure 24 diagramatically illustrates the relationship between the basal body and cell envelope of *E. coli*. Our conclusions are based on the results presented in this paper, the structures and dimensions of the basal body from *E. coli* (9), and the structures and dimensions of the *E. coli* cell envelope (10).

The L (for lipopolysaccharide) ring of flagellar basal bodies from *E. coli* specifically attaches to the cell envelope's outer membrane, a lipopolysaccharide-protein-phospholipid complex referred to as the L membrane. The evidence for this statement is as follows. (i) Purified L membrane is attached to flagellar basal bodies via the L ring. (ii) Osmotically shocked spheroplasts contain L membrane still attached to basal bodies via the L ring. (iii) Purified L membrane or purified lipopolysaccharide can be dissociated and then reconstituted onto flagellar basal bodies via

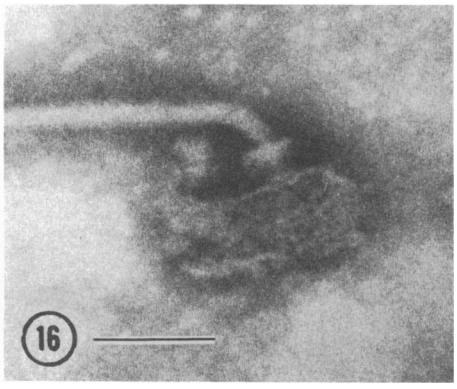
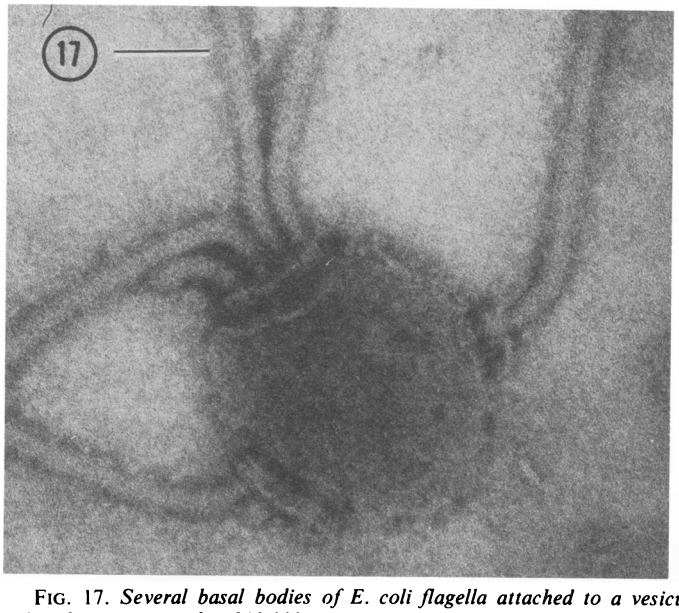


FIG. 15–16. *Basal body of an E. coli flagellum attached to a small fragment of cytoplasmic membrane. The S ring is visible above the cytoplasmic membrane. Phosphotungstic acid; ×265,000.*



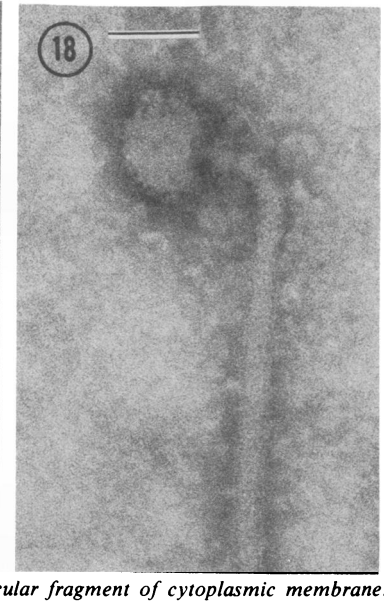


FIG. 17. *Several basal bodies of E. coli flagella attached to a vesicular fragment of cytoplasmic membrane. Phosphotungstic acid; ×210,000.*

FIG. 18. *Basal body of an E. coli flagellum attached to a spherical cytoplasmic membrane fragment, approximately 50 nm in diameter. Phosphotungstic acid; ×200,000.*

attachment to their L ring (DePamphilis, *manuscript in preparation*).

The M (for membrane) ring specifically attaches to the cell envelope's inner membrane, the lipoprotein complex known as the cytoplasmic membrane. The evidence is that osmotically lysed spheroplasts contain cytoplasmic membrane attached to the bottom ring of flagellar basal bodies.

Given the facts that the L ring is attached to the L membrane and the total thickness of the L membrane and peptidoglycan layer is 12 nm (10), the second ring from the top would be in register with the peptidoglycan layer to which it is presumably attached. We therefore refer to this ring as the P ring. Although there is no direct evidence for an association between the P ring and peptidoglycan layer, the fact that the P ring is always free of attached material after lysozyme treatment is consistent with the postulated attachment. An alternative is an association between the P ring and the lipoprotein component known to be covalently bound to the peptidoglycan (6).

The S (for "supramembrane") ring is visible just above the cytoplasmic membrane in osmotically lysed spheroplasts. Unlike the case of the L and M rings, our electron micrographs show no

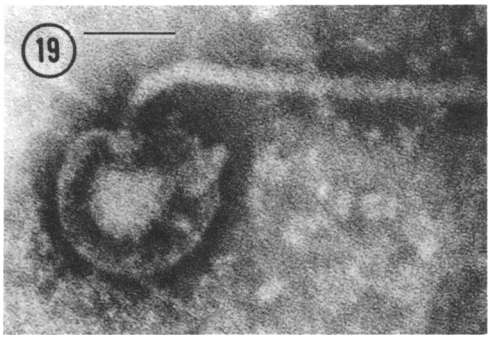


FIG. 19. *Basal body of an Escherichia coli flagellum attached to both a closed L membrane vesicle and a spherical cytoplasmic membrane fragment, approximately 45 nm in diameter. Phosphotungstic acid; ×200,000.*

material associated with the S ring, and, unlike the P ring, the S ring is not in register with any known cell envelope structure.

Spherical structures were sometimes found attached to *E. coli* basal bodies. These were shown to be artifacts resulting from vesiculation of fragments of either L membrane or cytoplasmic membrane that were specifically attached to either the L or M rings, respectively. We believe that reports in the literature [1, 14, 15, 24, 27, 28, 31; D. Abram, Bacteriol. Proc., p. 30, 1968 and p. 29, 1969; A. E. Ritchie and J. H. Bryner, Bacteriol. Proc., p. 29, 1969] of spherical basal body structures resulted from the same artifacts. Our work indicates that flagella do not have any structure penetrating the cytoplasm but that they terminate at the junctions of their M ring with the cytoplasmic membrane.

The differences in location and attachment of basal body rings suggest that each of the four rings is a chemically distinct component of the basal body. However, the chemistry of the basal body remains to be established.

The proposed attachment of *E. coli* flagellar basal bodies to the cell envelope (Fig. 24) appears to be a general model for gram-negative bacteria which have cell envelopes comparable in structure to that of *E. coli*. Photographs of flagellar basal

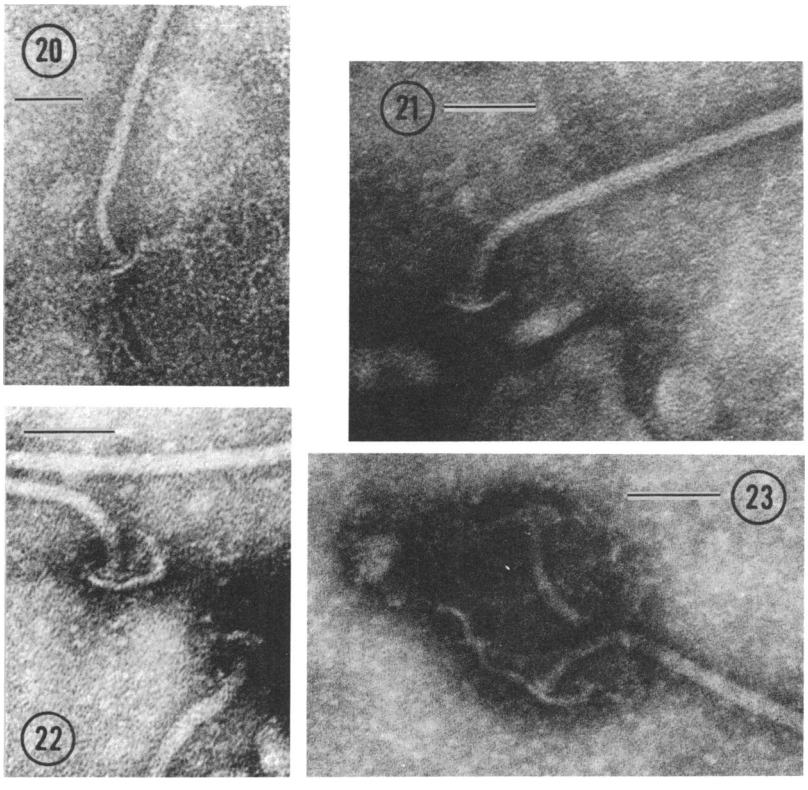


FIG. 20. *Basal body of a Bacillus subtilis flagellum attached to cytoplasmic membrane. One ring is visible above the membrane; the other is attached to the membrane. Phosphotungstic acid; ×145,000.*

FIG. 21–23. *Basal ends of B. subtilis flagella attached to fragments of cytoplasmic membrane. Only the bottom ring is attached to the membrane. Phosphotungstic acid; ×200,000.*

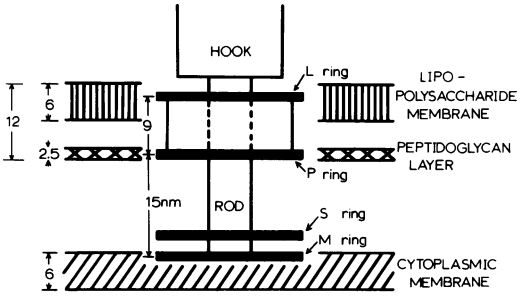


FIG. 24. *Model of the attachment of the flagellar basal body of Escherichia coli to the cell envelope. Dimensions are expressed in nanometers. The basal body rings are shown being thinner than the cell envelope layers. However, the dimensions of the flagellar basal end come from negatively stained preparations (9), whereas the dimensions of the cell envelope layers come from thin-sectioned material (10). Negative staining of material containing both basal body rings and cell envelope layers, such as Fig. 4, 5, 11, and 12, suggests that, in fact, the thickness of the basal body rings and the cell envelope layers is very similar. The space shown between the basal body and the cell envelope is only for clarity.*

bodies attached to cell envelopes from *Vibrio* (29), *Proteus* (1, 15, 31), *Rhodospirillum* (7), and *Ectothiorhodospira* (25) lysed by various methods are consistent with our model.

Flagellar basal bodies from *B. subtilis* are similar to those of *E. coli* except that they have only one pair of rings. These rings are analogous to the S and M rings of *E. coli* basal bodies on the basis of their position at the bottom of the rod, their 3.5-nm separation (9), and particularly their relationship to the cytoplasmic membrane as shown in this paper. Some of Abram's data (2) on a variety of *Bacillus* species are consistent with this point of view. The relationship between the structures of the flagellar basal body and cell wall of *B. subtilis* is more difficult to assess. Gram-positive bacteria have a single (possibly double; 21)-layered cell wall lacking an outer, lipopolysaccharide membrane (13). The thickness of this wall generally varies from 15 to 50 nm (13), with a thickening occurring in stationary phase of growth. The thickness of cell walls on exponentially growing *B. subtilis*, calculated from van Iterson's data (30), varies from 12 to 17 nm. The distance between the flagellar hook and S ring is approximately 15 nm, enough room to accommodate the cell wall in a manner analogous to *E. coli*. However, the cell wall in *B. subtilis* appears to lie in contact with the cytoplasmic membrane (30), and therefore could be attached to the basal body S ring.

One basal body function is clearly to anchor a flagellum to the cell. Cell walls of gram-positive bacteria (e.g., *B. subtilis*) evidently are capable of anchoring flagellar basal bodies without a need for the L and P rings found on basal bodies from gram-negative bacteria (e.g., *E. coli*).

A common feature of basal bodies from *E. coli* and *B. subtilis* is the presence of the S and M rings. This suggests that, if the basal body plays an active role in flagellar movement, one or both of these rings may be involved.

ACKNOWLEDGMENT

We thank June E. Sundquist for preparing the thin section shown in this paper.

This research was supported by Public Health Service grant AI-08746 from the National Institute of Allergy and Infectious Diseases. M. L. DePamphilis held a Public Health Service Predoctoral Fellowship from the National Institute of General Medical Sciences.

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