

Flagellated Ectosymbiotic Bacteria Propel a Eucaryotic Cell

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ABSTRACT A devescovinid flagellate from termites exhibits rapid gliding movements only when in close contact with other cells or with a substrate. Locomotion is powered not by the cell's own flagella nor by its remarkable rotary axostyle, but by the flagella of thousands of rod bacteria which live on its surface. That the ectosymbiotic bacteria actually propel the protozoan was shown by the following: (a) the bacteria, which lie in specialized pockets of the host membrane, bear typical procaryotic flagella on their exposed surface; (b) gliding continues when the devescovinid's own flagella and rotary axostyle are inactivated; (c) agents which inhibit bacterial flagellar motility, but not the protozoan's motile systems, stop gliding movements; (d) isolated vesicles derived from the surface of the devescovinid rotate at speeds dependent on the number of rod bacteria still attached; (e) individual rod bacteria can move independently over the surface of compressed cells; and (f) wave propagation by the flagellar bundles of the ectosymbiotic bacteria is visualized directly by video-enhanced polarization microscopy. Proximity to solid boundaries may be required to align the flagellar bundles of adjacent bacteria in the same direction, and/or to increase their propulsive efficiency (wall effect).

This motility-linked symbiosis resembles the association of locomotory spirochetes with the Australian termite flagellate *Mixotricha* (Cleveland, L. R., and A. V. Grimstone, 1964, *Proc. R. Soc. Lond. B Biol. Sci.*, 159:668-686), except that in our case propulsion is provided by bacterial flagella themselves. Since bacterial flagella rotate, an additional novelty of this system is that the surface bearing the procaryotic rotary motors is turned by the eucaryotic rotary motor within.

Numerous kinds of symbiotic associations occur between procaryotic and eucaryotic cells (3, 18, 36). In only a few cases, however, is the functional significance of the relationship known. We previously described the attachment sites of two types of ectosymbiotic bacteria to a devescovinid flagellate from termites (40, 43). Freeze-fracture and thin-section electron microscopy revealed that both the bacteria and the protozoan contribute structural specializations to the junctional complexes. It was therefore inferred that both partners must benefit from the association, but the presumed advantage accruing to each partner remained unknown.

Here we investigate the functional nature of the relationship between the bacteria and the devescovinid. However, we ask not what the protozoan does for its procaryotes, but what the bacteria do for the eucaryotic host. We show that one type of ectosymbiotic bacterium is flagellated and provides locomotion for the protozoan. This unusual motility system is analogous to the locomotory spirochetes attached to the Australian termite

flagellate, *Mixotricha* (8), except that in our case propulsion is provided by bacterial flagella themselves. A preliminary report of these findings has appeared previously (41).

MATERIALS AND METHODS

Organism

The protozoan is the same (as yet unnamed) devescovinid flagellate from *Cryptotermes caviifrons* used in previous studies on rotational motility and membrane fluidity (38, 39, 42, 44, 45). Wood containing termites was collected in southern Florida and stored in garbage cans under controlled conditions of temperature and humidity in the laboratory.

Microscope Preparations

Hindguts from several termites were teased apart in a drop of appropriate solution on a microscope slide. For most observations, 0.6% NaCl was used as the medium. The preparation was immediately sealed with a Vaseline-edged cover slip to protect the anaerobic hindgut fauna from the air. Devescovinids exhibit vigorous rotation of their anterior ends for at least several hours under these conditions (39). Extensive contact of the cells with a substrate, necessary to

induce gliding motility (see below), was achieved simply by flattening the cells with the cover slip.

Inhibitors

Stock solutions of amphotericin B and nystatin (kindly supplied by Dr. David Nelson, University of Wisconsin-Madison) were made in dimethyl sulfoxide (DMSO), and were added to the medium to give a final DMSO concentration of 1%. Controls consisted of 1% DMSO in 0.6% NaCl without antibiotics. 2,4-Dinitrophenol (DNP, Sigma grade II; Sigma Chemical Co., St. Louis, MO) stock solutions were also made in DMSO, and added to 0.3% NaCl, 0.1 M NaPO₄ (pH 7.0) to give a final DMSO concentration of 1%. 1% DMSO in this medium served as the solvent control.

Cinemicrography

Motility was filmed through a Zeiss Universal microscope with phase-contrast objectives (N.A. 0.75 or 1.3) using a Locam 16-mm ciné camera (Redlake Laboratories, Santa Clara, CA) at 25 frames/s on Plus-X Negative or Reversal film. Prints of selected frames were made from duplicate negatives using a modified photographic enlarger. Velocity measurements and tracings of bacterial swimming paths were made from films with a L-W Photo-Optical projector (L-W International, Woodland Hills, CA).

Video-enhanced Polarization Microscopy

Flagellar motility of the ectosymbiotic bacteria was visualized in collaboration with Dr. Shinya Inoué (Marine Biological Laboratory), using his polarization microscope and video techniques described previously (13). A $\times 100/1.35$ N.A. planapochromatic objective was used for this work.

Detergent Isolation of Ectosymbiotic Bacteria

Hindguts from several dozen termites were rapidly teased apart in a deep well of 0.6% NaCl. The suspension was filtered through cheesecloth to remove gut fragments, then centrifuged twice at low speed through 0.6% NaCl to exclude free-swimming bacteria and small flagellates. The resulting pellet of protozoa was resuspended in a small volume of 0.05% Nonidet P-40 (Particle Data, Inc., Elmhurst, IL), 0.1 M KCl, 0.02 M MgCl₂, 0.01 M NaPO₄ (pH 7.0) at 4°C for ~2 min. Cells were disrupted by repeated expulsions through a glass Pasteur pipette. The lysate was washed in cold salt solution without detergent, and examined by negative-stain electron microscopy.

Electron Microscopy

Drops of washed lysate were placed on Formvar-coated carbonized grids, washed with 0.6 M KCl, or 0.1 M KCl, 0.005 M MgCl₂, then rinsed with cytochrome c, and negatively-stained with unbuffered 1% uranyl acetate. For thin-sections, devescovinids were fixed and processed as described previously (44, 45). Grids were viewed with a Philips 300 electron microscope operated at 80 kV.

RESULTS

General Features and Rotational Motility of Devescovinids

The locomotory movements of the devescovinid are the subject of this report. As will be shown, this motility is distinct from, and completely unrelated to the remarkable rotational movements which first attracted attention to this cell (38, 39, 42, 44, 45).

The flagellate is 100–150 μm long, and missile-shaped when freshly isolated from termites (Fig. 1). Four flagella arise from the caplike anterior end of the cell: three flagella whip vigorously in an anterior-posterior direction, and a longer trailing flagellum propagates waves posteriorly.

The anterior end or head of the devescovinid continually rotates in a clockwise direction (viewed anteriorly) relative to the cell body at speeds of up to 0.5 rotation/s. Since the plasma membrane is continuous across the shear zone (42), this motility provides direct visual evidence for the fluid nature of cell membranes (44).

If the head is tethered, the body rotates in the opposite direction. Laser microbeam experiments show that rotational movements are caused by a rodlike axostyle complex which runs from the head through the body of the cell and generates torque along its length (39). Under *in vitro* conditions, the devescovinids gradually change shape, so that the posterior part of the axostyle projects caudally from the rounded cell body. Rotational motility continues *in vitro* for as long as the cells remain viable.

Ectosymbiotic Bacteria

Rod-shaped and fusiform bacteria live permanently attached to the surface of the devescovinid in a characteristic pattern (Figs. 1–3). The ultrastructure of the junctional complexes formed between the two kinds of bacteria and the devescovinid has been described previously (40, 43).

The rod bacteria are 2–3 μm long and 0.6–1.0 μm in diameter.

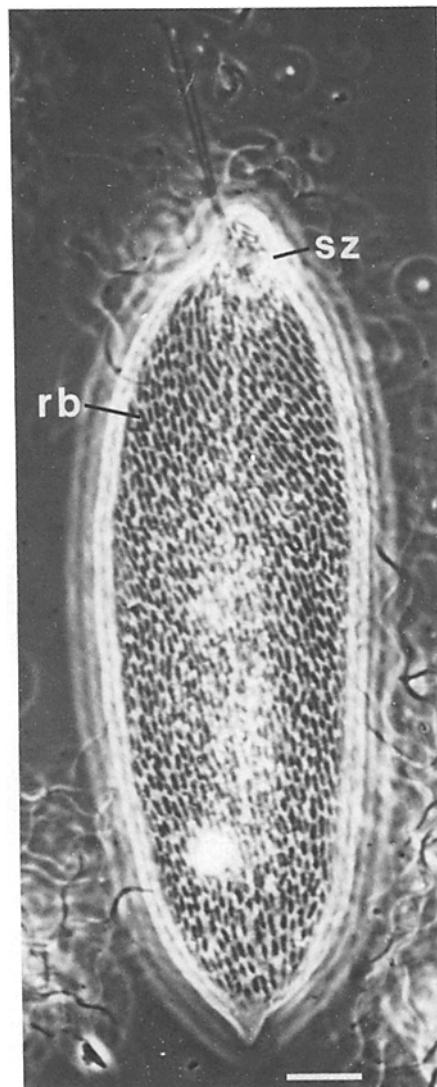


FIGURE 1 Pattern of ectosymbiotic rod bacteria (rb) on the surface of the devescovinid. The rod bacteria are arranged end-to-end in parallel rows which run helically on the body surface, but transversely on the anterior end or head. Between the head and the body is a bacteria-free zone of membrane (sz) that undergoes continual shear as the head rotates. Two of the protozoan's four flagella are visible extending from the head. The ectosymbiotic fusiform bacteria are not visible at this magnification. Bar, 10 μm . $\times 1,000$.

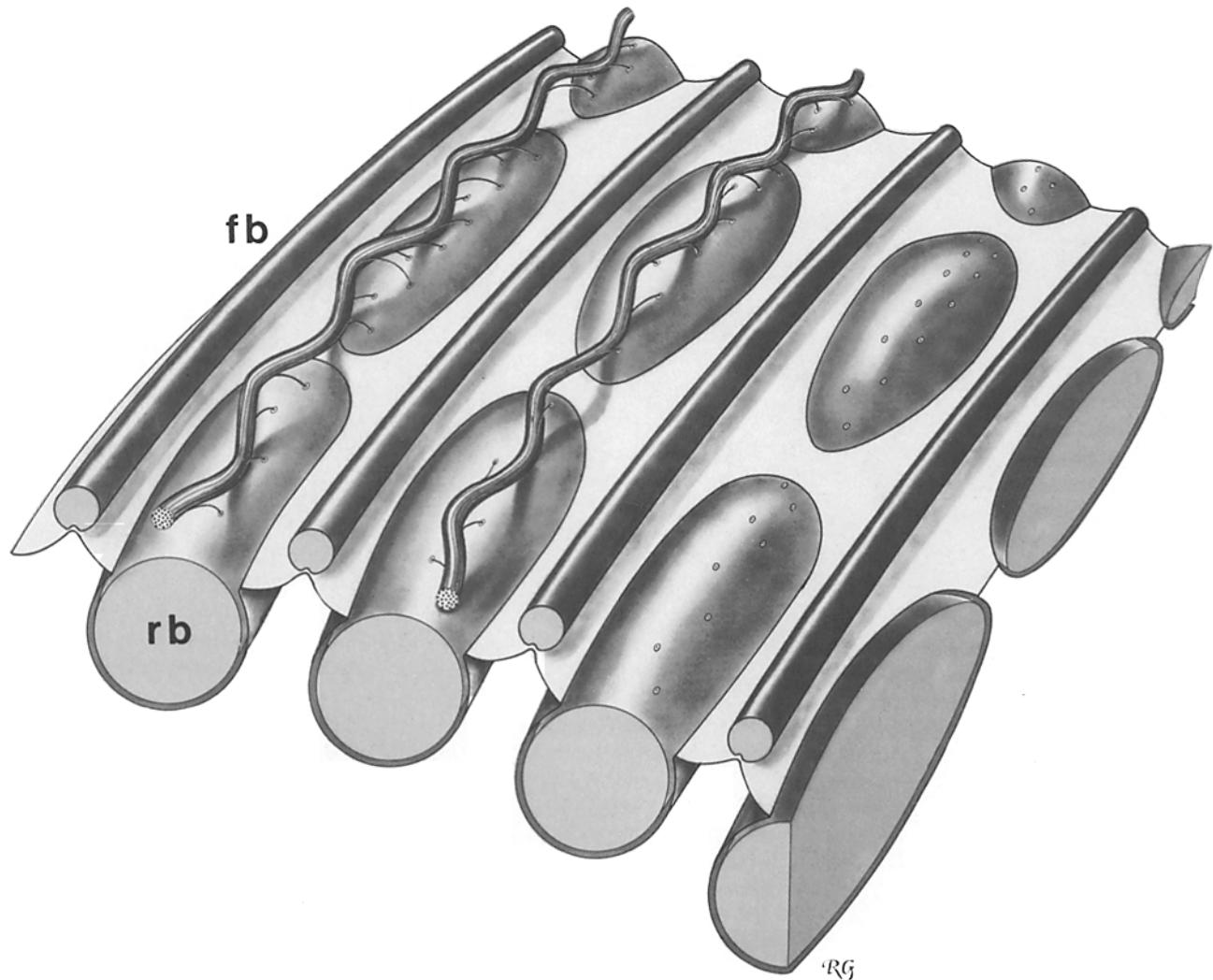


FIGURE 2 Reconstruction of a small area of the body cortex of the devescovinid, showing the attachment sites of the rod (*rb*) and fusiform (*fb*) bacteria and the alignment and synchronization of the rod bacterial flagella. Each rod bacterium lies in a pocket of the host membrane and bears about 12 flagella spaced at regular intervals along its surface exposed at the pocket opening (the pattern of flagellar insertions with filaments omitted is shown on the row to the right). The flagella of adjacent bacteria along a row form a continuous in-phase bundle which propagates helical waves posteriorly down the row (left two rows). As depicted here, the common flagellar bundle of each row consists of about 24 closely packed helical filaments with a pitch of $\sim 1 \mu\text{m}$, rotating in synchrony (based on polarized-light video-microscopy and electron microscopy). Flanking rows of fusiform bacteria are attached to surface ridges, and may act as guide tracks to align overlapping flagella in a uniform direction parallel to the row (see text).

They are arranged end-to-end in parallel rows which follow a helical path over the body surface (Fig. 1). 2,000–3,000 rod bacteria are attached to the surface of a single protozoan. Each rod bacterium lies in a deep invagination of the devescovinid's plasma membrane (Figs. 2 and 3). These membrane pockets do not completely enclose the bacteria, but leave part of their surface exposed to the surrounding medium.

The longer and more slender fusiform bacteria are 5–6 μm in length and $\sim 0.2 \mu\text{m}$ in diameter. They are arranged end-to-end in parallel rows which alternate with the rows of rod bacteria on the body surface (Figs. 2 and 3). The fusiform bacteria are attached to ridges of the devescovinid's surface by longitudinal grooves in their outer wall (Fig. 3).

Both types of bacterial possess multilayered cell envelopes typical of Gram-negative bacteria (Fig. 3).

Flagella of Rod Bacteria

The surprising feature of the rod bacteria is that they are flagellated, even though they normally never leave the surface

of the host (43). Negatively stained preparations of detergent-isolated rod bacteria show that each bacterium bears about 12 flagella (Fig. 4). The flagella arise only from one side of the bacterium, and are spaced at approximately equal intervals along its length. As a result, a uniform coat of bacterial flagella appears in profile around the edges of negatively stained whole devescovinids or surface fragments (Fig. 5). Fusiform bacteria in the same preparations do not possess flagella.

Negatively stained flagella have a contour length of 4–5 μm , and display sinusoidal profiles typical of flattened flagellar helices (Figs. 4–6) (2). The wavelength measures 1.0–1.25 μm , and three to four waves are usually present on individual filaments (Figs. 4 and 5). This two-dimensional projection of the native shape closely agrees with video-enhanced polarized-light measurements of flagellar bundles on living cells (see below).

At higher magnification, the flagella are found to be $\sim 180 \text{ \AA}$ in diameter and show a clear substructure of longitudinal rows or protofilaments, representing linear arrays of flagellin

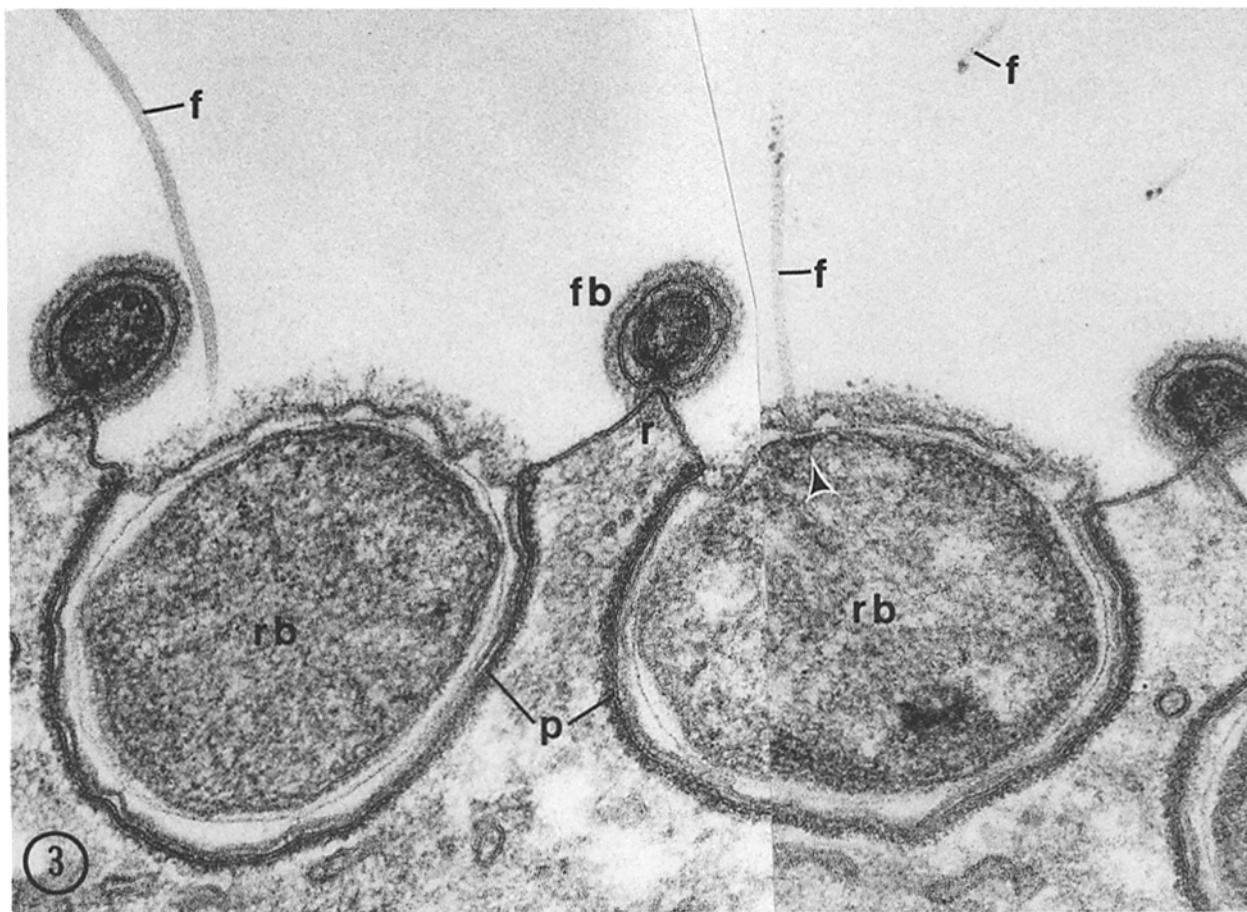


FIGURE 3 Thin-section cut transversely through the rows of rod bacteria (*rb*) and fusiform bacteria (*fb*) on the body surface of the devescovinid. The rod bacteria lie in specialized pockets (*p*) of the host membrane coated with dense material on the cytoplasmic side. Each rod bacterium bears flagella (*f*) and a thick glycocalyx on its surface exposed at the pocket opening; the insertion of one flagellum is evident on the rod bacterium to the right (arrowhead). Flagella are missing, and the glycocalyx is reduced or absent on the part of the bacterium surrounded by the pocket membrane. The alternating rows of fusiform bacteria are attached to ridges (*r*) of the devescovinid surface. Note that the cell walls of the fusiform bacteria are grooved to match the ridges of the host membrane. $\times 110,000$.

subunits (Fig. 6). This pattern resembles the type B structure of sheathless flagella described by Lowy and Hanson (21) for several different free-living bacteria.

Although less obvious, the flagella of the rod bacteria are also evident in thin-sections of intact devescovinids (Fig. 3). Such images show that the flagella arise only from that part of the bacteria surface which faces the openings of the membrane pockets (Figs. 2 and 3).

Thus, flagellation is restricted to the part of the bacterial surface exposed to the surrounding medium. This unilateral pattern of flagellation is a remarkable adaptation by the bacteria to the invaginated junctional complexes formed with the protozoan (43).

Locomotory Movements

Locomotory or gliding movements of devescovinids occur only under certain conditions, and in several forms. In densely packed protozoa from freshly opened hindguts, the devescovinids vigorously slither through the seething mass (Fig. 7). Individual devescovinids move head first in tortuous paths, at speeds of $\sim 100 \mu\text{m/s}$. These slithering movements are probably a close approximation of the kind of locomotion that takes place inside the termite hindgut. When devescovinids emerge from the densely packed mass and are no longer surrounded

by other protozoa, their speed of locomotion decreases, and they soon stop gliding. However, if such isolated devescovinids come into close contact with other cells or gut fragments, their gliding velocity immediately increases for as long as the chance contact is maintained. This is particularly evident when devescovinids undergo a temporary acceleration as they squeeze between other protozoa.

Nevertheless, except for these cases, or unless compressed between the slide and cover slip (see below), isolated devescovinids display little or no net locomotion (Fig. 8). Rotational movements of the head and flagellar activity continue vigorously in these stationary cells, however, indicating that the protozoan's own motile systems do not propel it.

If such nongliding devescovinids are gently flattened by pressure on the cover slip so that most of their body surface is in contact with a solid substrate, then locomotory movements reappear—often in bizarre forms. Most commonly, compressed cells glide smoothly forward in fairly straight paths at speeds of $100-150 \mu\text{m/s}$ (Fig. 9). Flattened devescovinids may also glide in circles, or simply spin like wheels (Fig. 9). Circling usually occurs head first, but cells sometimes glide backwards in circles. Spinning is characteristic of extremely compressed cells, and occurs in either a clockwise or counterclockwise direction at speeds of up to 1 rotation/s (Fig. 9). Less com-

monly, devescovinids may glide backwards or even sideways (Fig. 9).

Once initiated, these contact-induced locomotory movements continue indefinitely in the same pattern. For example, cells spinning clockwise never stop or spin counterclockwise, and cells gliding forward never glide in the reverse direction. The onset of gliding locomotion is not accompanied by any detectable modification in flagellar or rotational motility of the devescovinid. Nor are any undulations or other changes in form of the cell surface visible which might propel the organism.

The various types of gliding movements seen in flattened

cells undoubtedly reflect the same mechanism that causes slithering movements in densely-packed masses and, presumably, locomotion inside the termite hindgut. For convenience, these compression-induced locomotory movements are used in the following sections to investigate the causal basis of gliding motility.

Effect on Locomotion of Inhibiting the Flagellar and Rotational Motility of the Devescovinid

To determine their possible role in locomotion, the motility of the devescovinid's own flagella and rotary axostyle was

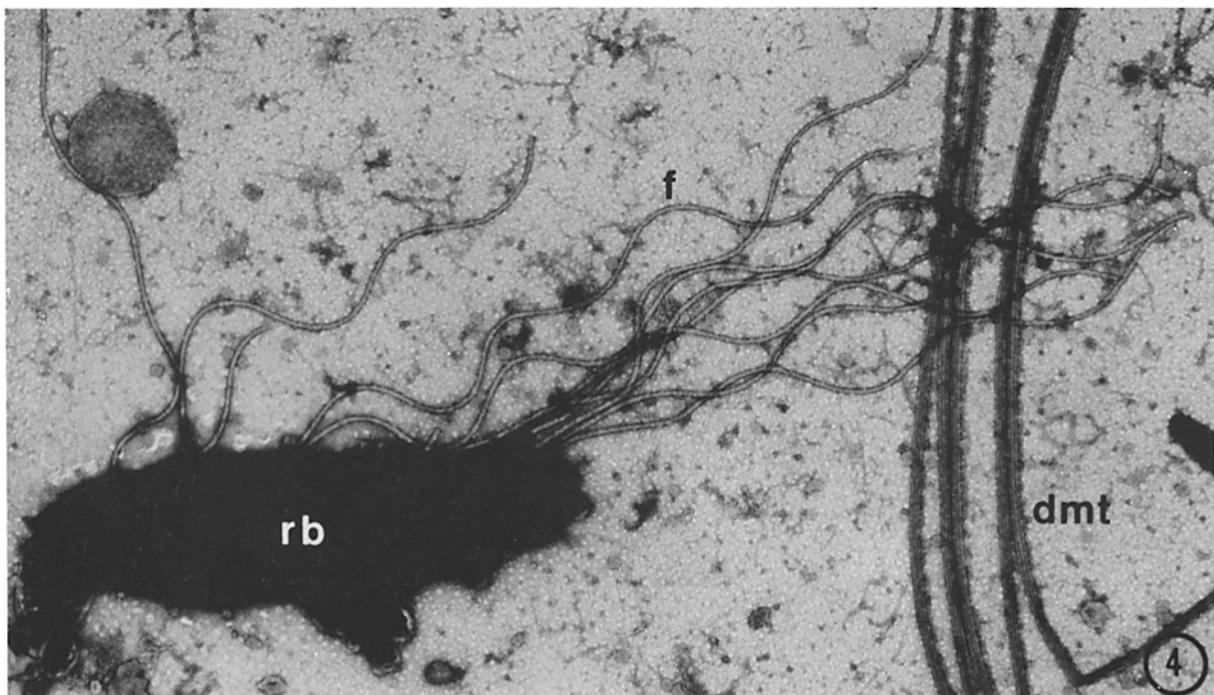


FIGURE 4 Negatively stained rod bacterium (*rb*) isolated from the surface of the devescovinid by detergent lysis. Each rod bacterium bears about a dozen flagella (*f*) on only one side, corresponding to its surface exposed at the pocket opening (cf. Fig. 3). The filaments display a sinusoidal profile and diameter typical of air-dried bacterial flagella. Doublet microtubules (*dmt*) from the devescovinid's own flagella are shown for comparison. $\times 25,700$.

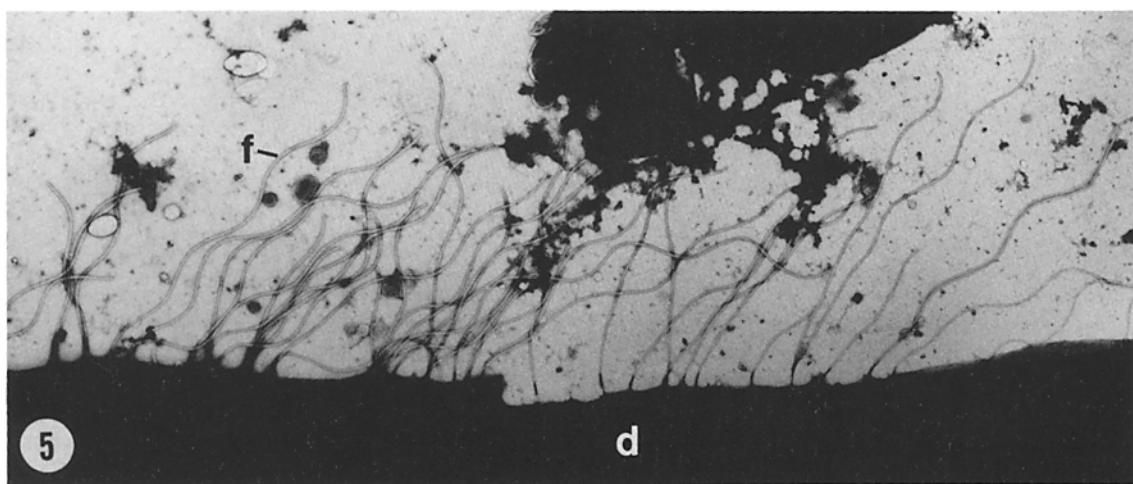


FIGURE 5 Negatively stained whole-mount preparation showing a uniform coat of bacterial flagella (*f*) projecting from a fragment of the devescovinid's surface (*d*). The flagella belonging to each bacterium are not distinguishable as a distinct group because of their even spacing and the end-to-end arrangement of adjacent bacteria (see text). $\times 15,000$.

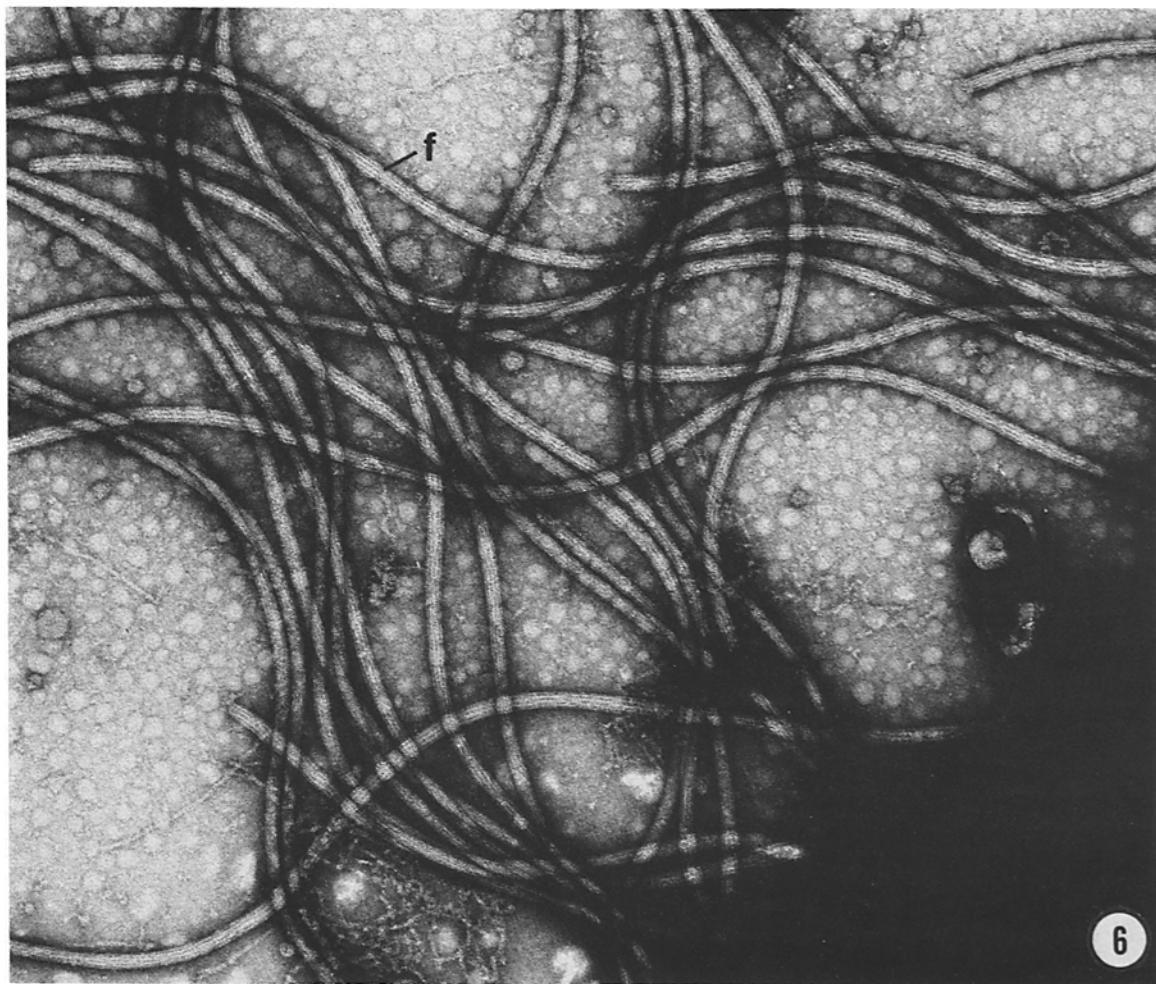


FIGURE 6 Negatively stained surface fragment of a devescovinid showing the rod bacterial flagella at higher magnification. The flagella are ~ 180 Å in diameter and display an obvious substructure of longitudinal lines typical of bacterial flagella. $\times 110,000$.

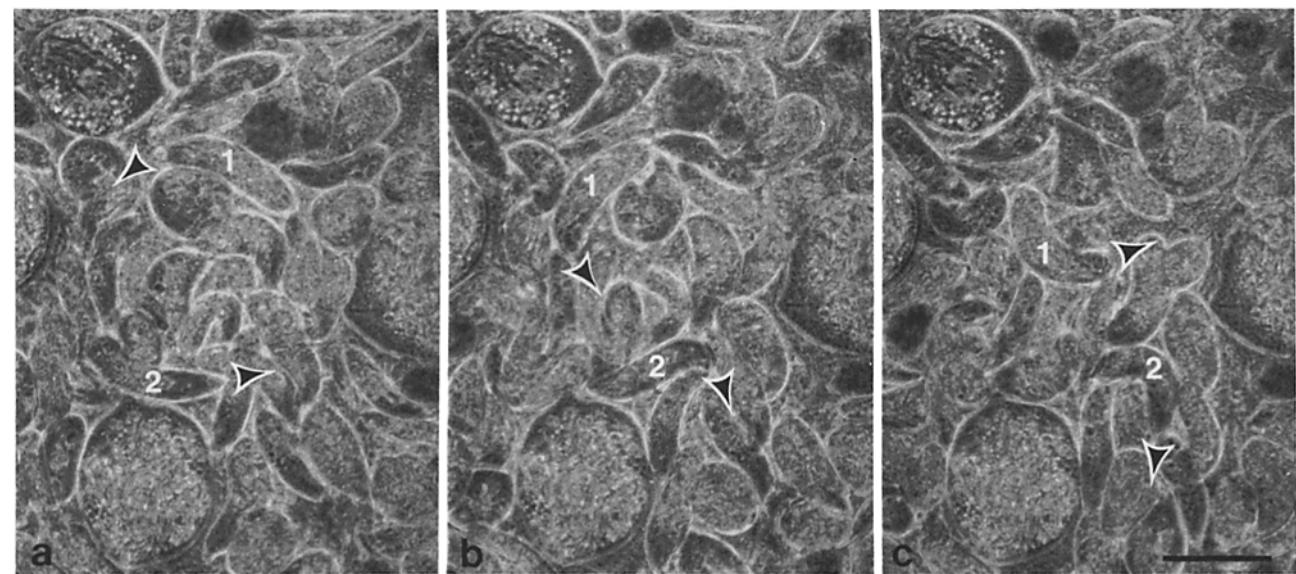


FIGURE 7 Ciné prints of devescovinids slithering through the crowded mass of protozoa from a freshly opened hindgut. Paths of numbered cells (arrowheads) are followed at 1-s intervals in successive prints. The larger hypermastigote flagellates are slightly compressed and immobilized by the cover slip. Bar, 100 μ m. $\times 140$.

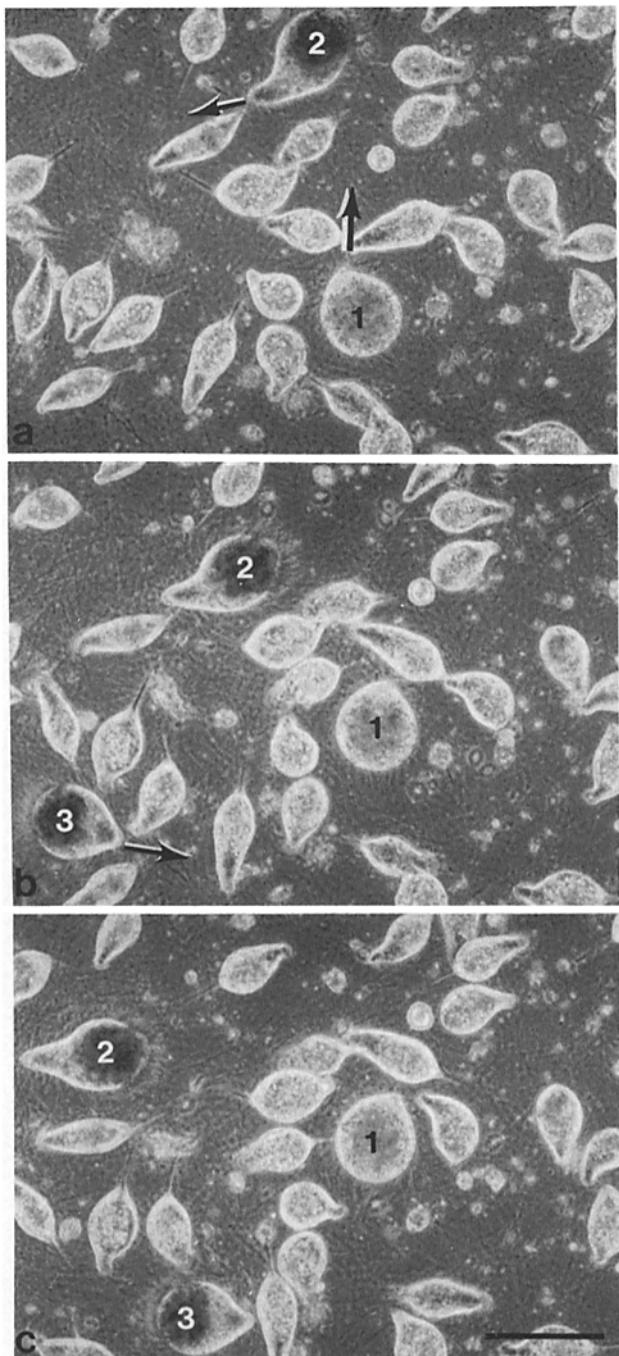


FIGURE 8 Ciné prints of noncompressed protozoa scattered at some distance from the densely packed mass. The devescovinids, most of which have caudally projecting axostyles typical of in vitro conditions, undergo little or no net locomotion, even though their heads rotate continuously and their flagella beat actively. In contrast, the large hypermastigote flagellates (arrows, numbered cells) swim vigorously through the medium, pushing stationary devescovinids out of their way. Time interval between prints is 2 s. Bar, 100 μ m. \times 150.

selectively inhibited without interfering with the activity of prokaryotic motile systems. Devescovinids were compressed in slide preparations containing 100 μ M amphotericin B or 50 μ M nystatin. These polyene antibiotics bind to membrane sterols, and thus are toxic to most eucaryotic cells, but do not affect bacteria since prokaryotic membranes lack sterols (32).

As expected, exposure to either polyene for 15–30 min did not affect the motility of free-living bacteria or spirochetes present in hindgut fluid. This same treatment, however, caused swelling and ballooning of the devescovinid's four flagella, which became completely inactive. Rotational movements of the anterior end of the cell usually stopped as well. Nevertheless, such devescovinids typically exhibited vigorous spinning and circling movements when compressed by the cover slip. These findings indicate that gliding is not powered by the devescovinid's own flagella nor by its rotary axostyle, but by a system resistant to polyene antibiotics.

Effect on Locomotion of Inhibiting Prokaryotic Motility

To investigate whether the flagella of the ectosymbiotic rod bacteria are responsible for locomotion of the host, the effect of inhibiting bacterial motility was determined. Advantage was taken of the fact that the immediate energy source for bacterial motility, unlike eucaryotic motile systems, is not ATP but a transmembrane electrochemical potential of protons (20, 27, 29). Proton ionophores such as DNP collapse the proton gradient driving rotation of bacterial flagella, thereby inhibiting bacterial motility (16, 17, 27). Uncoupling agents also inhibit the gliding motility of some prokaryotes (7). DNP should not immediately affect the energy metabolism of devescovinids, since termite flagellates do not carry out oxidative phosphorylation, but generate ATP glycolytically (30, 31).

We found that 10 mM DNP immediately inhibited all locomotory movements of the devescovinid: no slithering of the cells occurred in densely packed masses, nor were gliding movements induced after flattening cells with the cover slip. 10 mM DNP also inhibited the motility of most free-living bacteria, including the large spirochetes found in the hindgut. Lower concentrations of DNP (1 mM), or 1% DMSO controls without DNP, did not affect the locomotion of either the devescovinids or the free-swimming bacteria.

10 mM DNP did not inhibit rotation of the axostyle or beating of the devescovinid's own flagella. Similarly, other hindgut flagellates (*Stephanonympha*, *Snyderella*, *Foaina*) continued to swim actively in the presence of DNP.

Inhibition of bacterial and devescovinid locomotion by DNP is reversible: after 2-h exposure to 10 mM DNP, spirochete motility recovers and flattened devescovinids often resume gliding. This may be due to inactivation of DNP, since related trichomonad flagellates metabolically reduce DNP to 2-amino, 4-nitrophenol under anaerobic conditions (30).

Thus, inhibitory effects of DNP on the locomotion of devescovinids and free-living bacteria are closely coupled. These findings indicate that the ectosymbiotic prokaryotes—either the flagellated rod bacteria or the fusiform bacteria—provide the motive force for gliding movements of the devescovinid.

Movement of Isolated Vesicles Derived from the Surface of the Devescovinid

A fortuitous "microdissection experiment" provides further evidence that the adherent prokaryotes are responsible for locomotion, and points to the flagellated rod bacteria as the source of motility. In extremely flattened 1–2-h-old slide preparations, spherical membranous vesicles are found with rod and fusiform bacteria attached to their surfaces (Fig. 10). The presence of the ectosymbiotic bacteria shows that the vesicles are derived from the plasma membrane of the devescovinid,

but their mode of formation has not yet been observed. The vesicles vary in size and appear partially or completely empty of cytoplasm; the devescovinid's own flagella and rotary axostyle are entirely absent.

The striking feature of these isolated parts of the devescovinid's surface is that they rotate constantly in the same direction, like the spinning motions of whole devescovinids described above. Most significantly, the rotation speed of vesicles of similar size is related to the number of rod bacteria remaining on their surface (Fig. 10). Rotation rather than translation of the vesicles is evidently due to the circular arrangement of the attached bacteria (Fig. 10).

These observations clearly show that the devescovinid's own

flagella and rotary axostyle are not necessary for gliding motility: instead, locomotion appears to depend on the presence of the flagellated bacteria.

Independent Movements of Rod Bacteria over the Surface of Devescovinids

That the flagella of the rod bacteria are indeed functional is shown by the observation that individual bacteria can move independently over the surface of immobilized devescovinids. Translocation of rod bacteria is seen most readily on extremely compressed amphotericin-treated cells which have lost many bacteria during flattening (Fig. 11). The rod bacteria travel in

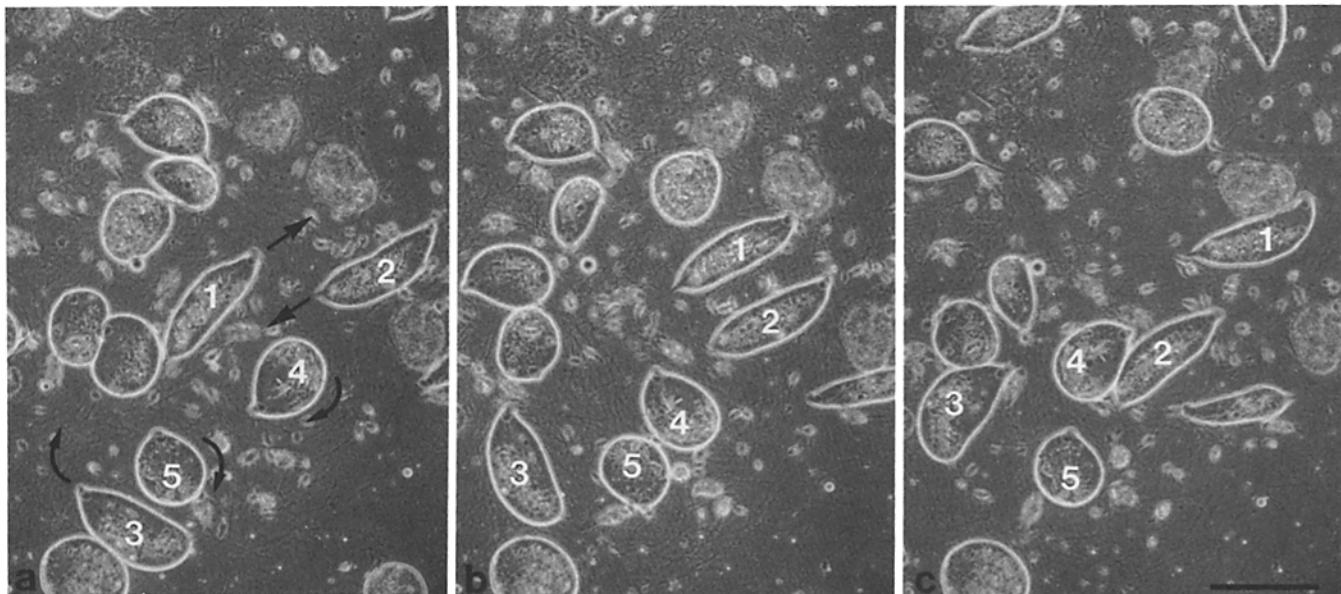


FIGURE 9 Ciné prints of a compressed slide preparation showing various types of gliding movements induced by flattening isolated devescovinids with the cover slip. Paths of numbered cells are indicated by arrows and followed in successive prints at 1-s intervals. Cell 1 glides forward in a fairly straight path. Cell 2 glides backward. Cell 3 circles head first. Cells 4 and 5 spin clockwise. Bar, 100 μ m. $\times 140$.

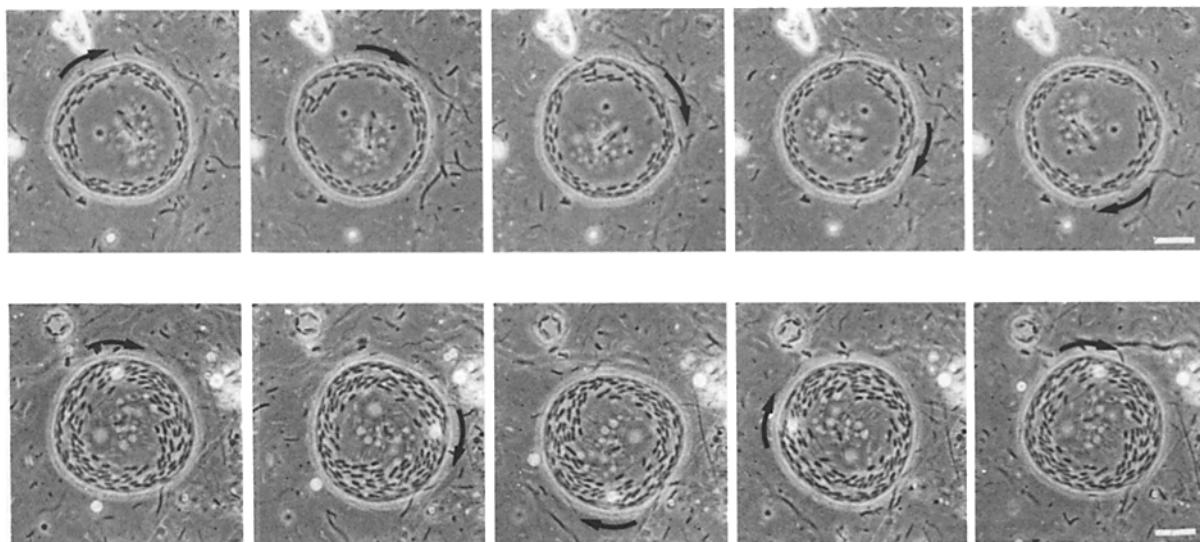


FIGURE 10 Rotation of membrane vesicles derived from the surface of the devescovinid. The vesicles in the two sequences are similar in size, but differ in the number of circularly arranged rod bacteria remaining on their surfaces. Time interval between prints is 0.5 s in both sequences. Lower sequence: a vesicle with many rod bacteria makes one full rotation in 2 s (arrows mark same position on vesicle). Upper sequence: a vesicle with about half the number of attached bacteria completes only one-half of a rotation in the same time (arrows). Bars, 10 μ m. $\times 525$.

various directions over the surface of the protozoan, at speeds up to 50–60 $\mu\text{m/s}$. Independent movements of fusiform bacteria on such cells have never been observed.

It cannot be determined by light microscopy alone whether the entire junctional complex moves with the rod bacteria through the membrane, or whether the bacteria have "popped out" of the membrane pockets and roam freely over the surface of the devescovinid. Although the rod bacteria are never observed to escape from the host by swimming off into the surrounding medium, the compressed conditions may restrict their freedom of movement.

Polarized Light Video Microscopy of Bacterial Flagellar Motility

Direct visualization of flagellar motility of the ectosymbiotic bacteria has recently been achieved using video-enhanced po-

larized-light microscopy (13). Bacterial flagellar activity is most evident on the surface of extremely compressed immobilized devescovinids, where disruption of the bacterial pattern allows clear observation of single bacteria (Fig. 12). Under such conditions, a linear series of alternating black-and-white birefringent stripes can be seen extending from one end of the bacterium. These bands of alternating contrast travel away from the bacterial body like a rotating barber pole; they represent a helical bundle of the bacterium's dozen flagella rotating together as a unit. By varying orientation of the specimen and/or compensator, each black-and-white region can be shown to correspond to a portion of the flagellar helix tilted in the opposite sense from its neighbor. The contrast is produced because the local flagellar axes alternately lie in the opposite and same quadrant as the slow axis of the compensator (13).

A pair of black-and-white birefringent stripes therefore rep-

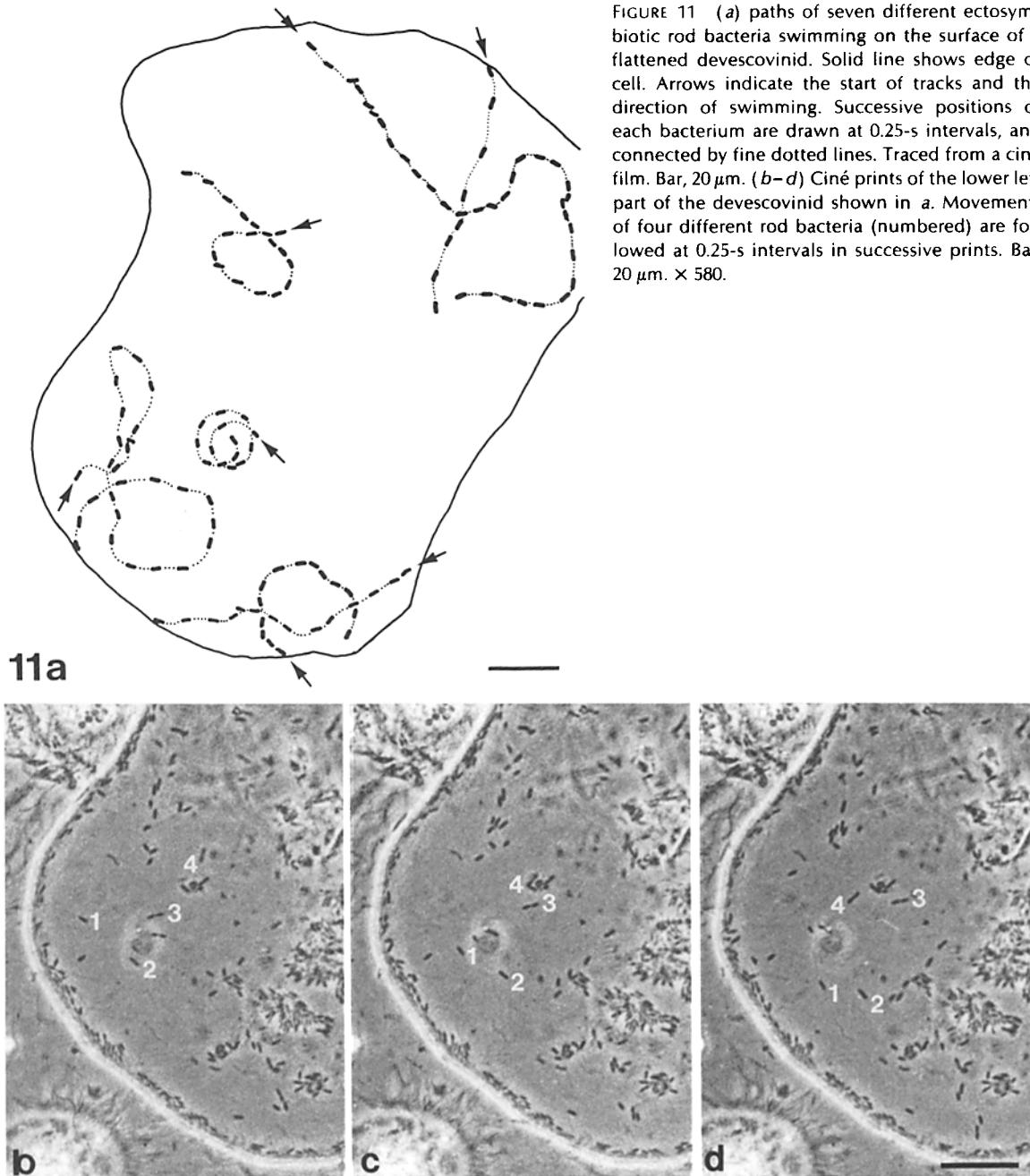


FIGURE 11 (a) paths of seven different ectosymbiotic rod bacteria swimming on the surface of a flattened devescovinid. Solid line shows edge of cell. Arrows indicate the start of tracks and the direction of swimming. Successive positions of each bacterium are drawn at 0.25-s intervals, and connected by fine dotted lines. Traced from a ciné film. Bar, 20 μm . (b-d) Ciné prints of the lower left part of the devescovinid shown in a. Movements of four different rod bacteria (numbered) are followed at 0.25-s intervals in successive prints. Bar, 20 μm . $\times 580$.

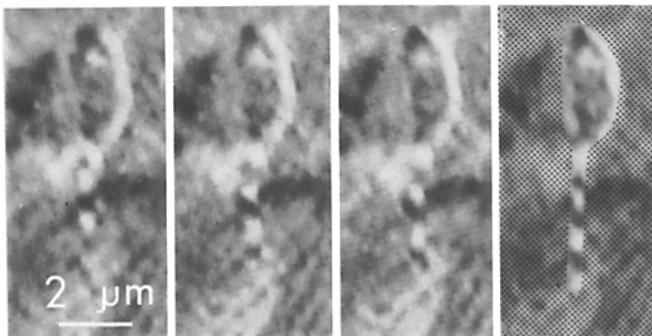


FIGURE 12 Birefringence of a rotating flagellar bundle on a single rod bacterium attached to the surface of the devescovinid. Regions of the flagellar spiral tilted to the right and the left show in black and white; a pair of black-and-white stripes represents one flagellar wavelength. Time interval between the first three frames, 17 ms; between frames three and four, 33 ms. Note that waves propagate away from the bacterial body. A stippled mask outlines the flagellar bundle and bacterium in the last frame. Polarization video microscopy, rephotographed through a Ronchi grating to eliminate scan lines (13). $\times 5,000$.

resents one wavelength of the flagellar helix. The flagellar wavelength is $\sim 1.2 \mu\text{m}$, and three to four waves are present on most flagellar bundles (Fig. 12). These measurements on living cells agree closely with those of negatively stained filaments (above).

Polarized light video microscopy of bacteria swimming over the surface of the devescovinid shows that the flagellar bundles point in a direction opposite to that of the swimming path, with birefringent waves traveling backward along the flagellar helix. Presumably, the flagella of these bacteria, like those of free-living species, are left-handed helices which rotate counterclockwise, resulting in the formation of an in-phase bundle with waves traveling from base to tip so that the cell is pushed from behind (24-26). The velocity of wave propagation on the flagellar bundles varies considerably, reflecting differences in the rotation speed of the flagella. Fig. 12 shows an example of a flagellar bundle rotating slowly at 3 Hz, with a wave velocity of $3 \mu\text{m/s}$.

In addition, waves often do not propagate smoothly and continuously, but proceed intermittently in a jerky manner down the bundle. Occasionally, wave propagation completely stops for brief periods, resulting in a static black-and-white stripe pattern on the bundle. Waves then resume traveling away from the body again. These interruptions in rotation of the flagellar helix are correlated with temporary cessations of forward swimming, confirming that the flagella are responsible for locomotion.

The reasons for the observed variations in wave velocity and the intermittent nature and occasional arrests of flagellar rotation are not understood. Most likely, these changes reflect temporary increases in the resistive torque experienced by the flagellar rotary motors under the extremely restricted conditions *in vitro*. No periods of dispersal of the flagellar bundle into separate filaments, as occurs during tumbling of free-living bacteria (25, 26), have been observed so far.

We have also observed compressed devescovinids with more intact bacterial patterns to see how the motility of the flagellar bundles is related to the end-to-end arrangement of bacteria into parallel rows on the surface of the host. In such cells, long lines of alternating black-and-white stripes, representing many

flagellar waves in series, travel as continuous streams down the rows of rod bacteria. The flagella of adjacent bacteria are thus aligned parallel to the row and overlap one another to a considerable extent. Because the waves pass uninterruptedly along the row, the flagella of adjacent bacteria must rotate in synchrony (cf. Fig. 2). Phase-matching between the flagella of neighboring bacteria is probably brought about by hydrodynamic forces and direct mechanical interaction (Fig. 2; Discussion).

DISCUSSION

This report demonstrates that the locomotion of a devescovinid flagellate from termites is caused not by the cell's own flagella, nor by its rotary axostyle, but by the flagella of thousands of rod bacteria which live on its surface. That the ectosymbiotic bacteria actually propel the protozoan was shown by the following criteria: (a) the bacteria, which lie in pockets of the host membrane, bear typical prokaryotic flagella on their surface exposed to the surrounding medium; (b) the devescovinid continues to glide when the activity of its own flagella and rotary axostyle are inhibited; (c) agents which inhibit bacterial flagellar motility, but not the eucaryote's motile systems, stop locomotion; (d) isolated membrane vesicles derived from the surface of the devescovinid rotate at speeds dependent on the number of bacteria still attached; (e) individual rod bacteria can translocate independently over the surface of compressed nonmotile cells; and (f) wave propagation by flagellar bundles of the ectosymbiotic bacteria can be visualized by video-enhanced polarization microscopy.

These results leave little doubt that the rapid gliding movements of the devescovinid are powered by the flagella of its adherent bacteria. The utilization of flagellated bacteria as a method of locomotion does not appear to have been reported in any other organism. The nearest example is the propulsion of the closely related protozoan, *Mixotricha*, by its associated locomotory spirochetes (8; see below).

Requirement for Solid Boundaries

A major question raised by the present findings concerns the reason why gliding movements occur only when the devescovinid is in close contact with other cells or with a substrate.

One likely explanation involves the effect of nearby solid boundaries on the alignment and phase-matching of flagellar bundles of neighboring bacteria. In free-swimming peritrichous bacteria, individual helical filaments rotate (4, 5, 19, 34) and are spontaneously brought together and synchronized to form an in-phase bundle as a result of hydrodynamic forces (1, 24-26). Indeed, both theoretical analysis and direct observations have shown that eucaryotic flagella and free-living spirochetes will, when undulating in proximity, exert mechanical forces upon each other that automatically synchronize their movements (9, 10, 23).

To propel the devescovinid, it is also necessary that most of the flagellar bundles of the attached bacteria be uniformly oriented in the same direction. Proximity to a substrate may bring adjacent flagella into common alignment for hydrodynamic reasons. Hydrodynamic alignment of flagella in the same direction as the rows of rod bacteria may be facilitated by the parallel, alternating rows of fusiform bacteria. These long slender bacteria adhere to ridges of the surface, and run along either side of the sunken rows of rod bacteria like elevated tracks. Proximity to solid boundaries may force the

flagella to lie in the channels formed by the bordering rows of fusiform bacteria (cf. Fig. 2).

The fusiform bacteria may therefore act as guides to direct the flagellar bundles of the rod bacteria in a posterior direction when the cells are densely packed. However, compression by a cover slip may sometimes result in alignment of the bundles in the opposite direction, so that they point anteriorly, thus explaining the occasional cases of backward gliding observed in vitro (cf. Fig. 9, cell 2).

Once oriented parallel to the row, the flagella of adjacent bacteria would overlap one another considerably due to the close end-to-end spacing of the bacteria in each row. Phase differences between overlapping flagella would then be expected to be reduced as a result of hydromechanical interactions, until the flagella of all the bacteria along a row rotate synchronously and propagate waves as one long continuous bundle (Fig. 2).

To investigate whether nearby solid boundaries indeed act to align the flagella in a common direction, we are using video-enhanced polarization microscopy to determine if the flagellar bundles on noncompressed stationary devescovinids are randomly oriented.

Another possible reason for the substrate dependence of locomotion is the propulsive advantage that can be derived from proximity to solid boundaries—the so-called wall effect (14, 15). Hydrodynamic calculations on model microorganisms show that the presence of a nearby wall produces a significant increase in the effective longitudinal resistive forces acting on a filament undulating parallel to the wall. By suitable alterations in the wave propagation velocity and wave shape of its flagella, an organism can take advantage of its proximity to a wall to swim faster while maintaining a constant power input to its flagella (14, 15, 28, 33). Essentially, a nearby boundary acts to increase frictional coupling, decrease slippage, and increase propulsive velocity by allowing more thrust to be exerted against the surrounding medium. It has been shown experimentally that the propulsive velocity of many flagellated bacteria is initially increased by small increases in the viscosity of the medium (11, 33). According to this explanation, the flagellar bundles may be uniformly aligned even in the absence of nearby walls, but simply not efficient enough to move the host. Proximity to a solid boundary may increase the propulsive efficiency above this threshold, thereby resulting in locomotion of the protozoan.

These explanations are not mutually exclusive, of course: proximity to a substrate may act to align flagellar bundles as well as to increase their propulsive advantage, with both effects being required for net locomotion of the cell. Future studies using video-enhanced polarization microscopy promise to show which parameters of bacterial flagellar motility are altered by contact with the substrate.

It should be noted that the swarming behavior of certain free-living bacteria (i.e., *Proteus*) is another type of flagella-dependent motility that requires contact with other cells and the presence of a surface (12, 35). All swarming bacteria possess peritrichous flagella and are able to swim in fluid media. The change in flagellar function induced during swarming is not understood, nor is it known whether this example of boundary-dependent flagellar propulsion is related to the motility described here.

Finally, the gliding motility of various nonflagellated bacteria occurs only when the cells are in contact with a solid surface (7, 12). The similar substrate-dependence of the deves-

covinid's locomotion and the presence of the fusiform bacteria on its surface raises the question of whether the fusiform bacteria are capable of gliding and, if so, whether they contribute actively to the locomotion of the host. No gliding movements of the fusiform bacteria were ever observed, even under conditions where the flagellated rod bacteria were able to move independently over the surface of the devescovinid. In addition, the velocity of bacterial gliding motility is typically very low (7)—about 100 times slower than the gliding speed of the devescovinid. It therefore appears that the bacterial-powered locomotion of the devescovinid is due solely to the flagellar motility of the rod bacteria. The fusiform bacteria may, at most, play only a passive role as “guide tracks” to align the flagellar bundles in a posterior direction.

How Bacterial Flagella Propel the Protozoan So Rapidly

The highest translation velocities reported for free-swimming bacteria are in polar monoflagellated species, ranging from $\sim 60 \mu\text{m/s}$ for *Pseudomonas* (33) to $\sim 140 \mu\text{m/s}$ for *Bdellovibrio bacteriovorus* (37). Peritrichously flagellated bacteria, such as *Escherichia coli*, *Salmonella*, and *Bacillus* typically swim at speeds of $20\text{--}30 \mu\text{m/s}$ (25, 33). The unilaterally flagellated bacteria on the devescovinid are able to move over the surface of squashed cells at twice this speed, or $50\text{--}60 \mu\text{m/s}$ (see Results).

One may ask how the flagellated bacteria propel the host at speeds of $100\text{--}150 \mu\text{m/s}$. The following considerations indicate that the answer lies in the geometry of the bacterial pattern, and possibly on wall effects, not on any novel properties of flagellar motility possessed by these bacteria.

Due to slippage, the velocity of wave propagation backward along the flagellar bundles must be greater than the forward propulsion speed of the protozoan. In free-living bacteria such as *E. coli* and *Salmonella* swimming at $20 \mu\text{m/s}$ in a medium of ~ 1 centipoise, the flagellar wavelength is typically $2.5 \mu\text{m}$, and the rotation speed of the flagellar bundle is 50 Hz (1, 24). The velocity of distal wave propagation along the flagellar bundle is therefore $125 \mu\text{m/s}$, or about six times greater than the translation speed of the bacterium itself.

In the case of the devescovinid with a translation velocity of $100\text{--}150 \mu\text{m/s}$ and a bacterial flagellar wavelength of $\sim 1 \mu\text{m}$, the minimum values for wave propagation velocity and rotation rate of the flagellar bundles, assuming no slippage, are $100\text{--}150 \mu\text{m/s}$ and $100\text{--}150 \text{ Hz}$, respectively. Such ideal conditions may be approached when the protozoan is close to a substrate, and may be the reason why nearly solid boundaries are needed for locomotion (see above). However, some degree of slippage seems unavoidable, even with a significant wall effect; consequently, the actual wave velocity and rotation speed of the bacterial flagellar bundles must be higher than these minimum values.

The maximum rotation speed reported for flagellar motors operating under essentially no load is $>150 \text{ Hz}$, using *E. coli* flagellar hooks (6). Such high speeds of rotation are due to a decrease in viscous drag on the flagellar motors. The following calculations show that the arrangement of rod bacteria on the surface of the devescovinid provides a means to reduce the resistive force experienced by each flagellar bundle.

The translational drag on the devescovinid and on a free rod bacterium will be proportional to their radii and to a shape factor. Since devescovinids and bacteria are of similar shape

(prolate ellipsoids with axial ratio of about 3), only their radii (roughly 15 vs. 0.4 μm , respectively) need be considered. The translational drag on the devescovinid will therefore be about 40 times that on a single free-swimming bacterium. However, since there are about 2,500 bacteria attached to the surface of the protozoan, the drag per flagellar bundle will be 2,500/40, or about 60 times smaller than on a flagellar bundle of a free-swimming bacterium.

This reduced drag on the flagellar bundles of attached bacteria should allow the flagellar motors to rotate considerably faster; the resulting increase in velocity of wave propagation along the bundles should be more than sufficient to propel the host cell at speeds of 100–150 $\mu\text{m/s}$, even with a moderate degree of slippage.

Relation to the Spirochete-*Mixotricha* Association

The bacterial-devescovinid motility system described here is comparable to the well-known symbiosis between the Australian termite flagellate *Mixotricha* and its associated locomotory spirochetes (8). Whereas *Mixotricha* is propelled by the helical undulations of its adherent spirochetes, the devescovinid's locomotion is powered directly by bacterial flagella themselves. In both associations, the prokaryotes are distributed in a specific pattern, and are attached to the surface of the host by specialized cell junctions (8, 43). In *Mixotricha* the spirochetes are inserted onto the posterior sides of projecting brackets of the cell surface. As a result the spirochetes are directed posteriorly and overlap one another to a considerable extent. The brackets themselves are distributed in such a way as to assure nearly perfect mechanical synchronization between adjacent spirochetes (22). Indeed, the "metachronal" waves which travel from anterior to posterior over the surface of *Mixotricha* represent direct continuations of individual sequences of helical bending waves.

Synchronization of neighboring spirochetes on *Mixotricha*, like the phasing of overlapping flagellar bundles on the devescovinid, therefore appears to be due to hydromechanical forces exerted between actively moving structures lying close to each other (8, 10, 23).

The use of ectosymbiotic prokaryotes as a means of locomotion has several important consequences, as pointed out by Cleveland and Grimstone (8). It apparently results in continual, undirected movement, as in fact observed in *Mixotricha* and the devescovinid. Cleveland and Grimstone (8) suggested that this method of locomotion could have evolved only in a "sheltered, constant environment such as the termite gut provides, in which it is unnecessary to search for food and avoid predators and unfavourable conditions".

Nevertheless, under *in vitro* conditions free-swimming spirochetes and flagellated bacteria from the hindgut do not swim constantly forward in an invariant pattern, but often display motor responses similar to those shown by free-living bacteria to environmental stimuli (S. L. Tamm, unpublished observations). Whether or not they do so in the hindgut, these bacteria are thus capable of altering their pattern of movement. It is therefore possible that the bacterial-powered motility of *Mixotricha* and the devescovinid may show behavioral responses to certain, as yet unknown stimuli encountered in the insect's gut. If so, it would be interesting to know whether the sensory receptors for such responses reside in the bacteria or the protozoan, and if the latter, how the eucaryote controls the motility of its symbiotic prokaryotes.

Several differences between the two motility systems should be noted. *Mixotricha* glides at a considerably slower speed ($\sim 20 \mu\text{m/s}$; Tamm, unpublished observations) than does the devescovinid, in spite of the much larger size of its locomotory "organelles." In addition, the locomotion of *Mixotricha*, unlike that of the devescovinid, does not require proximity to solid boundaries, nor does its gliding velocity increase upon contact with a substrate (Tamm, unpublished observations). Further analysis of the motility of *Mixotricha* should help to understand these differences.

On the basis of observations on the flagellar apparatus and axostyle of *Mixotricha*, Cleveland and Grimstone (8) proposed that this protozoan is a trichomonad flagellate, closely related to devescovinids. Our discovery of a second case of a bacterial-protozoan motility-linked symbiosis—in a devescovinid—is, in hindsight, not too surprising. Indeed, symbiotic associations between bacteria and protozoa reach their highest level of development among the devescovinid flagellates of termites (3, 18). Relationships similar to the one described here may therefore be a widespread occurrence in this group. In support of this possibility, transmission electron micrographs of *Hyperdevescovina balteata* from *Ceratokalotermes spoliator* show numerous flagellar filaments emanating from the exposed edges of the adherent rod bacteria (Tamm, unpublished observations). Further studies on the locomotion of various devescovinids should reveal interesting new examples of how symbiotic prokaryotes propel eucaryotic cells.

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