Fine Structure of the Motile Cells and Flagella in a Member of the Actinoplanaceae (Actinomycetales)

Charles E. Bland

DEPARTMENT OF BIOLOGY, EAST CAROLINA UNIVERSITY, GREENVILLE, NORTH CAROLINA 27834

Abstract. The motile cells (sporangiospores) of an undescribed member of the Actinoplanaceae are studied by electron microscopy as shadowed, negatively stained, and sectioned preparations. The rod-shaped spores exhibit a typically bacterial internal structure. However, a single tubular structure (rhapidosome) is positioned just inside the site of flagellar attachment of each spore and is oriented perpendicular to the direction of the flagella. Flagella arise from basal discs and pass through the plasma membrane and the two-layered cell wall to become associated with other flagella to function as a posteriorly directed unit. Each flagellum consists of a helical band or ribbon which dissociates into 5 or 6 subfibrils.

Sporangial structure and development in various genera of the Actinoplanaceae[5-7] have been described.[2,21,22] Flagellar number, and position on spores, of several members of this family have been reported,[5,12,13,16,20] and conditions favoring flagellar formation were studied.[14] However, no one has until now investigated the fine structure of the swimming spores, or the attachment mechanism and fine structure of the flagella.

Materials and Methods. Organisms: Stock cultures of strain JA-97 (culture collection of Dr. J. N. Couch) were maintained on Czepek agar. Growth in Petri plates on hay-infusion agar,[27] final concentration of 3–4% (w/v) gave optimal sporangial production in 5–7 days.

Spore discharge and collection: 5–7-day-old hay-infusion cultures were flooded with sterile distilled water to give a maximum concentration of motile sporangiospores in 30 min–1 hr, and centrifuged at 1800 × g for 5 min to yield a small pellet of spores. The supernatant was decanted and the spores were resuspended in about 3 ml of sterile distilled water for study by one of the following methods:

Enzymatic lysis: Spore samples were mixed with the purified bacteriolytic enzyme of *Myxobacterium* AL1[3,8] to give a final concentration of 100 μg/ml, then incubated at 35°C for 30 min and mounted for negative staining or shadowing.

Ultrasonic treatment: Spore suspensions were treated 1–5 min at about 82 mA in an Acoustica DR50AH ultrasonicator equipped with tank AT-210 (Acoustica, Los Angeles, Calif.).

Negative staining: Spore suspensions were mixed 1:1 with 2% (w/v) potassium phosphotungstate (pH 7.0) containing 0.01% bovine serum albumin, and mounted on collodion-coated, carbon stabilized, 200-mesh copper grids. Drying was hastened by gentle fanning of the grids.

Shadowing: Spores mounted by the agar-block technique of Sharp[33] or placed drop-wise directly on collodion-coated, carbon stabilized, grids were shadowed with a platinum–palladium alloy.

Thin sectioning: Spore suspensions were fixed on ice in 3% (v/v) glutaraldehyde–0.1 M sodium cacodylate buffer, pH 7.4 (1:1) and washed overnight in cacodylate-buffered sucrose (0.2 M) at 6°C. Centrifugation gave a pellet of spores which was resuspended in a small drop of barely-molten 3% agar and postfixed for 1 hr in Kellenberger's standard fixative,[18] washed in Kellenberger buffer containing 0.5% uranyl acetate for 15 min, dehydrated through a graded EtOH series, passed through two changes of propylene oxide, and embedded in Epon 812.[25] Sections cut on a Porter-Blum MT-1 ultramicrotome equipped with a DuPont diamond knife were collected on uncoated, 200 mesh, copper grids to be poststained for 1–10 min with 2% (w/v) uranyl acetate, lead citrate,[30] or a combination of the two.

Sporangia growing on agar were processed as described.[2]

Electron microscopy: A Zeiss EM-9A electron microscope was used for examination of specimens. Micrographs were taken on Kodak Kodalith LR film at instrumental magnifications ranging from ×7,000–40,000.

Results. Swimming spores appeared as short rods measuring 1 × 1.5 μm with 18–26 polar flagella of approximately 2–3.5 μm in length (Figs. 1 and 2). Flagella on spores mounted by the agar-block technique were characteristically spread in a fan-like configuration about the spores (Fig. 1). However, when spores were mounted directly and dried down rapidly, the flagella on each spore were grouped together as a unit and were directed along one side of the spore (Fig. 2). This was observed in over 90% of the spores mounted by this method, but only rarely when the spores were mounted by the indirect agar-block technique.

In negatively-stained preparations, each flagellum arose from a button-like structure of 25 × 50 nm (Figs. 5 and 6). Mesosomes,[9] 1–4 per spore, (Figs. 3 and 4) varied in size but appeared to consist of a series of highly coiled membranes.

In thin section, the motile cells exhibited a bilaminar cell wall, consisting of an inner, electron-dense, layer of 12 nm and an outer, less electron-dense, layer of 20 nm thickness. (Figs. 7 and 10). Occasionally the inner, electron-dense, wall layer appeared as two electron-dense regions separated by a less dense region (Fig. 10). The striate outer-wall layer was made up of vertically oriented repeating subunits spaced about 12 nm apart (Fig. 7). The bilaminar wall characteristic of motile spores differed from that of spores not discharged from sporangia; walls of the latter consisted of a single electron-dense layer of 12 nm thickness (Fig. 11). A plasma membrane of 10 nm lay immediately inside the inner wall layer of the motile spores. The nuclear material was typically bacterioid, consisting of finely fibrillar chromatin surrounded by a less electron-dense ground substance (Fig. 7). The mesosomes appeared to arise near the plasma membrane of the spores (Fig. 8) with a placement immediately inside the site of flagellar attachment observed frequently (Fig. 9). Also associated with the flagellar end of the spores was a vertically-striated tubular structure, rhapidosome,[23] which measured approximately 300 × 25 nm (Fig. 7). The rhapidosome lay 10–200 nm inside the plasma membrane in an orientation perpendicular to the direction of the flagella. A repeating subunit of 14 nm gave the structure a striate appearance. Oblique sections through the rhapidosome indicated the tubular nature of the structure (Fig. 9). Similar structures were observed in spores contained in sporangia (Fig. 11). The button-like structures visible at
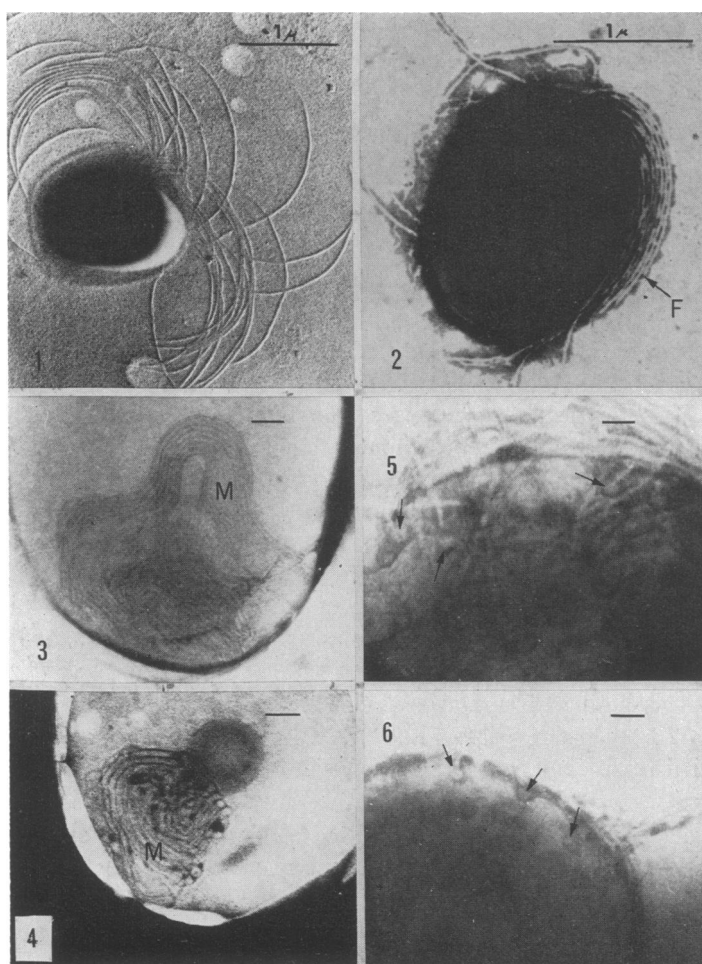
FIGS. 1–6. Whole mounts of motile spores. (Fig. 1), Shadowed with Pt–Pd. (Figs. 2–6) Negatively stained with phosphotungstic acid. (Fig. 1), Spore mounted by the agar block technique. (Fig. 2), Spore mounted directly and dried rapidly; note flagella united along one side of spore. (Figs. 3 and 4), Mesosomes as seen in intact cells. (Figs. 5 and 6), Flagellar bases (*arrows*) at anterior end of spore. Abbreviations: *F*, flagella; *M*, mesosome. *Line* represents 100 nm, except where indicated.

the flagellar bases of intact, negatively stained, cells (Figs. 5 and 6) were seen in thin sections to lie immediately inside the plasma membrane. (Fig. 10). From this structure, each flagellum passed through the plasma membrane and the two-layered cell wall to become associated with other flagella. Indications that flagella were firmly attached to the plasma membrane were seen in negatively-stained preparations of lysed spores in which flagella remained attached to the plasma membrane after it collapsed inside the disintegrated spore wall (Fig. 12).

Free flagella of negatively-stained lysed and ultrasonically-treated spores terminated usually in a basal knob of 25 × 50 nm (Figs. 13 and 14). These
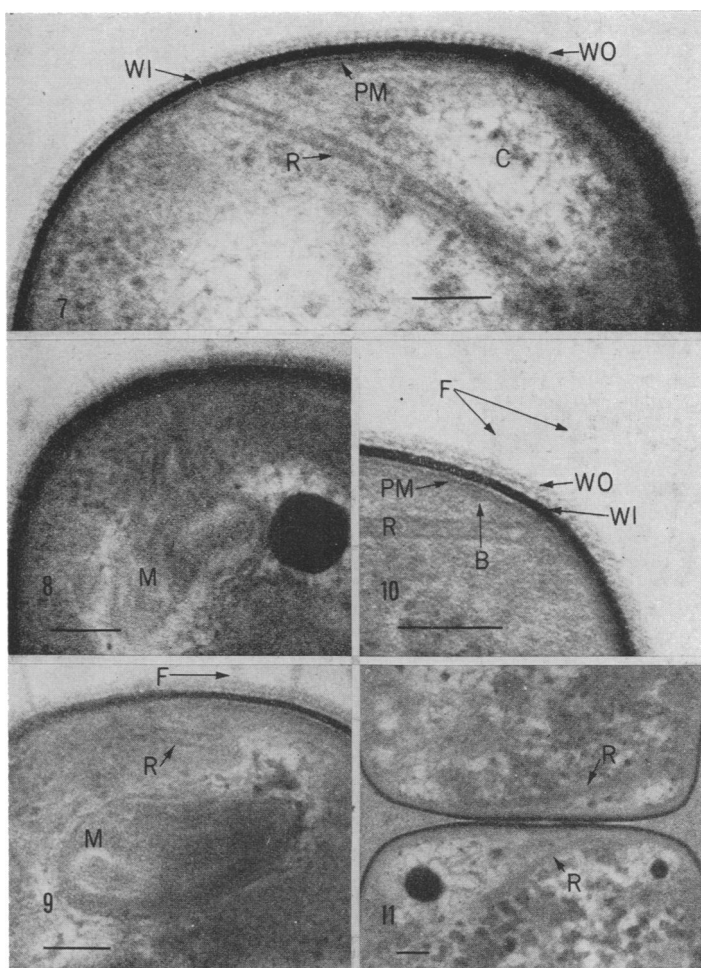
FIGS. 7–11.   Thin sections of spores of JA-97.   (Fig. 7), Rhapidosome in anterior end of spore.   (Figs. 8 and 9), Mesosomes at flagellar end of spores.   (Fig. 10), Flagellar attachment through cell wall and plasma membrane.   (Fig. 11), Rhapidosomes as seen in nondischarged spores.   Abbreviations: *WO*, outer wall layer; *WI*, inner wall layer; *PM*, plasma membrane; *R*, rhapidosome; *C*, chromatin, *M*, mesosome; *F*, flagella; *B*, flagallar base. *Line* represents 100 nm.

knobs resembled closely the knob-like structures seen in negatively stained intact cells (Fig. 5 and 6) and in thin sections (Fig. 10).   Fragments of the plasma membrane were often visible as spherical structures surrounding the basal knob (Fig. 13).   Approximately 8-nm distal to the basal knob was a double-layered disc (Fig. 13) which apparently originated at the inner wall layer of the spore. Fragments of the outer wall layer, as well as the flagellum, adhered external to the disc (Fig. 14).   The proximal 100 nm of each flagellum was bent at an angle of about 90° to form the "hook" that is characteristic of bacterial flagella in general (Figs. 13–15).   The flagellar shaft, 20–25 nm in diameter, was made up of a compact helix of subfibrils.   Each subfibril, 4–8 nm in diameter, coiled
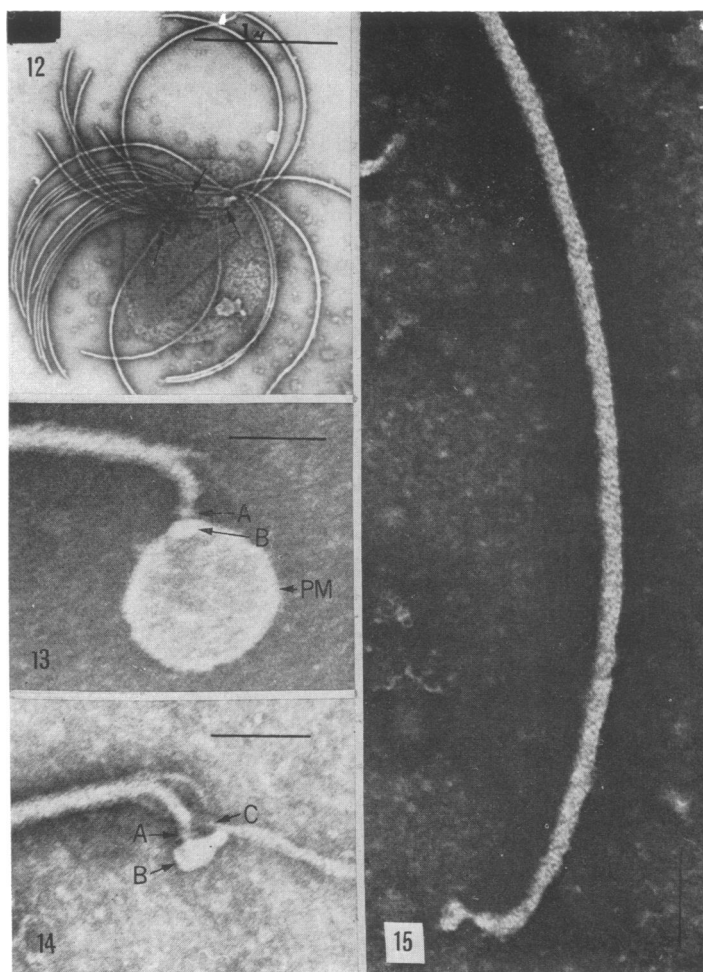
FIGS. 12–15. Negatively-stained preparations of isolated flagella. (Fig. 12), Flagella attached to fragments of the plasma membrane (*arrows*). (Figs. 13 and 14), Basal attachment process of flagella. (Fig. 15), Helical subunits as seen in a flagellum. Abbreviations: *A*, flagellar disc; *B*, flagellar basal bulb; *C*, cell wall and flagellar debris; *PM*, plasma membrane. *Line* represents 100 nm, except where indicated.

at an angle of about 45° (Fig. 15). After enzymatic digestion or ultrasonic treatment, each flagellum uncoiled distally into a helical band or ribbon with a diameter of about 20 nm (Fig. 16). In many instances, each helical band further dissociated into 4–6 subfibrils, with each having a diameter of roughly 4 nm (Fig. 17–19). The subfibrils, were bound together in a unit of four to six to form a flat ribbon, which in turn coiled tightly at an angle of 45° to make up the flagellum proper. The fine structure of an isolated flagellum is diagrammed in Fig. 20.

Discussion. Koch's report[20] that the numerous flagella found on motile cells of *Actinoplanes* function during swimming as a posteriorly-directed unit are
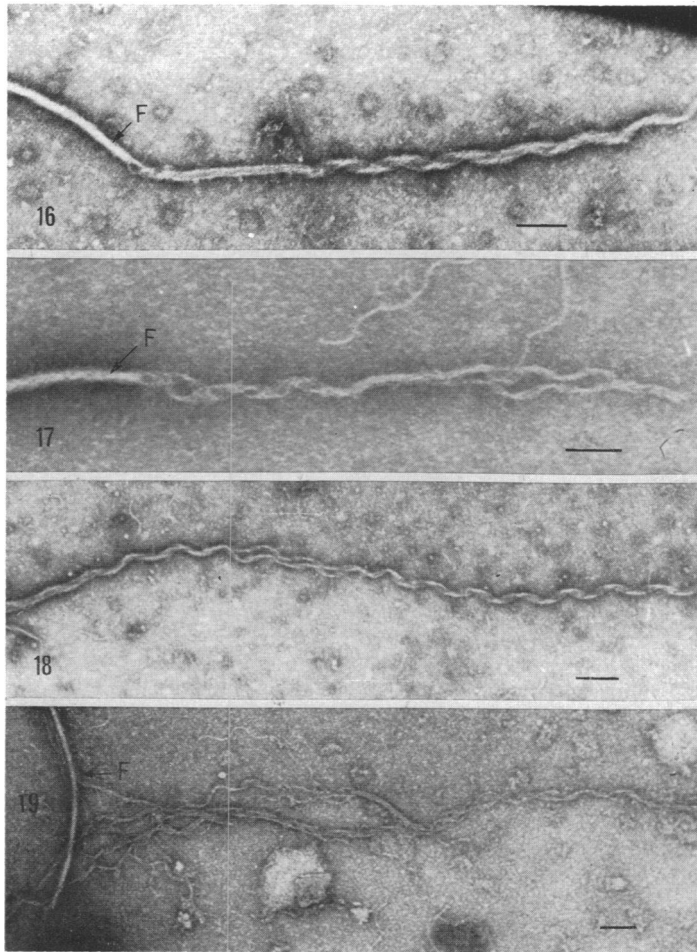
FIGS. 16–19. Negatively-stained preparations of ultrasonically-treated isolated flagella. (Fig. 16), Flagellum uncoiling. (Figs. 17–19), Flagella breaking down into individual subfibrils. Abbreviations: *F*, flagellum. *Line* represents 100 nm.
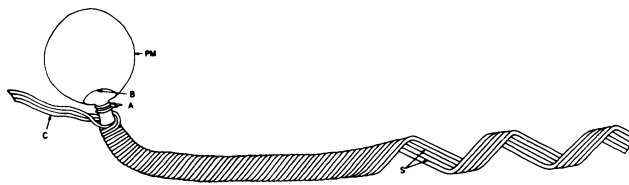

FIG. 20. Diagram illustrating the fine structure of the flagellar base and shaft. Abbreviations: *A*, flagellar collar from spore wall; *B*, flagellar basal bulb; *C*, debri from flagellar shaft and the cell wall; *S*, flagellar subfibrils.

confirmed here with a new member of the Actinoplanaceae, JA-97. The fan-like or dispersed configuration of flagella described for spores of several Actino-

planaceae,[13,16] and for many genera of eubacteria, has probably resulted from the slow drying of specimens during preparation for electron microscopy. In actively motile cells, however, flagella function as a unit in providing locomotion.[20,26] In support of this, Couch (personal communication) observed by dark-field microscopy swimming spores of the genus *Actinoplanes* in which the flagella were united to function as a posteriorly directed unit.

The tubular bodies seen in spores of JA-97 resemble closely the rhapidosomes first reported[4,23] to occur in *Saprospira grandis*. Similar microtubular components have been described for several bacteria and mycobacteria. Suggestions as to their nature have been as follows: defective phage tails,[4,11,23,24,28,31] anchors to the nucleoplasm around which rotation takes place during replication,[39] products of metabolic disturbances caused by bacitracin,[31] and aids to locomotion.[38] A relationship has been reported between rhapidosomes and the nucleoplasm of some species.[24,39] Besides this association, most investigations have reported a scattered distribution of several rhapidosomes per cell. No relationship between nucleoplasm and rhapidosomes was observed in spores of JA-97. However, because of their precise location, orientation, and occurrence in all spores, the rhapidosomes seen in JA-97 are thought to be a normal, and probably functional, cell component. The relationship between rhapidosomes and flagella, and the presence of such structures prior to flagellar formation, may indicate a function in flagellar synthesis, or possibly flagellar action. Might the rhapidosome of JA-97 have some relationship to the polar plate or polar membrane reported by various authors[3,15,17,26,29,37] in gram-negative bacteria?

Since the work of van Iterson,[36] there has been considerable controversy concerning the attachment mechanism of bacterial flagella and whether the basal granules reported by many investigators to occur at flagellar bases were actually structures or were artifacts (discussed in ref. 37). Such structures were reported as artifacts resulting from the adherence of cytoplasmic debris to the flagellar shaft.[35] However, these authors do describe "a real entity at the flagellar terminus" consisting of a cluster, 60 nm in diameter, of "ribosome-like granules." The button-like structure seen at the base of flagella in JA-97 are no doubt comparable to these structures since such structures were identified here in negatively-stained whole cells, in thin sections, and on isolated flagella. Although similar structures have been described by other authors,[1,15,26,29,32,37] the function of flagellar knobs at the proximal terminus of bacterial flagella is unknown at present.

As reported for many bacteria, flagella of JA-97 spores consist of a series of helically-wound subfibrils. Such subfibrils were suggested to function as three helical, or five parallel, strands in making up the flagellum proper.[19] Similarly, a flagellar model consisting of a triple helix of 5-nm subfibrils wound about a hollow center was suggested on the basis of low-angle x-ray diffraction studies.[34] However, flagella of JA-97, when ultrasonically treated, uncoil distally into helically-wound ribbons which may be broken down further into four to six subfibrils. This corresponds with the results of Abram *et al.*, (1964, Abst. Biophys, Soc. WC4) in which flagella of *Bacillus pumilus* were shown to break up into six coiled fibers. Such subfibrils are likely comparable to fimbriae[19] or subfibrils seen in section in OsO_4-fixed flagella.[10]

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