January 15, 1998

Cosmopolitan Distribution of the Large Composite Microbial Mat Spirochete, Spirosymplokos Deltaeiberi

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Summary Inocula from organic-rich black muds immediately underlying intertidal laminated microbial mats dominated by *Microcoleus chthonoplastes* yielded large, variable diameter spirochetes. These unusual spirochetes, previously reported only from the Alfacs Peninsula at the delta of the Ebro river in northeast Spain, contain striking arrays of cytoplasmic granules packed into their protoplasmic cylinders. On several occasions, both in summer and winter, the huge spirochetes were recognized in samples from mats growing in the Sippewissett salt marsh at Woods Hole Massachusetts. They were also seen in similar samples from microbial mats at North Pond, Laguna Figueroa, Baja California Norte, Mexico. The identity of these spirochetes was confirmed by electron microscopy: number and disposition of flagella, composite structure, measurements of their distinctive cytoplasmic granules. The granules, larger, more conspicuous and present in addition to ribosomes, are hypothesized to contain ATPases. As culture conditions worsen, these spirochetes retract into membrane-bounded round bodies in which they form refractile inclusions. From morphology and behavior we conclude the North American spirochetes from both Atlantic and Pacific intertidal microbial mats are indistinguishable from those at the delta of the Ebro river. We conclude a cosmopolitan distribution for *Spirosymplokos deltaeiberi*.

Key words *Spirosymplokos deltaeiberi* · Spirochete ultrastructure · Spirochete round bodies · Cytoplasmic granules · Microbial mats

Introduction

A bloom of unique microbial mat spirochetes led to the characterization and naming of these eubacteria *Spirosymplokos deltaeiberi* [4]. On the basis of ultrastructural details and accompanying morphometrics, *Spirosymplokos deltaeiberi* was distinguished from all other genera of spirochetes [8]. Like the other spirochete genera, *S. deltaeiberi* is a Gram-negative, motile and helically shaped bacterium in which overlapping periplasmic flagella can be described by the formula n:2n:n [7]. A unique aspect of *S. deltaeiberi* behavior is that it tends to swim into the light of the microscope field, and thus to follow cyanobacteria. Also it actively withdraws its protoplasmic cylinder inside its outer membrane. Live, variable diameter, spirochetes form round bodies that contain within actively moving spirochetes and/or refractile, membranous structures. We suspect the latter are viable propagules [8]. Eighteen morphometric features of the Spanish mat *S. deltaeiberi* were tabulated (see Table 1 in [4]). A light micrograph of an enrichment culture (Fig. 1), the distinctive granulated cytoplasm of a single 5:10:5 (Fig. 2A) and a composite, variable diameter (Fig. 2B) spirochete from Ebro delta are shown here.

Our original studies compared modern mat communities to ancient laminated or microfossiliferous cherts [1, 13, 14]. They were designed to grow and test the survival and preservation characteristics of distinctive mat microbiota under anoxic conditions: *Paratetramitus jugosus* and other mat amoebomastigotes [2], including those that form chromidia [6], cyst-forming ciliates [10], and morphologically-distinguishable spirochetes [15]. In the course of this work we noticed *Spirosymplokos*-like organisms, which we sampled for electron microscopy and other investigation.

Materials and methods

Field Sites BAJA CALIFORNIA, MEXICO: Samples were collected near tidal channels east of the dunal complex at North Pond (Charco del Norte), Laguna Figueroa, Baja California Norte, Mexico. This long dunal complex extends 16 km in the NE-SW direction (latitude 30°40'N, longitude 116°0'W) and fewer than 3 km across. Commercial salinas (salt works) abound in the south; the northern lagoon, periodically flooded or exposed, is unworked and undeveloped by people [12]. All samples had well-developed mat communities of *Microcoleus chthonoplastes* underlain by a thick layer of organic-rich mud.

NEW ENGLAND, USA: Samples were collected from the Great Sippewissett Salt Marsh (latitude 41°35'30"N, longitude 70°38'50"W) in the summers of 1991, 1993 and 1995, January 1993 and December 1997. The site, on the southeast shore of Buzzard's Bay, harbors salt marsh grasses and other plants (e.g., *Spartina alterniflora, Spartina patens* and *Salicornia* sp.) where a barrier rock wall and dunes separate the microbial mats from the channel. Diurnally flooded and desiccated by tidal activity, the mats are located just west of the main channel entry to Quahog Pond (Site 2, map of Fig. 1B in [15]). The mats, best developed from June until February, display thickness up to 3 cm. They are underlain by sand or mud, depending primarily on season and location. The community is vertically stratified and minimally displays five layers. A surface brown-orange diatom layer, a green cyanobacterial layer (primarily *Microcoleus, Spirulina, Oscillatoria* and variable quantities of *Lyngbya*), a pink sulfur bacterial layer *(Thiocapsa roseopersicina, Chromatium)*, a salmon-colored layer mainly of *Thiocapsa pfennigii*, a dark greenish thin layer of *Chlorobium* and other green sulfur phototrophs have been documented [9].

Samples, culture conditions and observations The acidities and salinities of the field sites were measured with Hydrion paper and an optical refractometer respectively. Samples of mat in flat Pyrex dishes or in glass jars tightly closed were returned to the laboratory for study. Mats stored in plastic dishes invariably yield fewer healthy spirochetes. Alternatively small pieces, about a millimeter cubed, were placed directly in a cellobiose-trypticase peptone-sodium thioglycolate medium in 80% sea water (BA-2 medium of [3]) to which was added stock of filter-sterilized rifampicin, a protein synthesis inhibiting antibiotic (final concentration ranged from 5–20 µg/ml as noted). The antibiotic media of these tubes, filled to the brim and tightly closed to prevent entry of extraneous oxygen, tend to select for survival of some cyanobacteria, spirochetes, sulfide and anoxia tolerant small ciliates and a variety of tiny unidentified spirilla and rods. Spirochetes were enriched from mat samples in glass jars left in the light at laboratory temperatures for as long as three months. Live microbes were observed using an inverted Nikon phase contrast microscope, a compound microscope
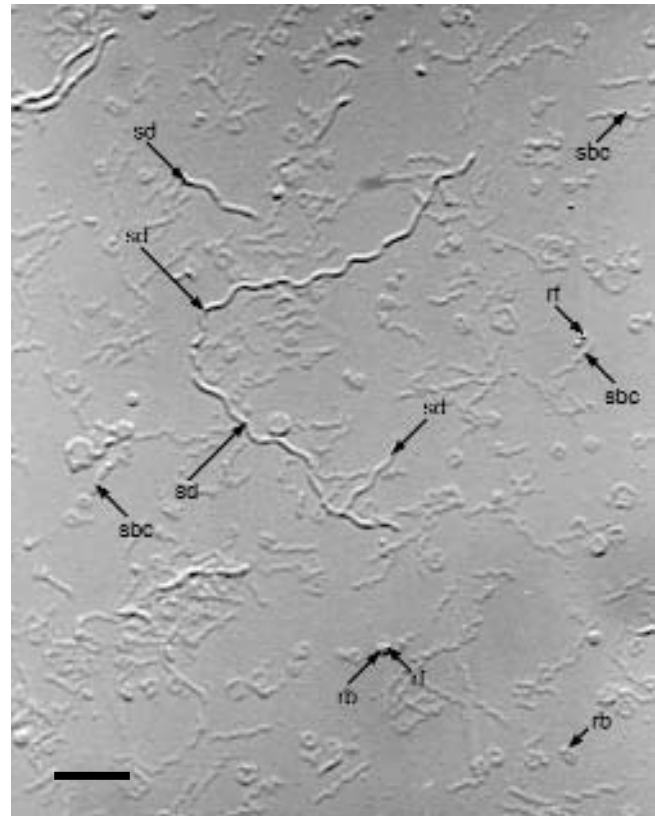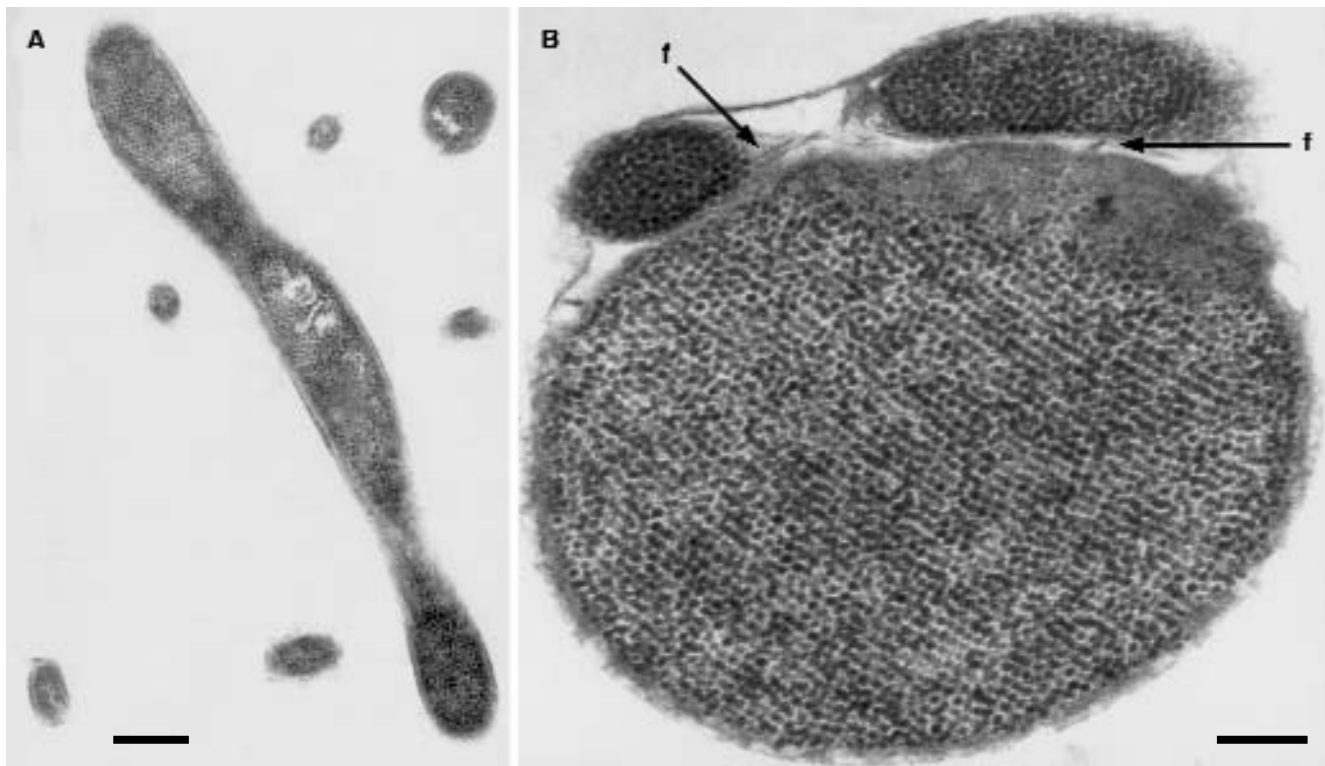

Fig. 1 *Spirosymplokos deltaeiberi* (sd) from Spain, showing detectability of these large spirochetes when live. Nomarski DIC in mixed enrichment culture with *Spirochaeta bajacaliforniensis* (sbc). Round bodies (rb), refractile structures (rf), bar = 10 µm

fit with darkfield condensor and a Nikon Microphot DIC (Nomarski, fluorescence and phase contrast) to which 35 mm camera back and either a Sony CDC or an Optronix videocamera were mounted. Microbial behavior was recorded on 3/4" Sony-U-matic videotape.

Ultrastructure As soon as appropriate population densities of spirochetes were noted the mixed culture samples were fixed in 2.0-2.5% glutaraldehyde using methods for transmission electron microscopy developed by D. Chase and J. F. Stolz [12] and described in [15].

Results

Field studies The salinities of the field samples were well within the ranges of sea water standards: 3.3 to 3.4%. The pH varied from about 7.0 at the mat surface to closer to 5.0 in the lowest layers. Sippewissett winter collections, especially in January 1993, were characterized by high wind velocities (15-30 mph) and intemperate (subzero Fahrenheit) conditions. Most notable were the well-developed winter microbial mats that included brightly red colored

Fig. 2 *Spirosymplokos deltaeiberi* from Spain, electron micrographs. (A) Single (noncomposite) Ebro delta spirochete with 5-10-5 (or 6-12-6) flagella insertions in longitudinal and (upper right) tranverse section. Both continuity of flagella with cytoplasmic granules and density of the array of granules are clearly seen; bar = 5 μm. (B) Composite Ebro delta spirochete in membrane-bounded round body that shows typical low number of periplasmic flagella (f), packed arrays of protoplasmic-cylinder granules and variable diameter; bar = 1 μm

communities of phototrophic bacteria that apparently thrive under more than 2 millimeters of ice. The microbial mats of Laguna Figueroa under study are subject to rapidly varying salinities from those of rain water to crystalline salt. Yet mats of Laguna Figueroa never suffer ice cover. Daily periods of high winds are generally noted at both Sippewissett and Laguna Figueroa.

Observations of live spirochetes Samples incubated with well-developed underlying black muds tended to yield spirochetes more quickly and in greater profusion than those underlain with sand.

The spirochete described as SI-5 (Fig. 4 in [15]) has now been seen in enrichment samples collected in 1991, 1993, 1995 and 1997 and handled by the methods described here. Populations reaching densities adequate for videographing of live material were seen in all samples prior to 1997; blooms adequate for electron micrographs were especially apparent in June of 1993 (at site 2 of [15]) and July of 1995. The pink mat layers were particularly prominent, the peach layer absent or reduced and the quantities of rifampicin (between 10–20 μg/ml) in the tubes in which the most dense populations of large spirochetes developed. In favorable mixed-culture preparations jerky swimming, orientation toward the microscope light, refractile structures, round bodies and

swellings, variable diameters and ranges in sizes of the loosely coiled large spirochete were observed and videographed.

Culture conditions and morphological analysis At the Sippewissett salt marsh, one evening in June 1993 collections were made at low tide of mat material that had a highly developed *Microcoleus* layer. Three days later 20 ml tubes containing BA-2 rif (10 μg/ml) medium were inoculated with 1 mm³ mat pieces cut through all the vertical layers. Five days later these tubes were examined and seen to contain thin membranous round bodies with spirochetes curled up and moving inside of them. Many moving refractile bodies and swimming spirochetes with refractile bodies in them (like those in Fig. 1 above from the Ebro delta) were also observed. A few loosely-coiled long spirochetes were also seen. The following day, a total of nine days after collection of the field sample, a few tubes had high population densities of loosely coiled large spirochetes and round bodies containing these spirochetes. Several fresh 20 ml glass culture tubes were inoculated with 1 ml of the spirochete-rich sample and a 1 mm³ fresh piece of mat from the same locality added to new BA-2 medium. The new medium was made up with a higher concentration of rifampicin (20 μg/ml) to help eliminate the extraneous bacteria. Two days later two of these
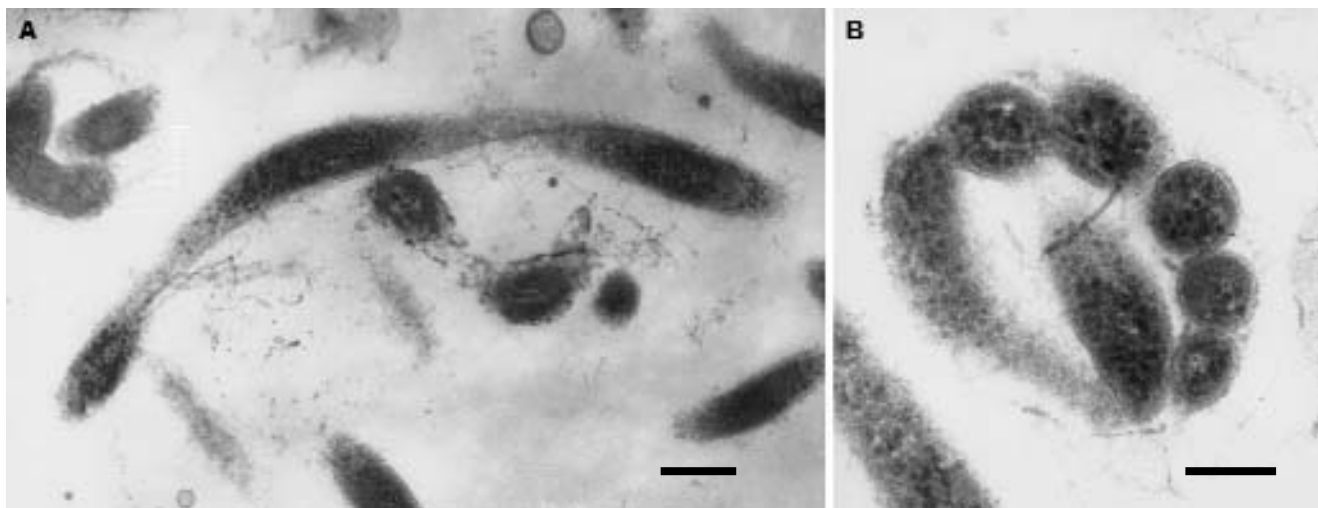
Fig. 3 Large spirochetes, collected from Sippewissett salt marsh mats, bars = 5 μm. (A) Granulated, non-composite spirochete, mats collected December 1994. (B) Membrane-bounded round body with granulated cytoplasm

tubes had excellent blooms of loosely coiled spirochetes, swimming refractile bodies and round bodies containing spirochetes. Very few small rods, vibrios or spirilla were present nor were any ciliates, amoebae or mastigotes seen. Three similar spirochete-rich tubes were pooled. The liquid was sedimented in a desk centrifuge, the supernate discarded and the pellet resuspended and spun again in Eppendorf tubes. The resuspended thick pellet was fixed in "Baja cocktail" [3] to a final concentration of 2.0% glutaraldehyde. The features seen in live material, still photographs of live spirochetes and in the ultrastructural preparations are listed in Table 1; micrographs in addition to those previously published (as Fig. 4, page 579 of [15]) are shown here (Figs. 3 and 4).

Our experience with samples taken from Laguna Figueroa, Baja California Norte, Mexico is similar. We have recognized the large, composite, variable diameter spirochete in samples designed to select for the much smaller *Spirochaeta bajacaliforniensis* on at least three occasions. Apparently, conditions that are ideal for *S. bajacaliforniensis* are too anoxic or otherwise not suitable for the large loosely-coiled variable-diameter spirochete. After about one week in BA2-rif tubes if not transferred to fresh medium, blooms of the small spirochetes tend to subside. In some cases they are replaced with the large spirochetes but even more frequently no large, loosely coiled spirochetes develop at all in these cultures. On favorable occasions between six days and three weeks after inoculation of BA2-rif media tubes with vertical microbial mat sections we have seen and photographed cultures that clearly contain the stages of the loosely coiled large spirochetes: round bodies with moving spirochetes inside, refractile structures, jerky swimming behavior, orientation toward the light as well as twitching round bodies and swimming refractile forms (Figs. 3–6). Although one large one is common, as many as five or six swellings on a single long, variable-diameter spirochete have been documented (Fig. 5). The single and composite granulated spirochetes with 4:8:4 or 3:6:3 periplasmic flagellar arrangements seem so frequently associated and similar to each other as to be stages of the same organism (Fig. 6). The composite, variable diameter and granulated structures of the typical membrane-bounded round bodies are in Fig. 6B and 6D. Measurements of granules in the protoplasmic cylinders of these granulated spirochetes, whether from New England or Baja California, showed their diameters to be 27±7 nm independent of the size of the spirochete. In several micrographs, smaller, less well-stained and more irregular spheres, presumably ribosomes, are seen in the same sections (Figs. 4A and 6B). The protoplasmic granules but not the putative ribosomes usually stain darkly and some granules appear continuous with the spirochete flagella (Fig. 2B).

On several occasions large quantities of loosely coiled spirochetes have been observed to swim alongside gliding filaments of cyanobacteria, such as *Lyngbya* or *Microcoleus*. In preliminary results we have tested the Bellcore U-tube apparatus with incubations of either pure cultures of appropriate cyanobacteria or mixed cyanobacteria taken from mat material. On one side of a 0.22 Millipore filter is placed the cyanobacterial mixture (H-X medium [16]) and on the other side BAD rifampicin medium. Mixed-culture samples known to contain relevant spirochetes are inoculated into the BA-2 side. This arrangement provides useful enrichment culture conditions for mat spirochetes, especially the flat Bellcore tubes under investigation now. Several kinds of spirochetes swim through the filter,
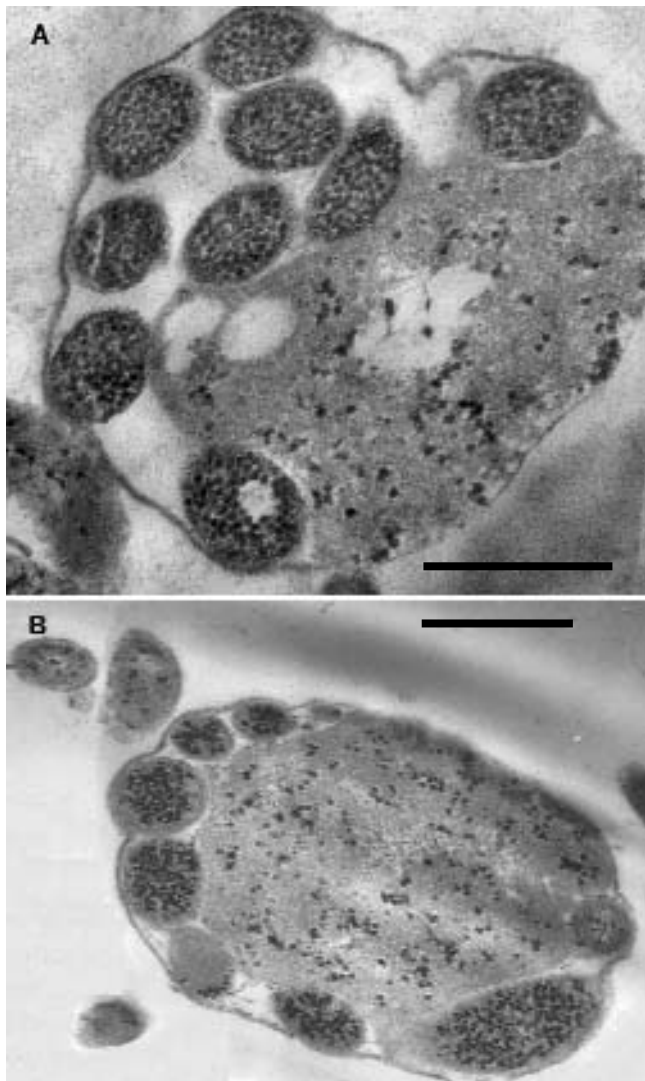
Fig. 4 Large composite spirochetes from Sippewissett salt marsh (bars = 5 μm). (A) Parental protoplasmic cylinder disintegrating in healthy rounded composite granulated spirochete round body. Single spirochete emerging at lower left. (B) Variable diameter (lower right) of composite spirochete in rounded membrane-bounded body

accumulate and presumably feed, at the cyanobacterial mat interface.

Discussion and conclusions

We conclude based on a consistent set of criteria, that *Spirosymplokos deltaeiberi* are normal and regular inhabitants of stratified microbial mat communities both at the great Sippewissett salt marsh of Cape Cod and North Pond in Laguna Figueroa, Baja California Norte, Mexico. The criteria include: large, loosely coiled morphology with variable diameter, composite structure, conspicuous 27±7 nm granules

in the protoplasmic cylinder often continuous with the 3-4 flagella per terminus. The behavior of these spirochetes is also distinctive (jerky swimming, the formation of swellings along the loose coil, oriented swimming toward the lit microscope field, retraction of the protoplasmic cylinder into the outer membrane covering). The life history of the Ebro delta composite spirochete from Spain (depicted in Fig. 7 of [8]) conforms precisely to the observations here of the North American mat spirochetes. Whether from Spain, New England or Mexico, these large mat spirochetes form blooms, with certain unpredictability but most conspicuously some 7–10 days after removal from the field and placement of mat pieces in rifampicin-rich medium designed for the maintenance of *Spirochaeta bajacaliforniensis*.

Table 1 Criteria for identification of *Spirosymplokos deltaeiberi* in North American samples*

Diameter	0.4–3 μm, variable in a given cell
Flagella (at one terminus)	3–5
Sillon, crenulations	absent
Coatings of inner and outer membranes	absent
Ratio diameter of protoplasmic cylinder to diameter of cell	0.90–0.99
Angle protoplasmic cylinder subtended by flagella	10–90°
Flagellar bundle	absent
Length	25–100 μm
Amplitude	1.1 μm
Wavelength	5.5 μm
Cytoplasmic tubules	absent
Polar organelle	absent
Rosettes	absent
Granulated cytoplasm	present
Composite structure (greater than 1 protoplasmic cylinder per periplasm)	present
Round bodies with moving spirochetes inside	present
Refractile structures in medium	common
Jerky swimming and stopping	present
Phototropic swimming	present
Growth in axenic culture	no

*Determined by light (phase contrast, darkfield, Nomarski DIC and brightfield) microscopy or transmission electron microscopy or both

All morphometric observations made on the loosely coiled, jerkily moving spirochetes that display these criteria from videos, still photographs, electron microscopic thin sections and especially live material indicate that by any criterion the Sippewissett and Laguna Figueroa spirochetes fall well within the published range of features attributed to *Spirosymplokos deltaeiberi* from Spain.

Fig. 5. Variable diameter, probably four flagella of granulated large spirochete from Laguna Figueroa, Mexico. Refractile body (rf) probably forming at arrow, bar = 5 μm

Conspicuous arrays of packed cytoplasmic granules (Figs. 2–6) coupled with a paucity of flagella lead us to hypothesize that the granules contain ATPase. *Cristispira*, *Pillotina* and all other vigorously swimming spirochetes that exceed 1.0 μm in diameter have far more numerous flagella. Fluorescence microscopic studies of protoplasmic cylinders with antiATPase antibodies against *Spirosymplokos deltaeiberi* are in progress to test this hypothesis.

The most parsimonious conclusion is that the composite spirochete is cosmopolitan and forms resistant propagules (Figs. 5D and 7 in [8], and Fig. 5D in [4]). These probably correspond to the arrow (in Fig. 5). We suggest that the propagule-forming life history is especially advantageous in the severe weather faced by inhabitants of the inconstant intertidal salt marsh environment. These conditions and genetic capabilities have led to a wide, at least northern hemisphere, distribution of the free-living spirochete: *Spirosymplokos deltaeiberi*.

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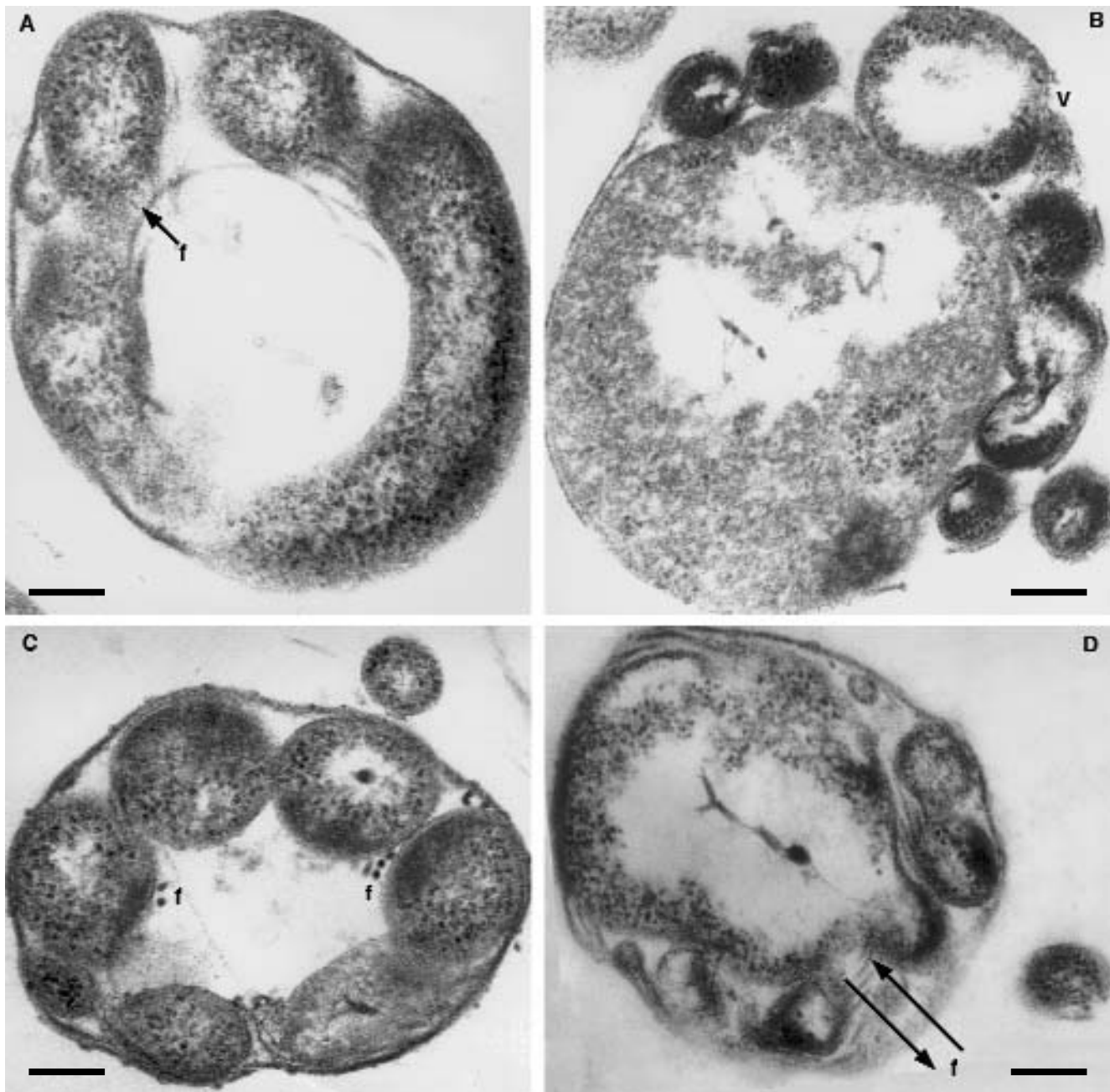
Fig. 6 Variable diameter, composite spirochetes from Laguna Figueroa Mexico, bars = 1 μm. (A) Round body in which the tendency of the spirochete to fragment is seen, f = flagellum. (B) Fragmenting variable diameter (v) spirochetes, granulated cytoplasm of the parental form can be seen disintegrating. Release of the single spirochetes can be seen at the right. (C) Eight flagella, typically the size of the cytoplasmic granules, are seen here in transverse section of a healthy membrane-bounded round body. Four flagella (f) and composite granulated structure in this round body. (D) Round body with disintegrating parental cytoplasm apparently budding off small spirochetes at arrow

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