Cristispira From Oyster Styles: Complex Morphology of Large Symbiotic Spirochetes

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Abstract

Crystalline styles (digestive organs) of bivalve mollusks provide the habitat for highly motile bacteria. Styles from freshly-collected oysters, *Crassostrea virginica*, were studied by electron microscopy; *Cristispira* spirochetes were abundant in these organs. Detailed study reveals these spirochetes to be among the most complex prokaryotic cells known. More than 600 periplasmic flagella and an adhering outer lipoprotein membrane (e.g., a 270° sillon) form the ultrastructural basis for the "crista," first described by light microscopy. Unique rosette structures corresponding to the "chambers" or "ovoid inclusions" of light microscopy were detected at the periphery of all protoplasmic cylinders. Polar organelles and linearly aligned flagellar insertions are conspicuous. In size and complexity, *Cristispira* more resembles *Pillotina*, *Diplocalyx*, *Clevelandina* and *Hollandina* (large spirochetes symbiotic in termites) than it does *Treponema*.

Cristispira pectinis (Gross, 1910), the type species; *Spirillum ostrea* (Noguchi, 1921); and another, less frequent bacterial symbiont are the predominant inhabitants of the dense style matrix. The ultrastructure of the spirillum and an electron micrograph of the third bacterium are shown.

Keywords: bacterial consorts, bacterial symbionts, *Clevelandina*, *Crassostrea virginica*, crest, crista, *Cristispira*, *Cristispira pectinis*, crystalline style, *Diplocalyx*, flagellar bundles, *Hollandina*, morphometric analysis, ovoid bodies, oyster symbionts, *Pillotina*, polar organelles, rosettes, *Spirillum ostrea*, spirochete morphometrics, spirochete ultrastructure, style matrix, *Treponema*

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1. Introduction

Cristispira is only known to maintain healthy high populations and divide by transverse fission when symbiotic in animals. Most *Cristispira* are found in the crystalline styles of bivalve algivorous marine mollusks, primarily oysters (Kuhn, 1981). Attempts to isolate these large bacteria into culture have failed. *Cristispira* was first described in 1882 by A. Certes as a kinetoplastidic protist, *Trypanosoma balbianni*, which was thought to have a chambered body in which each chamber was equivalent to a single cell. It later was renamed by Gross (1910). The modern understanding of the organism comes from Noguchi (1921), who, detecting it in oysters, clams (*Venus myenaria*), and mussels (*Modiolus*), compared *Cristispira* to spirochete bacteria. After observations of live material, many preservation experiments (methyl alcohol fixative with Giemsa staining was most successful) and attempts to culture these oyster symbionts, Noguchi (1921, p. 312) wrote: "...it is possible that the crista of *Cristispira* is a highly modified form of flagella." We recognized *Cristispira* in our studies of the ultrastructure of the crystalline styles, and confirm Noguchi's and Ryter and Pillot's (1965) interpretations about the spirochete nature of *Cristispira*. We also resuscitate Noguchi's introduction of *Spirillum ostrea* into the biological literature, augmenting a brief ultrastructural description of these bacterial consorts (Sieburth, 1979).

First called an undulating membrane, then a "crest," Ryter and Pillot (1965) showed *Cristispira*'s protrusion to be a bundle of bacterial flagella. The number comprising the bundle was estimated to be over a hundred (Ryter and Pillot, 1965). Given the limited knowledge about the physiology, behavior, and ecology of these spirochetes, the literature has been well reviewed (Kuhn, 1981; Sieburth, 1979; Breznak, 1984). While morphological detail is limited in the smaller spirochetes (e.g. *Spirochaeta*, *Treponema*), it provides a sufficient basis for taxonomy in the large and complex prokaryotes (e.g. *Clevelandina*, *Diplocalyx*, *Hollandina*, *Pillotina*; (Bermudes et al., 1988). On the basis of observations of live organisms and numerous light and electron micrographs, we detail the ultrastructure of these obligate marine microbial symbionts in oyster styles.

2. Materials and Methods

I. Oyster styles

Oysters, *Crassostrea virginica*, were obtained from three sources: Marine Resources at the Marine Biological Laboratory in Woods Hole, MA and Waquoit Bay Shellfish Company in Falmouth, MA supplied material for live

observation and cellulolytic experimentation, and samples for electron microscopy were collected from the Pettaquamscutt Estuary, Narragansett, RI.

Oysters were dissected within 2 hr of removal from their natural habitat, and styles, yellow or clear in color when present, were removed with forceps. Each style was placed on a 22 mm × 55 mm cover slip, examined for the presence or absence of *Cristispira* under an Olympus ITM2 inverted microscope, and then scored + or −, as appropriate.

II. Light microscopy

Bright and darkfield light micrographs of live *Cristispira* were taken of styles examined with an Olympus IMT2 inverted BHS-2 or Zeiss phase contrast compound microscope to which a 35 mm cameraback was mounted.

III. Electron microscopy

Fresh styles, fixed in a solution of 3% glutaraldehyde in 0.025 M phosphate buffer at pH 6.8, post-fixed in 1% OsO_4, were dehydrated to acetone, and embedded in Spurr's resin. Further details of electron microscopic and morphometric procedures are described (Johnson and Sieburth, 1976; Bermudes et al., 1988).

3. Results

To observe *Cristispira* it is essential that oysters are freshly harvested. Observations are optimal within the first 2 hr; by 6 hr or so after removal from the natural estuarine habitat the styles degenerate or are sloughed, rendering study of these mollusk symbionts impossible. Spirochetes tend to be either plentiful or absent in the style. Although it has been suggested that *Cristispira*-containing styles are not present during the coldest months, we have found dense populations in all seasons at Woods Hole, MA. In oysters from the Pettaquamscutt Estuary, the style and its microbiota were present in those living at 8° but not at 4°C (Sieburth, 1979). Mature oysters were used in these studies; the mean length of the styles was approximately 3 cm (Fig. 2A). Because of the large size of *Cristispira*, the presence of these large spirochetes (but not the other style bacteria) was easily determined in intact styles by inverted light microscopy.

Note: Figs. 2 and 4A from Sieburth, 1979. Used with permission from Oxford University Press.

Both high magnification light and electron microscopy revealed three symbiont types regularly present in the thick-style matrix: the large *Cristispira* spirochete, a smaller spirillum, *Spirillum ostrea* Noguchi (Fig. 1) and occasionally another unidentified Gram-negative bacterium (Fig. 2). *Cristispira* is generally more abundant by at least an order of magnitude. Masses of them were present in the styles chosen for electron microscopy.

Spirillum ostrea is a blunt-ended bacterium measuring about 0.5 μm in diameter and 10–20 μm long. Lophotrichously flagellated, it bears 4–5 flagella at the two termini of the cell (Fig. 1C). *S. ostrea* displays from 6–8 waves per cell (Fig. 1A). Vigorously motile, the spirillum apparently deforms the dense matrix of the style as it moves (Fig. 1A). The very close lengthwise adherence of the outer lipid membrane of the Gram-negative cell wall to the mollusk style suggests direct exchange of materials between the spirillum and the noncellular matrix of the bivalve's organ (Figs. 1A,B).

Although the orientation of the population of spirilla in the elongated style is not known, *Cristispira* tend to align longitudinally in the style matrix. The edge of the style is shown in Fig. 3B. There, and in the transverse section of Fig. 4A, the tendency of *Cristispira* toward longitudinal alignment is apparent.

In highly motile *Cristispira*, the bundle of flagella is difficult to discern. Later the spirochete degenerates, this "crest" flares and swells, and becomes more obvious (Fig. 3C).

The flagellar bundles, aligned along the long axis of *Cristispira*, very nearly contact the matrix along their length (Fig. 4). In fact, the matrix is in greater contact with the outer lipid membrane than the outer membrane is with its inner membrane (Fig. 4B). The periplasmic space in *Cristispira* is minimal (15 nm) except where it expands to about 400 nm because it is filled with periplasmic flagella. Between 6 to 16 waves per *Cristispira* are commonly observed. Morphometric data on the wavelength and amplitude (Table 1) was derived from measurements of Figs. 4, 6, 7 and others like them.

The most striking feature of the protoplasmic cylinder of *Cristispira* is its unique organelles, the peripheral rosettes (Figs. 4C,D). These structures align the entire periphery of the organism along its length except where flagella insertions are present (Figs. 5A,B,7B). Although the individual spheres of the rosettes generally measure about 100 nm in diameter, the number of spheres and relations between those comprising the rosettes vary such that the ultrastructure of the rosettes is not constant. From as few as 2 to as many as 9 electron-luminous components can be resolved in each rosette (Figs. 5C–G). Fortunate transverse sections reveal a seven-fold symmetry in some (Fig. 6B).
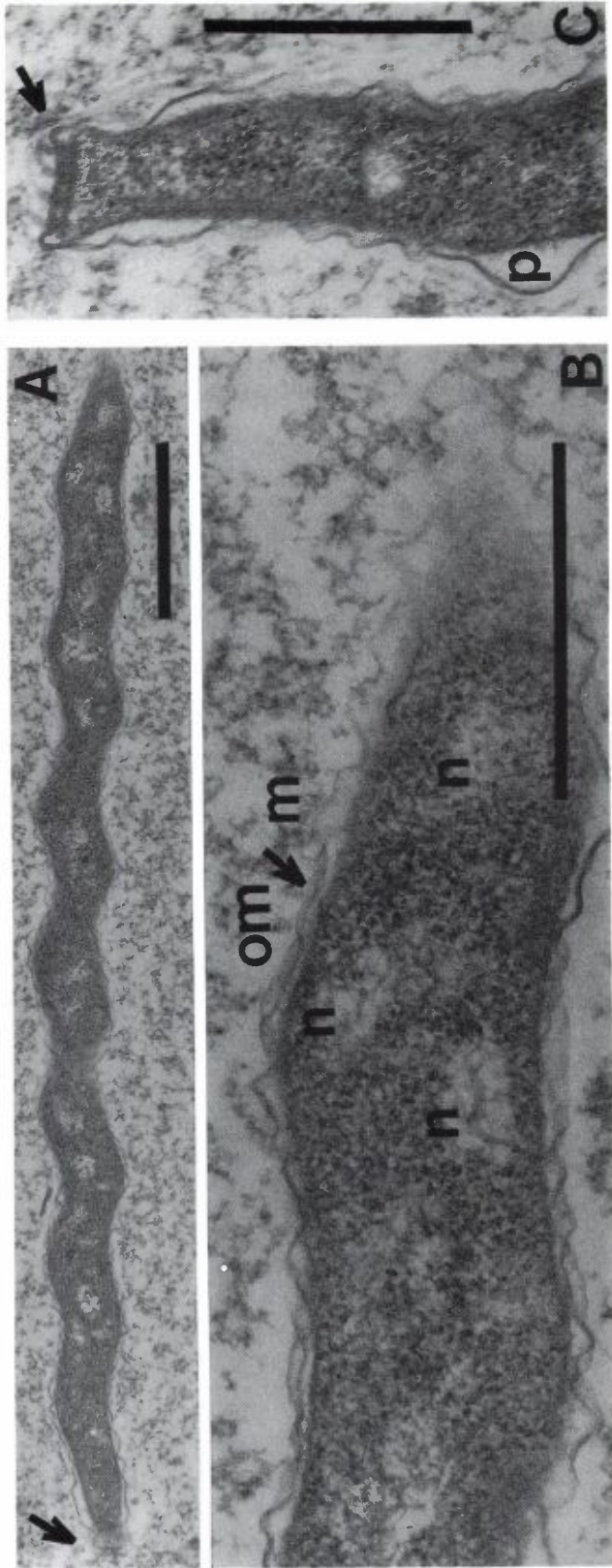
Figure 1. *Spirillum ostrea* (electron micrographs)

A. Morphology of *S. ostrea* in longitudinal section, showing deformation of matrix material (arrow; bar = 1 μm).

B. Proximity of matrix (m) material to outer membrane (om) (arrow); distribution of nucleoids (n) (bar = 0.5 μm).

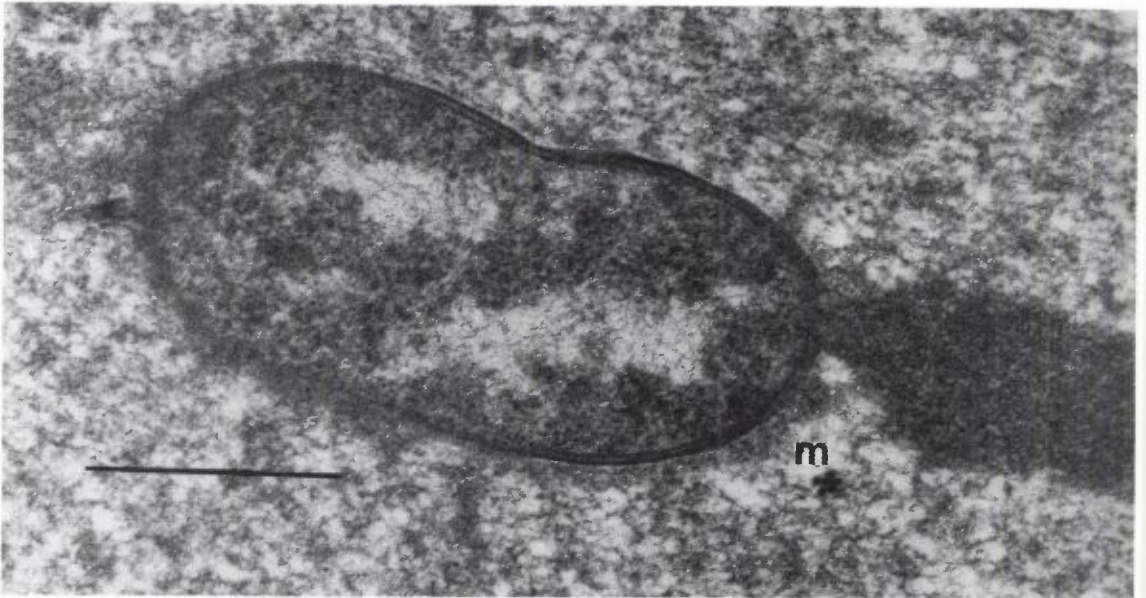C. Five flagellar insertions and their flagellar (arrow) at the blunt end; periplasm (p) (bar = 0.5 μm).

Figure 2. Unidentified Gram-negative rod-shaped bacterium in oyster style. m = matrix, w = wall, n = nucleoid (bar = 0.5 μm).

The polar organelle, a structure first described in spirilla and subsequently noted in many types of motile flagellated bacteria, is clearly present in *Cristispira* (Tauschel, 1985). It lies conspicuously beneath the flagellar bundle (Fig. 6A) and was not observed beneath non-flagellated periplasm (Fig. 6B). *Cristispira*, like all flagellated bacteria, displays disk-shaped flagellar insertions. About 40 nm in diameter and embedded in the outer membrane of the protoplasmic cylinder, they are spaced about 60 nm from center to center along the flagellated periphery.

Cristispira, which moves in either direction by rotation and translation, apparently deforms the style matrix as it proceeds (Fig. 6A,C). A thickening of the cell wall, probably corresponding to the peptidoglycan layer, can be seen in Fig. 6D. This corresponds to a fused outer coat of inner membrane with inner coat of outer membrane (Table 1), but the best-developed coats seem to be limited to the termini of the cell. The extensive surface area contact of the outer membrane to the oyster style matrix on the flagellated side is seen in Figs. 6A,B and 7B.

From 608 to 706 flagella, counted in micrographs of transverse sections of *Cristispira*, comprise the "crest" or "crista" as seen in the light microscope (Fig. 7B) is typical. The cell periphery (protoplasmic cylinder) is crowded
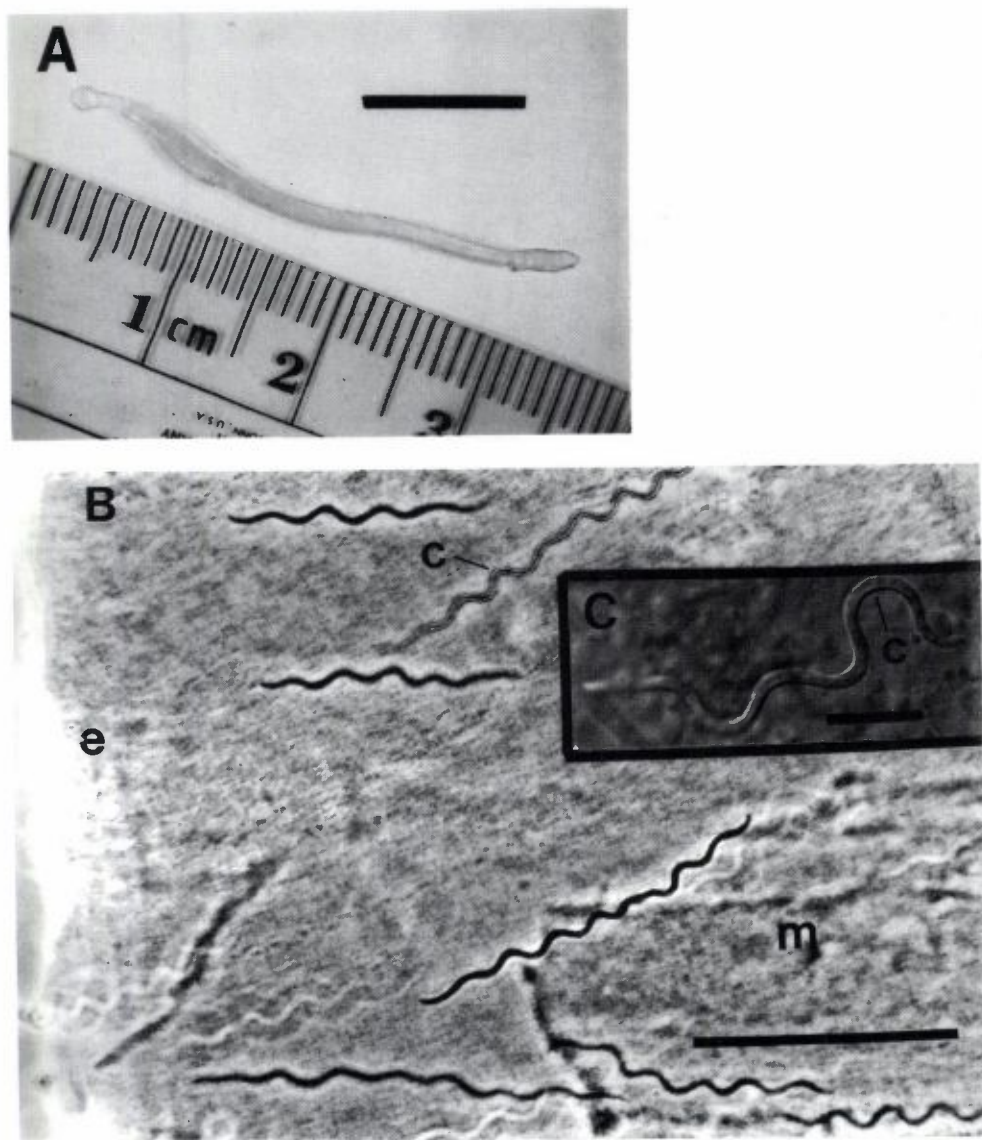
Figure 3. *Cristispira* (light micrographs)
    A. Isolated style from *Crassostrea virginica* (bar = 1 cm).
    B. Over eight *Cristispira* embedded within the style matrix (m); edge of style (e) is marked; conspicuous "crest" (c) noted (bar = 30 μm).
    C. Healthy *Cristispira* shortly after removal from style, "crest" (c) becomes more conspicuous as spirochetes degenerate (phase-contrast photomicrograph; bar = 10 μm).
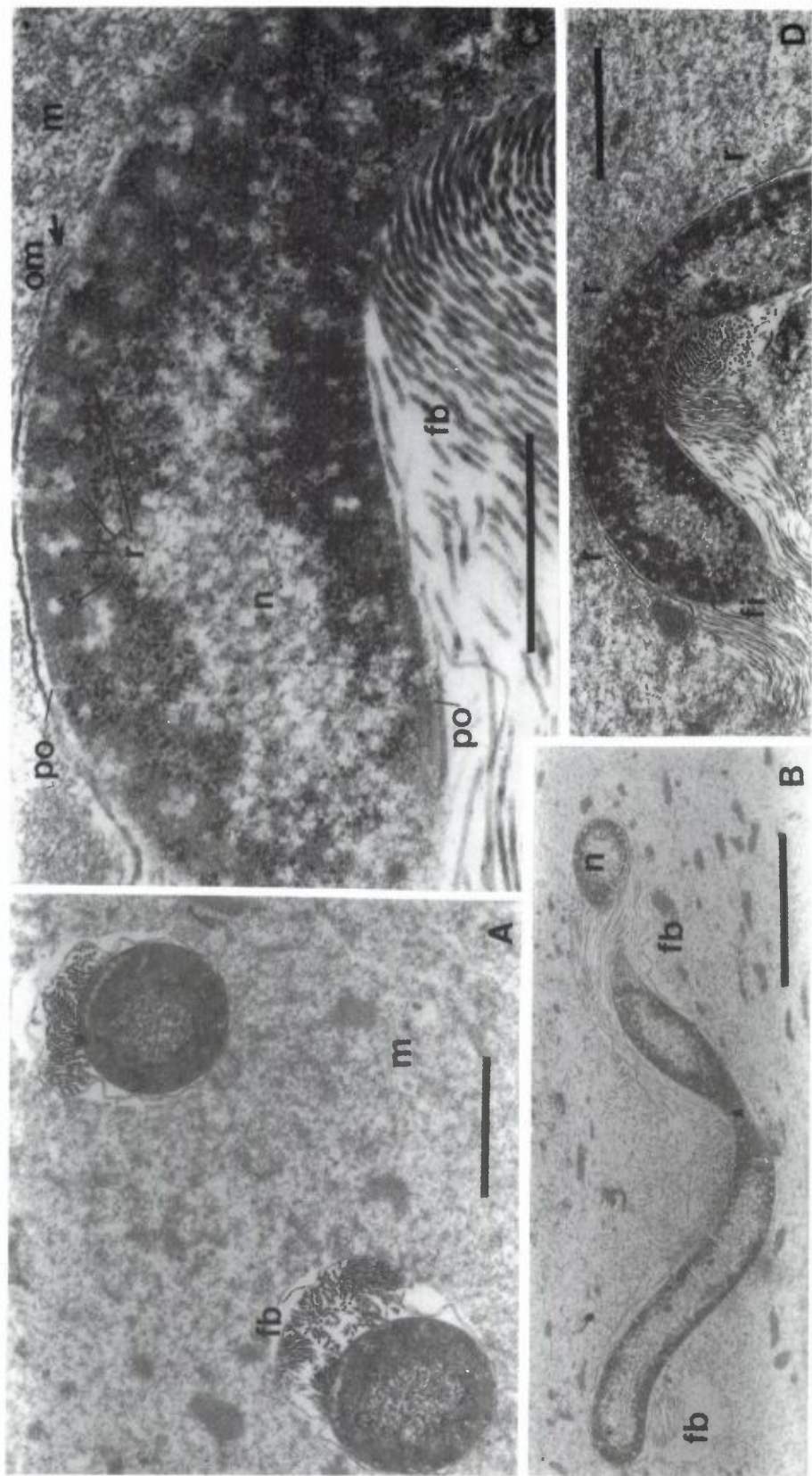
Figure 4. A. Two *Cristispira* longitudinally aligned in the style (as in Fig. 3B). Flagellar bundles (fb) seen in transverse section with the matrix (m) (bar = 1 μm) (Sieburth, 1979).

B. Flagellar bundles (fb) follow the contours of the helix; amplitude and wavelength (criteria 10, 12, and 13) can be determined from electron micrographs such as this. The nucleoid (n) is indicated (bar = 5 μm).

C. Flagellar bundles (fb) are peripheral in location but not in the same plane as the rosettes (r). Polar organelle (po), nucleoid (n) and (at arrow) proximity of outer membrane (om) to matrix (m) are clearly visible in this micrograph (bar = 0.5 μm).

D. Rosettes (r) stud the periphery of *Cristispira* except where the flagella (f) and their insertions (fi) are present (bar = 1 μm).
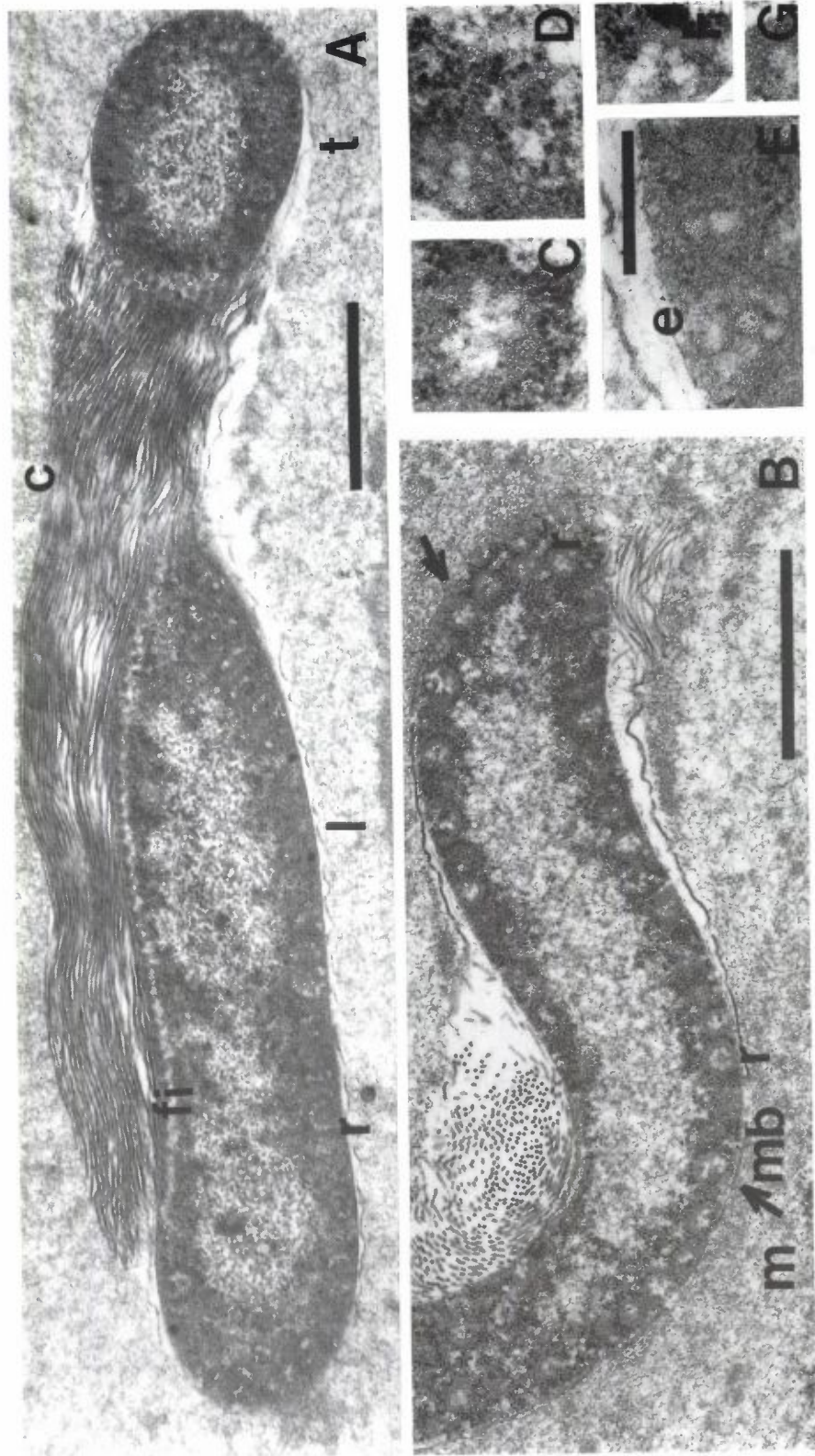
Figure 5. A single *Cristispira* seen both in transverse (t) and longitudinal (l) section. The crest (c) is clearly visible. Plane of flagella insertions (fi) is opposite to the peripheral rosettes (r) (bar = 1 μm).
B. *Cristispira* membrane (mb) is in contact with style matrix (m) for nearly a wavelength; rosettes (r) are marked (arrows; bar = 1 μm).
C–G. Rosettes seem to have from as few as two (G) to nine or more (E) electron luminous components (e) (bar = 100 nm).
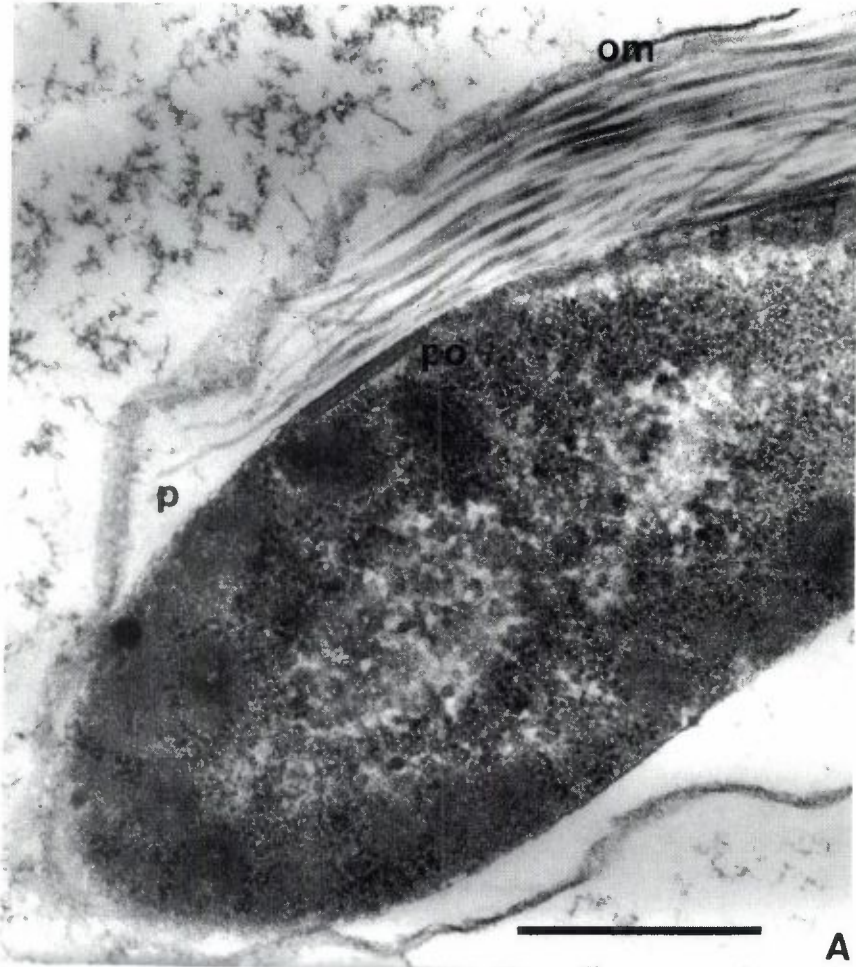
Figure 6A. The bilayer structure of the polar organelles (po) is opposite the periplasm (p). The periplasm itself is bounded by the outer membrane (om) (bar = 0.5 μm).

with rosettes and polar organelles, the ribosomes fill the space just proximal to it; the nucleoid, which extends continuously down the lumen nearly to each terminus, lies proximal to the ribosomes. Cytoplasmic tubules, common in *Pillotina* and other large spirochetes, have not been seen in any sections of *Cristispira* (Margulis and Hinkle, 1991).

Based on micrographs, a longitudinal external view (Fig. 8B) and transverse EM cross-section of *Cristispira* is depicted (Fig. 8A).
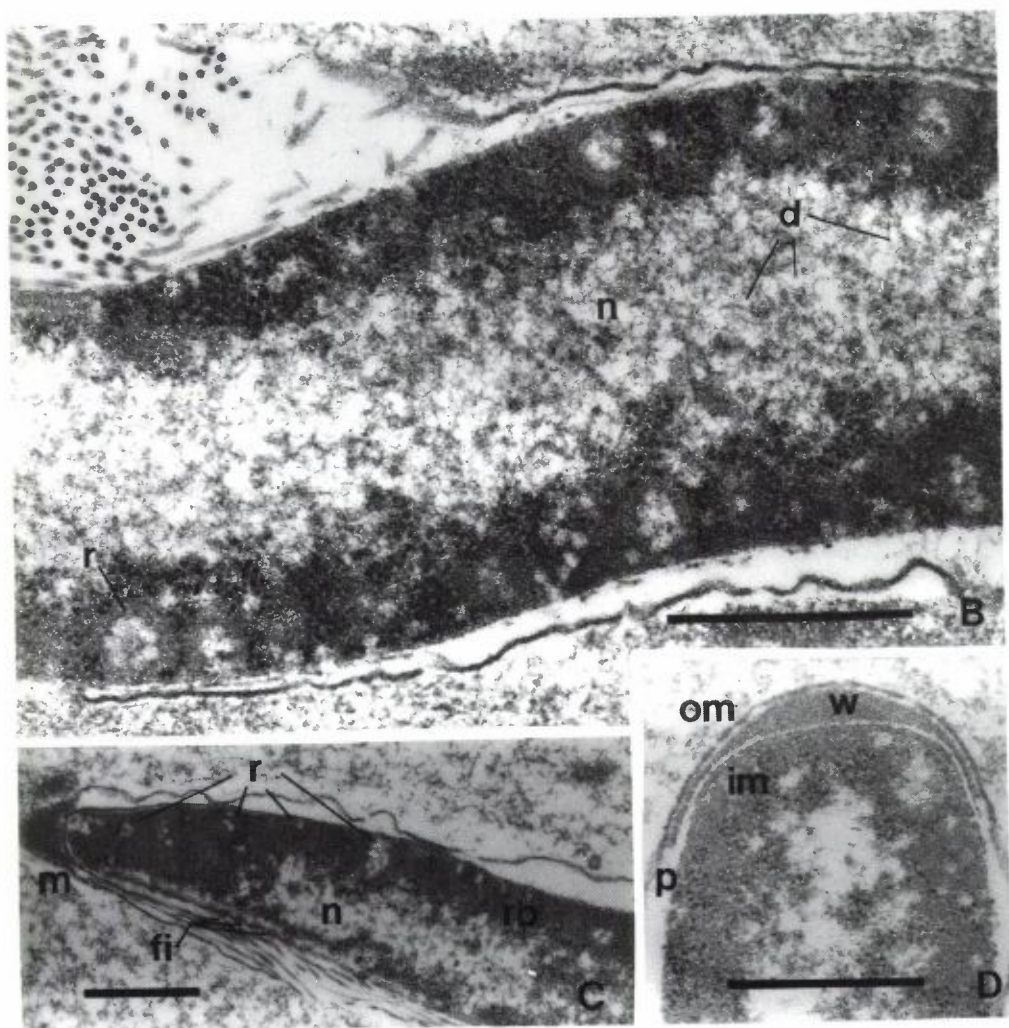
Figure 6B. Large central nucleoid (n) containing DNA fil rils (arrows, near d) extends the
length of *Cristispira*. Seven-fold symmetry i; seen in this rosette (r) (bar =
0.5 μm).
C. Style matrix (m) density apparent in front of the pointed tip of *Cristispira*.
Rosettes (r), ribosomes (rb) and flagellar insertions (fi) lie peripheral to the
nucleoid (n) (bar = 0.5 μm).
D. The dense substance in the periplasm (p) between the inner membrane (im)
and the outer membrane (om) may be peptidoglycan wall (w) material (bar =
0.5 μm).

Table 1. *Cristispira pectinis* morphological criteria*

		Measured	Estimated ranges for genus/comments
1.	Diameter	1.4–2	0.5–3.0 μm
2.	Number of flagella	608, 706	[100–200–100] to [300–600–300]
3.	Sillon	270° continuous (Fig. 7B)	200–320° sillon angle=angle subtended by flagella (criterion 9)
4.	Crenulations		absent
5.	OCOM/OM*	0–0.5	coat absent or likely to be matrix derived; matrix attached
6.	OCIM/OM	0	absent or in fuzzy patches
7.	OCIM/IM	0–3	OC more conspicuous at cell extremities
8.	Diameter PC/cell diameter	0.63	invariant for population
9.	Angle PC subtended by flagella	270°	from 1/4 to 1/3 diameter of protoplasmic cylinder
10.	Presence of flagellar bundle	+	line C–D (Fig. 1E in Bermudes et al., 1988)* angle from 85–130°
11.	Length	50–84 μm	30–150 μm
12.	Amplitude	5–6 μm	4–12 μm
13.	Wavelength	5–10 μm	5–15 μm
14.	Cytoplasmic tubules	–	absent; ("ovoid inclusions" or rosettes conspicuous)
15.	Polar organelle	+	See Fig. 6A
16.	Rosettes**	+	Composite structure comprised of between 2–8 units (Fig. 5B–G)

* See Bermudes et al., 1988 for details of criteria
  Abbreviations: OCOM = outer coat of outer membrane; OM = outer membrane; OCIM = outer coat of inner membrane; IM = inner membrane; PC = protoplasmic cylinder.
** Rosettes are comprised of spherical units (each about 12–14 nm in diameter) that make up the structure (from 50–100 nm). Rosettes are found at the periphery of *Cristispira* at intervals ranging between 0.25 and 0.40 μm.
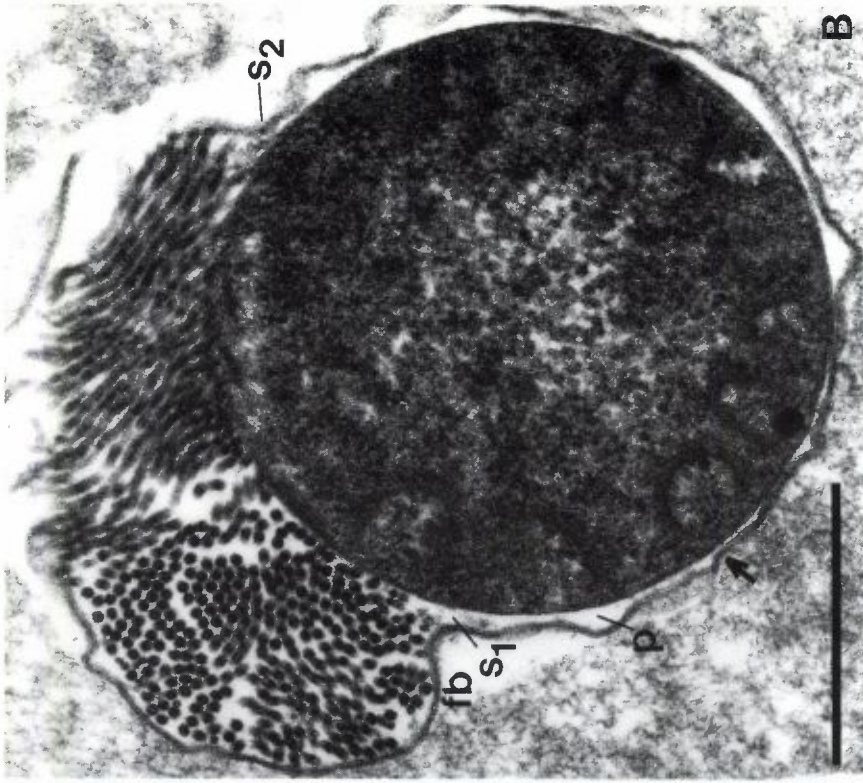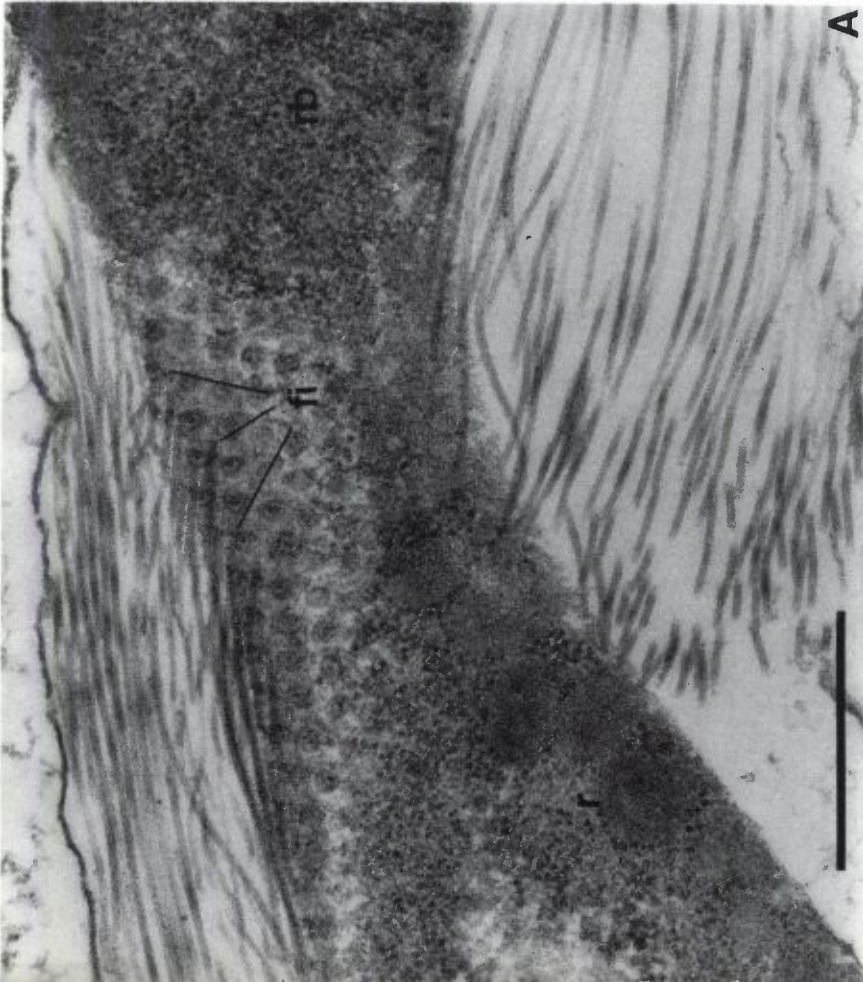
Figure 7A. Regularly spaces rows of flagellar insertions (fi), more centrally-located ribosomes (rb), and rosettes (r) on the opposite periphery can be seen at high magnification (bar = 0.5 μm).

Figure 7B. Numbers of flagella, angle of protoplasmic cylinder subtended by flagella and presence of flagellar bundle (fb) (criteria 2, 9, 10) are estimated from transverse sections. Angle of sillon measurement between S_1 and S_2 (bar = 0.5 μm), p = periplasm, matrix-outer membrane connected at arrow.
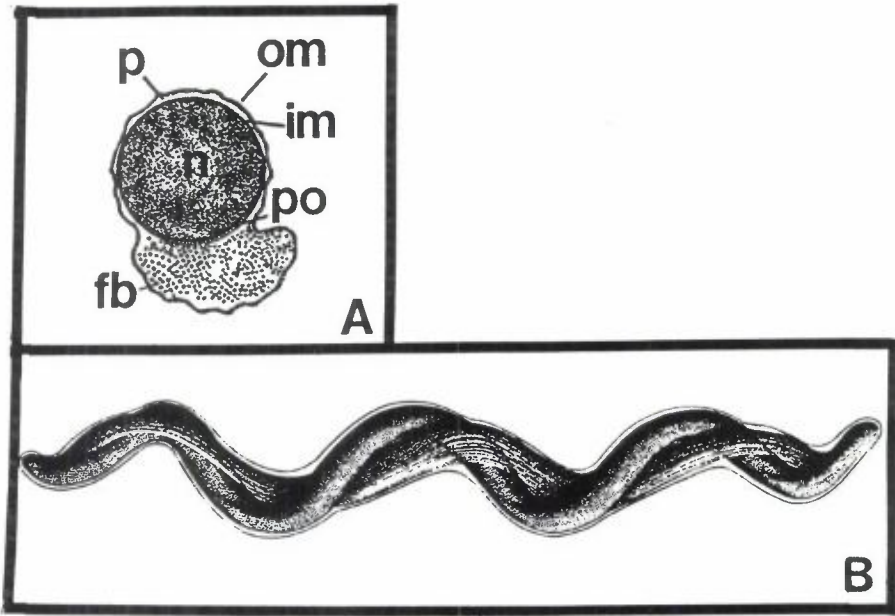
Figure 8. *Cristispira* based on electron micrographs (drawing by Christie Lyons).
    A. Transverse section: fb = flagellar bundle, po = polar organelle, om = outer membrane, im = inner membrane, p = periplasm, n = nucleoid
    B. External view showing how flagella form "crest"; 3 wavelengths shown.

4. Discussion

Light microscopic observations of live and stained preparations led Noguchi (1921) to accurately assess the place of *Cristispira* in the living world. The crest, a structure that develops during disintegration of *Cristispira*, is, as Noguchi suspected, a state of organization of the moving bundle of typical–but periplasmically located–bacterial flagella.

The function of the unique rosettes is not known. As *Cristispira* moves, the relative positions of the spherical components change, indicating either a passive or active role in motility. Cytochrome oxidase, along with ATPase, has been localized to the polar organelle of the flagellated stage of *Sphaerotilus natans* (Tauschel, 1985). If cytochrome oxidase is located at the polar organelle in *Cristispira* the metabolism of this spirochete might be aerobic or microaerophilic. As yet, the reaction of *Cristispira* or *S. ostrea* to gaseous oxygen is unknown. The proximity of the flagella insertions to the polar organelle suggests this structure also has a role in motility generation in *Cristispira*.

In spite of nearly annual efforts of members of the Marine Biological Laboratory microbiology course (Woods Hole, MA) as well as many attempts by numerous microbiologists, notably Noguchi (1921), to culture style microorganisms under varying conditions of nutrients, style fluid, viscous media, and anaerobiosis, no success of any kind has ever been reported. We were able to maintain, but not culture, *Cristispira* for several days on human serum. The association of *Cristispira pectinis* and *Spirillum ostrea* with each other and with algal debris suggests that they form a consortium in which the bacteria depend upon the syntrophy of algal constituents. Methanogenic bacterial consortia, ubiquitous in the sea, are often enriched using the osmoprotectant glycine betaine (GBT) and its methylated amine degradation products. Aerobic methylotrophs, anaerobic sulfate-reducing bacteria and methanogens comprise this type of consortia (Sieburth, 1988). The use of similar enrichment procedures with GBT and dimethyl sulfonioproprionate (DMSP), another algal osmolyte, might provide an innovative approach to the cultivation of the symbiotic bacteria of mollusk styles.

The style milieu provides the complete physical and nutritional environment for these bacteria. Since oysters shed their styles soon after removal from their habitat and even storage in sea water leads to disintegration of the style matrix, it is likely that *Cristispira* and *S. ostrea* require rapid reestablishment of the symbiosis in another mollusk host in order to grow and reproduce. The close proximity of the bacterial membranes to the style matrix material strongly suggests a trophic relationship of the bacteria with the products of digestion by the mollusks. Pelycepod-style cellulases presumably reduce algal and plant cell walls and other fibrous carbohydrate polymers to smaller particles that are further broken down in vacuoles of the mollusk's digestive cells. According to Store and Morton, 1958 (p. 136):

> The crystalline style which may be regarded also as providing a mechanism for the orderly dispersal of finely divided plant and detrital materials through stomach and intestine... contains cellulase.

The provision of glucose and other sugars via mollusk cellulase digestion is likely to be the source of carbon for both types of symbiotic bacteria. Fermentation of cellobiose, glucose, xylose, and other hydrolytic products of cellulase is the most common mode of nutrition for spirochetes. Attempts to assess cellulolytic activity in styles with and without spirochetes are underway (R. LaMontagne and M. LaMontagne, unpublished report).

In Woods Hole, MA, the edible oyster *Crassostrea* seems to host the most dense populations of *Cristispira*. Both Noguchi's and our own observations

found morphologically indistinguishable cristispires but with much less frequency in other bivalve mollusks such as *Venus myenaria* and *Modiolus modiolus.* *Modiolus* is known to contain carbohydrases including cellulase (Store and Morton, 1958). For unknown reasons the sperm and seminal plasma of *Modiolus* contains carbohydrases including cellulases (Store and Morton, 1958). Perhaps *Cristispira* or *S. ostrea* that move into this nutrient-containing tissue ensure their propagation to the next generation of mollusk host.

Because they never have been grown in culture, like the symbiotic spirochetes of termites and wood-eating cockroaches, morphometric analysis of these complex bacteria currently suffices to distinguish the genera (Bermudes et al., 1988). To the list of 15 characteristics to be sought in spirochete analysis, we add the presence of rosettes, structures so far detected only in *Cristispira pectinis* (Gross, 1910), the type species of the genus. Our description also reinstates *Spirillum ostrea* (Noguchi); apparently because of the lack of any work after Noguchi, this symbiotic spirillum was omitted in the 7th and 8th editions of Bergey's Manual (Sneath et al., 1986). Both *Cristispira pectinis* and *Spirillum ostrea* now deserve status as revived names in the bacteriological literature.

Acknowledgements

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