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Scanning Electron Microscopy of *Cristispira* Species in Chesapeake Bay Oysters

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Scanning electron microscopy was employed to observe the physical interactions between *Cristispira* spp. and the crystalline style of the Chesapeake Bay oyster (*Crassostrea virginica* Gmelin 1791). *Cristispira* organisms were found associated with both the inner and outer layers of the posterior two-thirds of the style. The spirochetes possessed blunt-tipped ends, a cell diameter range of 0.6 to 0.8 μm, and distended spirochetal envelopes which followed the contour of the cells. Transmission electron microscopy showed that the distension of the envelope was probably due to the containment of numerous axial filaments. In addition, they were found to possess two distinct spiral shapes which were dependent on whether their location was inside or on the surface of the style.

Cristispira is a genus of large spirochetes colonizing the crystalline style of a variety of mollusks. They have been characterized on the basis of their host species and the fact that they possess no known pathological involvement with these hosts. Morphologically, they are distinguished from other pathogenic and nonpathogenic spirochetes by the presence of a ridge or crestlike structure called the "crista." The crista can be visualized by bright-field (4), dark-field (13), and electron microscopy (15). Transmission electron microscopy shows the crista to consist of a closely packed bundle of numerous axial filaments bound between the outer sheath and the protoplasmic cylinder (14). Certes (4) was the first to document the presence of these spirochetes and observed them inhabiting the digestive tract of several species of oysters of the genus *Ostrea*. However, it was Gross (3) who, in 1910, proposed the genus *Cristispira*. Studies of *Cristispira* spp. have been hampered by the inability to achieve in vitro growth (2, 6, 12, 13). It is probable that the spirochetes found associated with the style are closely tied to the functional activity of the style. Therefore, it was felt that a study of the physical interactions between *Cristispira* spp. and the crystalline style of the oyster would provide additional information about this unusual ecological relationship. One technique useful in studying these interactions is scanning electron microscopy (SEM). This paper describes SEM observations of the physical interactions between *Cristispira* spp. and the crystalline style of the American oyster, *Crassostrea virginica* (Gmelin 1791).

MATERIALS AND METHODS

Collection of colonized styles. Crystalline styles were obtained from hand-dredged oysters collected from various oyster beds located in the upper Chesapeake Bay. The oysters were immediately opened when removed from the water, and the presence of a style was determined by making a lateral incision into the visceral mass initiating from the mantle cap, continuing along the anterodorsal border (border opposite the gills), and terminating dorsal to the cloacae. If a style was found, the anterior end was grasped with forceps and gently removed from the surrounding tissue.

The freshly excised style was placed on a clean glass microscope slide and examined for the presence of *Cristispira* organisms by bright-field microscopy (×100). Colonized styles were then prepared for SEM as described below.

SEM. Freshly excised and colonized styles were prefixed in 1% glutaraldehyde-filtered bay water (pH 6.0; salinity, 13.9 ppt) for 3 h at 4°C. Bay water used in this study was first adjusted to the desired salinity by the addition of sea salts (Marinemix, Hawaiian Marine Imports, Inc., Houston, Tex.) and then filtered through a 0.2-μm membrane filter (Millipore Corp., Bedford, Mass.). The styles were then gently washed with three changes of filtered bay water (pH 6.0, 13.9 ppt) and postfixed in 1% osmium tetroxide-filtered bay water (pH 6.0; 13.9 ppt) for 24 h at 25°C. The styles were once again washed with three changes of filtered bay water and then dehydrated with a graded series of ethanol (30 to 100%), critical-point dried with CO_2, and sputter-coated with gold or gold palladium. Specimens were viewed in a JEOL (JSM T-20) SEM or an AMR 1000 SEM. Micrographs were recorded with Polaroid film (type 55 P/N, 4 by 5 in. [ca. 10 by 12.5 cm]) or Kodak commercial 4127 film.

Transmission electron microscopy. Negatively

stained specimens of whole cells for electron microscopy were prepared as follows. A colonized style excised from an oyster by the method described above was placed in a 1-dram (ca. 3.9-g) screw-capped vial containing the filtered bay water (pH 6.0; 13.9 ppt). The vial was then placed in ice until it was returned to the laboratory (approximately 3 h, during which time the style dissolved). The cell suspension was then centrifuged at 3,000 × *g* for 10 min. The supernatant was discarded, and the pellet was resuspended in 0.1 ml of the filtered bay water. A drop of the cell suspension was placed on a 0.25% Formvar carbon-coated copper grid (300 mesh). Excess fluid was withdrawn from the grid surface with filter paper, and the grid was immediately stained with 1% (wt/vol) sodium phosphotungstate (pH 7.3) for 15 to 20 s. Excess stain was removed from the grid, and the grid was air dried before the specimen was examined in a Siemens IA transmission electron microscope operating at 80 kV.

RESULTS

Style morphology. The style possessed an overall club-shaped appearance, with the food tassel end (anterior) tapering towards the style sac end (posterior) (Fig. 1). The wrap-around appearance of the style (Fig. 1) is thought to be an artifact produced during specimen preparation, since other styles examined did not demonstrate this appearance. Close examination of the styles (Fig. 2) revealed a spongy inner layer covered by a much smoother outer layer.

Spirochete-style interaction. SEM revealed these organisms to possess blunt-tipped ends, a cell diameter range of 0.6 to 0.8 μm, and distended spirochetal envelopes which followed the contour of the cells (see Fig. 5). Negatively stained specimens suggested that the distension of the envelope was probably due to the containment of the numerous axial filaments found associated with the cell (Fig. 3 and 4).

Critsispira organisms were seen in all areas of the crystalline style except the area encompassing the food tassel end. They were seen associated with both the inner and outer layers of the style (Fig. 5, 6, and 7). Closer examination of the spirochetes within these two layers revealed some spirochetes to be tightly coiled, whereas others were loosely coiled. Those organisms observed just below the outer surface and, possibly, within the style inner matrix were tightly coiled (Fig. 7 and 8), whereas those emerging through and adhering to the style surface were loosely coiled (Fig. 7, and see Fig. 10). Spirochetes observed in the interior of the style occupied pore-like openings and were seen to protrude from these openings (Fig. 9). The inner portion of these openings or tunnels displayed a smoother appearance than that seen for the style inner matrix, thus suggesting these openings or tunnels resulted from the movement by the spirochetes within the inner layers of the style. Other marine microorganisms were observed along with *Cristispira* organisms, primarily at the posterior end of the style (Fig. 10).

DISCUSSION

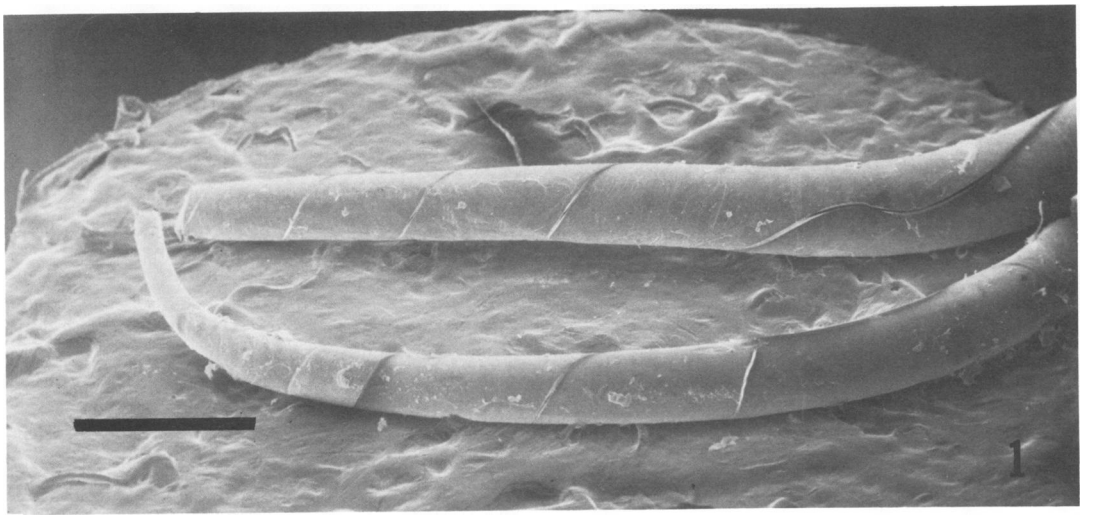
The observations reported here were of *C. virginica* (Gmelin 1791) with its host-associated spirochetes. These spirochetes, as determined from their characteristic appearance, fine structure, and unusual habitat, are believed to be members of the genus *Cristispira*, and the conclusions drawn here are likely to be pertinent to other *Cristispira* spp.-style interactions found in other species of mollusks which harbor these organisms. Furthermore, evidence presented in this study correlates nicely with the work of Ryter and Pillot (14) and with the description presented in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (10). Principally, the reports of early investigators centered around taxonomic, staining, and morphological properties (3–6, 12, 14). The conclusions which evolved from these early studies were that *Cristispira* organisms are spiral shaped and live in association with the intestinal tract of bivalve mollusks and that these organisms, because of their shape and possession of axial filaments, are spirochetes and should be classified as such.

The early literature described a structure which was referred to as the crista. Earlier investigators believed this structure to be an undulating membrane (4). However, the studies of Ryter and Pillot (14) and the observations reported here indicate that the crista is a distension of the outer layer of these organisms created by the numerous axial filaments (Fig. 3 and 4) found between the outer layer and the protoplasmic cylinder.

More recently, Ingham (E. R. Ingham, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, N10, p. 230) reported using SEM and observed *Cristispira* organisms adhering to the outer surface of the oyster style.

The findings presented here, however, showed not only surface-adhering spirochetes but also spirochetes within the inner matrix of the style (Fig. 5, 7, and 10). A possible reason for the differences observed may simply be the time required for the spirochetes to colonize the style. The longer the feeding oyster is left undisturbed, the greater likelihood of the style being formed, and therefore, a greater degree of colonization of the style may occur. However, the nature of this relationship is not well understood.

Judd (9) observed *Cristispira* organisms within the crystalline style of *Amphidemsa australe*. He used a carbohydrate-specific periodic



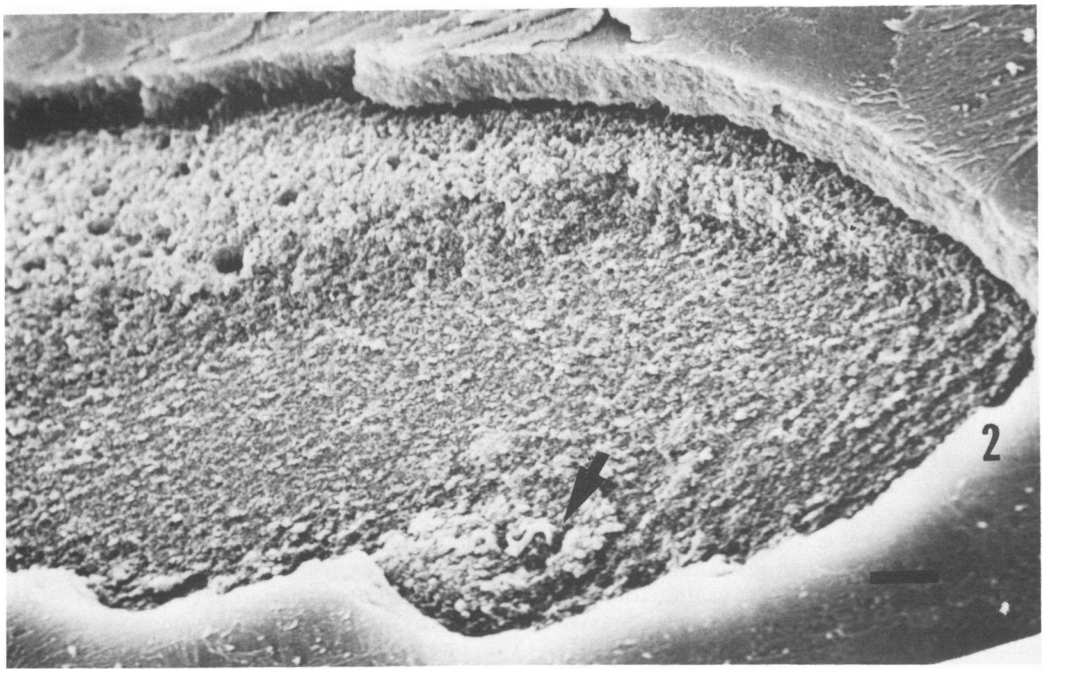
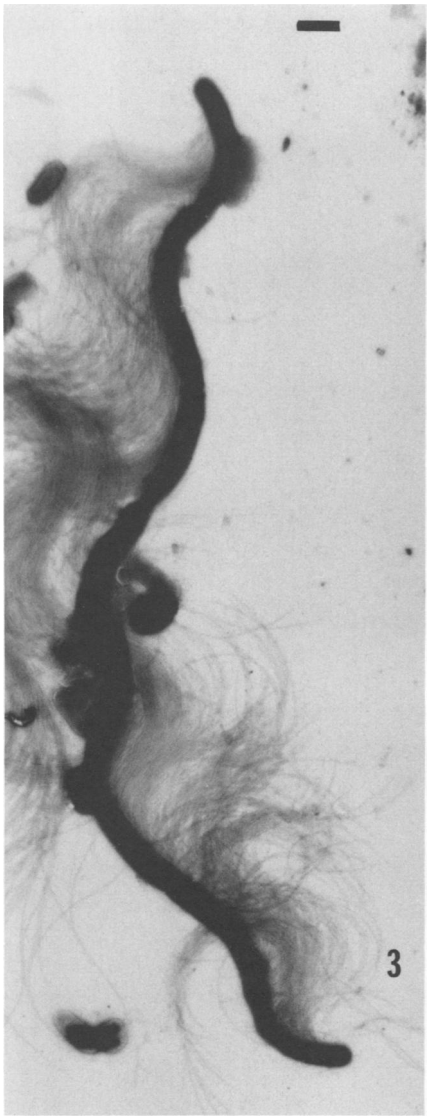


FIG. 1. *Scanning electron micrograph of the crystalline style of C. virginica which was cut into two pieces to facilitate mounting onto the specimen stub. Bar = 1.0 mm.*

FIG. 2. *Scanning electron micrograph of a crater-like area in a portion of the style illustrating its layered nature. Arrow points to spirochete observed in crater. Bar = 10 μm.*

acid-thiosemicarbazide-silver proteinate staining technique (PATSCSP). Using this stain, he concluded that the style was composed of carbohydrates and that the reactions he observed with the PATSCSP stain were indicative of glycoproteins. Judd believed that *Cristispira* organisms enzymatically removed the carbohydrate moiety from these glycoproteins. His interpretation was based on clear areas surrounding spirochetes in thin sections of styles. Although his interpretation may be correct, the zones of clearing may also be due to the inability of the spirochete envelope to take up the PATSCSP stain. Still another possibility is suggested by



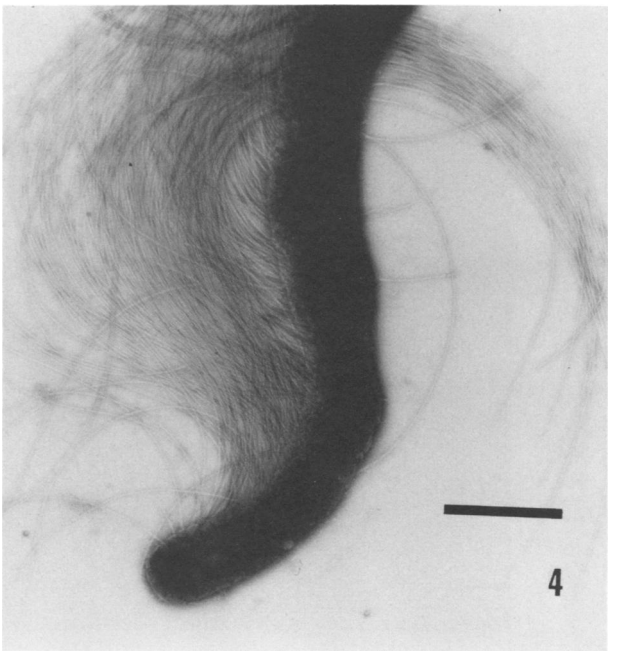
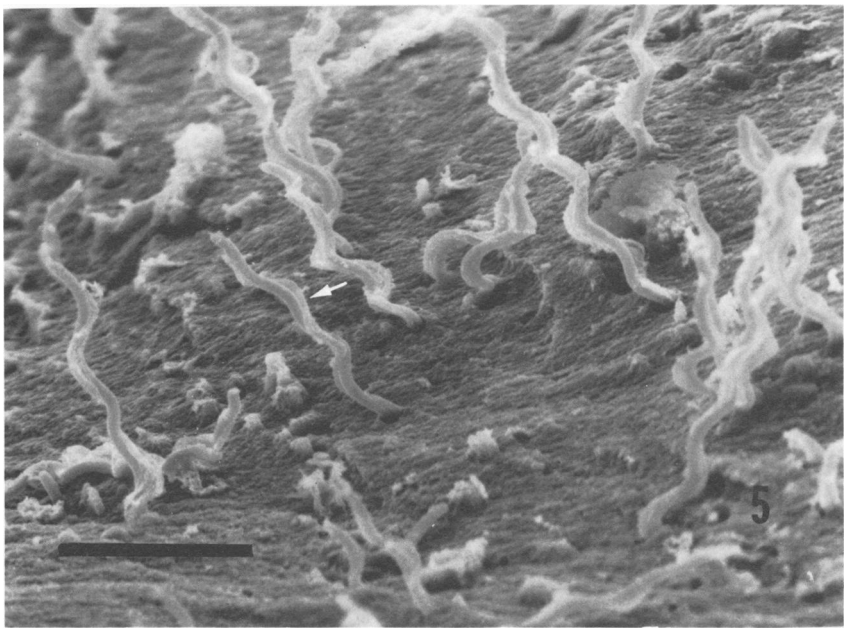


FIG. 3. *Electron micrograph of a negatively stained specimen. Note the numerous axial filaments. Bar = 1.0 μm.*

FIG. 4. *Electron micrograph of a higher magnification of one end of the spirochete seen in Fig. 3. Bar = 1.0 μm.*

close examination of Fig. 9. The clear areas reported by Judd could very well be the tunnels which resulted from the spirochetes' movement through the style.

The two spiral shapes of *Cristispira* organisms observed in different areas of the style lend support to the studies of Greenberg and Canale-Parola (7). They studied the effect of viscosity on the motility of several spirochetes. Their studies revealed a correlation between cell coiling and the ability of spirochetes to swim in viscous environments. The two different spiral shapes observed in the present study are probably examples of spirochetes moving in two different viscous environments. *Cristispira* organisms on the surface and emerging from the inner layers come in contact with a less viscous environment; thus, a tightly coiled morphology is not required for motility. Conversely, those organisms found within the style require a tightly coiled morphology to translocate in the more viscous environment. It is possible that the different cell types observed could represent two different populations of cells. However, the scope of this study does not permit us to determine this. Another possibility exists that the two coiling morphologies observed could be artifactual, resulting from SEM preparation procedures. This seems unlikely because the organisms were immediately fixed in 1% glutaralde-



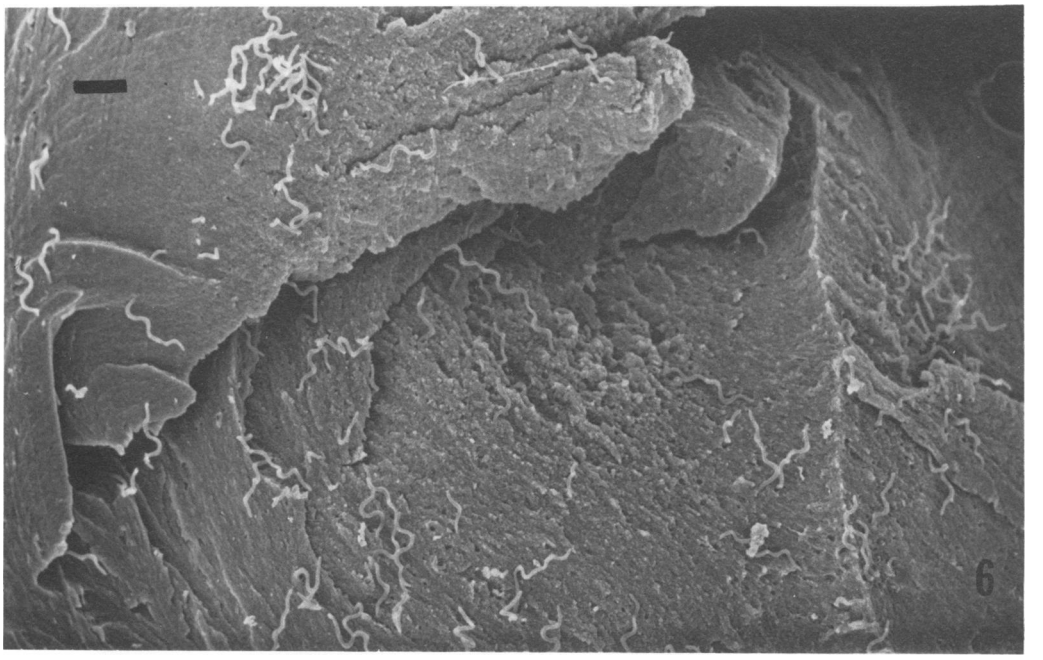
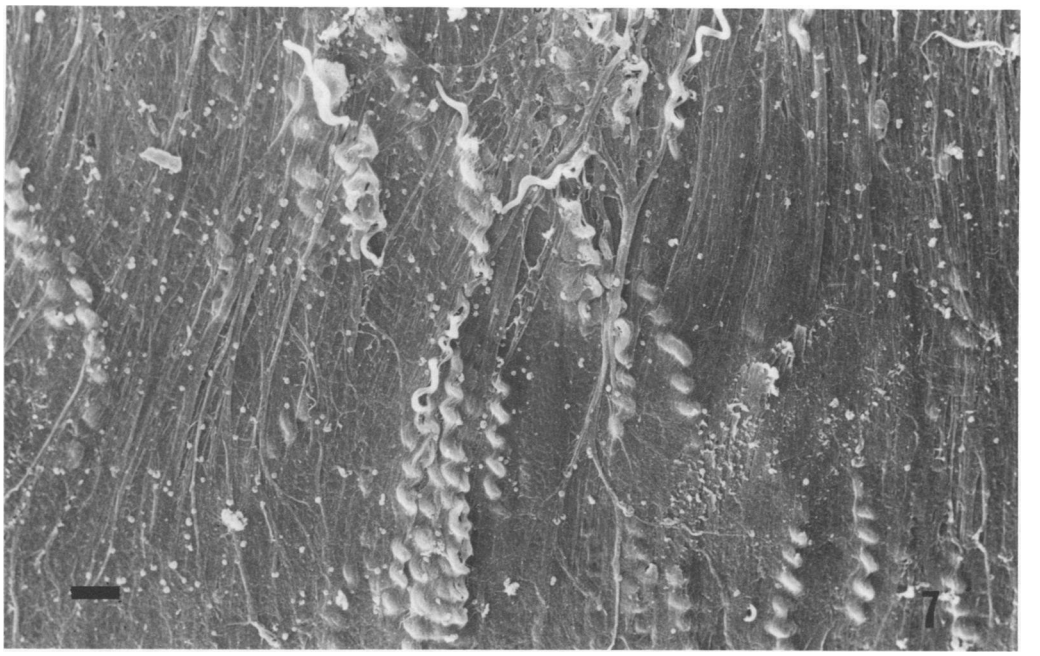


FIG. 5. *Scanning electron micrograph of spirochetes observed within the inner matrix of the style. Arrow points to crista. Bar = 10 μm.*

FIG. 6. *Scanning electron micrograph of spirochetes observed associated with a point where the style was cut. Note that the spirochetes are found throughout the inner matrix. Bar = 10 μm.*

hyde followed by osmium. This double-fixation procedure should have preserved the true morphology of the organism (8). It is possible that the style may have shrunk during dehydration



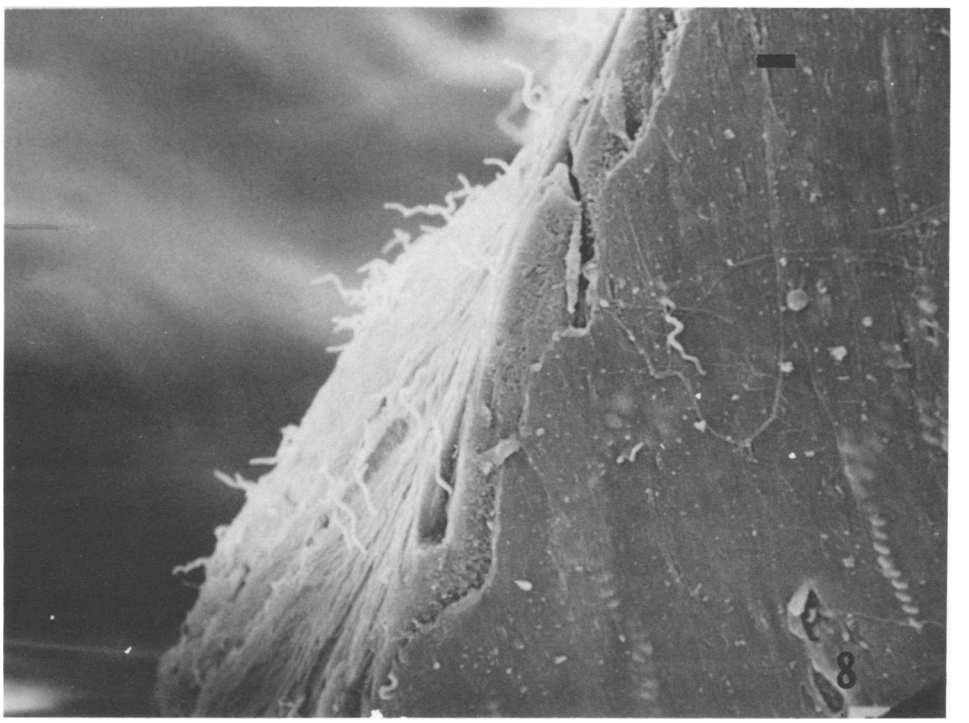
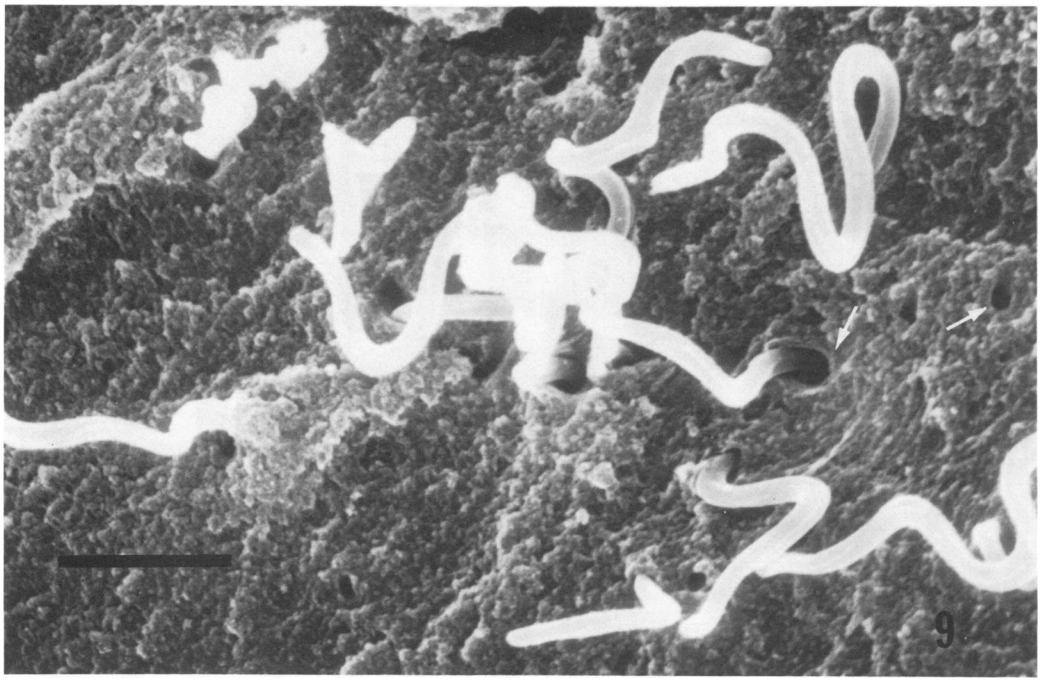


FIG. 7. *Scanning electron micrograph of the spirochetes observed associated with the outer layer of the style. Note the differences in coiling between the spirochetes on the surface and those below the surface of the style. Bar = 10 μm.*

FIG. 8. *Scanning electron micrograph of spirochetes associated with the style where it had been cut. Note the layering of the style and the way the spirochetes extend away from the inner surface of the style, demonstrating proper drying of the specimen. Bar = 10 μm.*



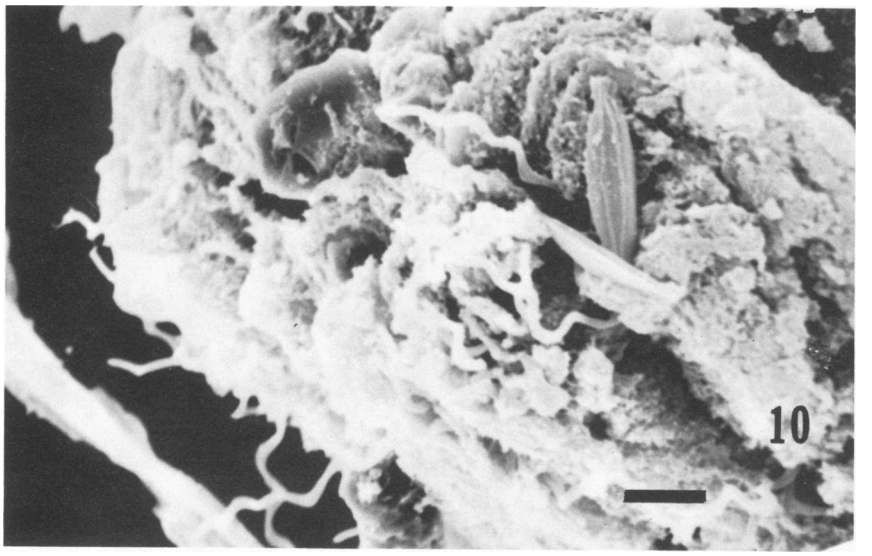


FIG. 9. *Scanning electron micrograph illustrating the pore-like openings occupied by the spirochetes (arrows). Bar = 5 μm.*

FIG. 10. *Scanning electron micrograph of the posterior end of the style. Note the loosely coiled shape of spirochetes adhering to the surface in addition to other marine microorganisms. Bar = 10 μm.*

and critical-point drying; however, the organisms should have maintained their prefixation morphology during this process.

The findings presented here generally corroborate those of other investigators. Most importantly, they confirm that *Cristispira* organisms adhere to the outside of the style besides being associated with the inner layers. Therefore, their movement within the style suggests they obtain energy from either the style itself or some part

of the style. However, the reason this organelle harbors *Cristispira* organisms remains unknown.

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