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Ultrastructure of Red-Sore Lesions on Largemouth Bass (*Micropterus salmoides*): Association of the Ciliate *Epistylis* sp. and the Bacterium *Aeromonas hydrophila**

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SYNOPSIS. Epizootic outbreaks of red-sore disease in several reservoirs in the southeastern United States have been reported to cause heavy mortality among several species of fish having sport and commercial value. The etiologic agent is said to be the peritrich ciliate *Epistylis* sp.; secondary infection by the gram-negative bacterium *Aeromonas hydrophila* produces hemorrhagic septicemia which results in death. However, in recent studies on the largemouth bass *Micropterus salmoides*, *Epistylis* sp. could be isolated from only 35% of 114 lesions from 114 fish, while *A. hydrophila* was found in 96% of the same lesions. Transmission and scanning electron microscopy of lesions associated with red-sore disease indicate that neither the stalk nor the attachment structure of *Epistylis* sp. have organelles capable of producing lytic enzymes. Since other investigators have shown that *A. hydrophila* produces strong lytic toxins, and in absence of evidence to the contrary, it is concluded that *Epistylis* sp. is a benign ectocommensal and that *A. hydrophila* is the primary etiologic agent of red-sore disease.

Index Key Words: Red-sore disease; *Micropterus salmoides*; *Epistylis* sp.; *Aeromonas hydrophila*; scanning electron microscopy; transmission electron microscopy.

IN the southeastern United States, the gram negative-bacterium *Aeromonas hydrophila* (Chester) appears to be intimately associated with the peritrich ciliate *Epistylis* sp. in causing 'red-sore' disease. Common among centrarchid and other game fishes, the disease may reach epizootic proportions, resulting in massive fish kills (9, 10), i.e. Dean (2) reported that more than 34,000 fish died of the disease in Badin Lake, North Carolina, in 1973.

The external pathologic changes include scale erosion and hemorrhea of the pit-like lesions. These lesions may cover 75% of the body surface of a largemouth bass and ultimately result in hemorrhagic septicemia and death. Esch et al. (3) showed a correlation between thermal loading and a striking seasonal periodicity in infected fish in Par Pond, a cooling reservoir for a nuclear production reactor near Aiken, South Carolina. They also demonstrated a significant relationship between the body condition of the fish and the probability of being infected with red-sore disease.

The primary invader in red-sore disease is said to be the stalked, colonial ciliate *Epistylis* sp. (10), with secondary involvement by the gram-negative bacterium *A. hydrophila*. Rogers (10) has suggested that the stalk of *Epistylis* sp. produces an enzyme that causes scale erosion, allowing secondary invasion of waterborne *A. hydrophila*. Other researchers (7) maintain that the stalk does not produce lytic enzymes; *Epistylis* sp. would thus be incapable of producing the lesion which is considered typical of the disease. Though *A. hydrophila* and *Epistylis* sp. appear to be intimately associated in the 'red-sore' phenomenon, the exact nature of their relationship to each other and to the host, *Micropterus salmoides* Lacépède, is unresolved. In this study, transmission and scanning electron microscopy have been used to examine the nature of these relationships.

MATERIALS AND METHODS

Fish were collected by a combination of electrofishing and angling at Par Pond, Aiken, South Carolina (3). Immediately

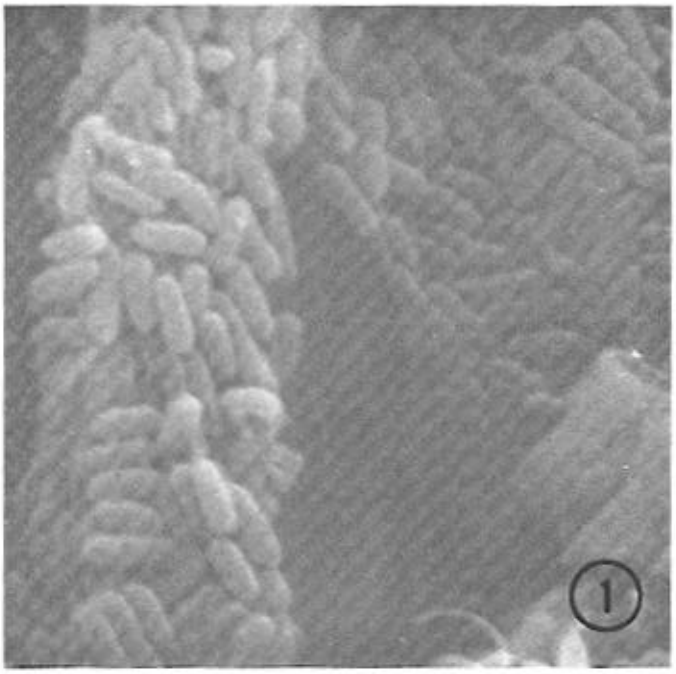

Fig. 1. Scanning electronmicrograph of stalk from largemouth bass lesion covered with rod-shaped bacteria. × 10,000.

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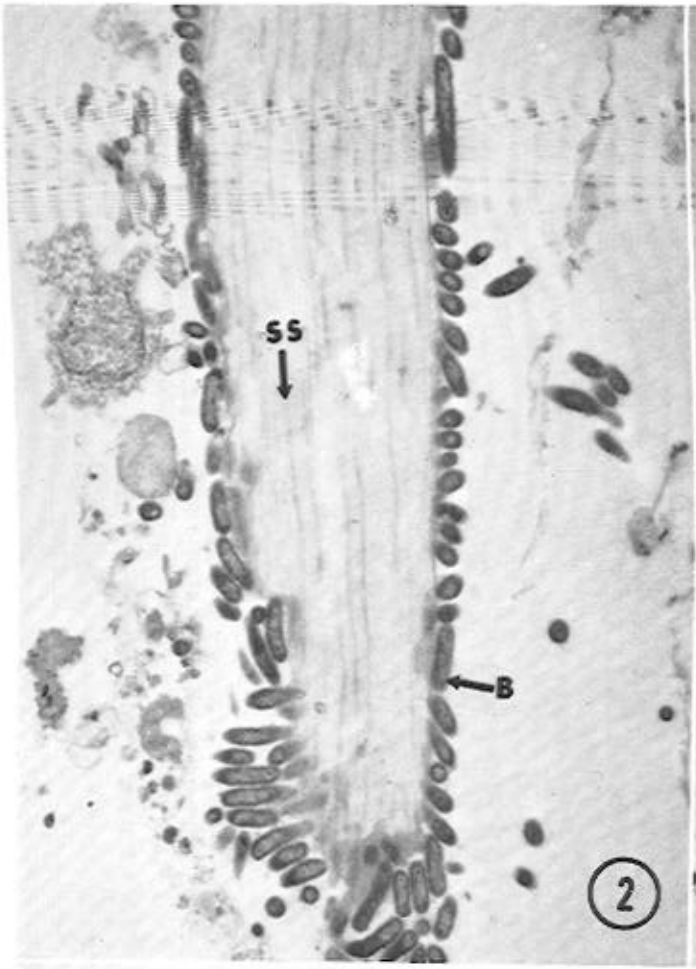
Figs. 2-4. [Transmission electronmicrographs of *Epistylis* sp. from a largemouth bass lesion. × 4,250.] 2, 3. Stalk in longitudinal (Fig. 2) and transverse (Fig. 3) section, showing bacteria (B) surrounding stalk, and striated stalk fibers (SS). 4. Section of zooid (Z) and stalk (S) showing bacteria (B) on stalk but not on zooid.

Fig. 5. Bacteria (B) within *Epistylis* sp. oral ciliature, cilia (C). × 9,200.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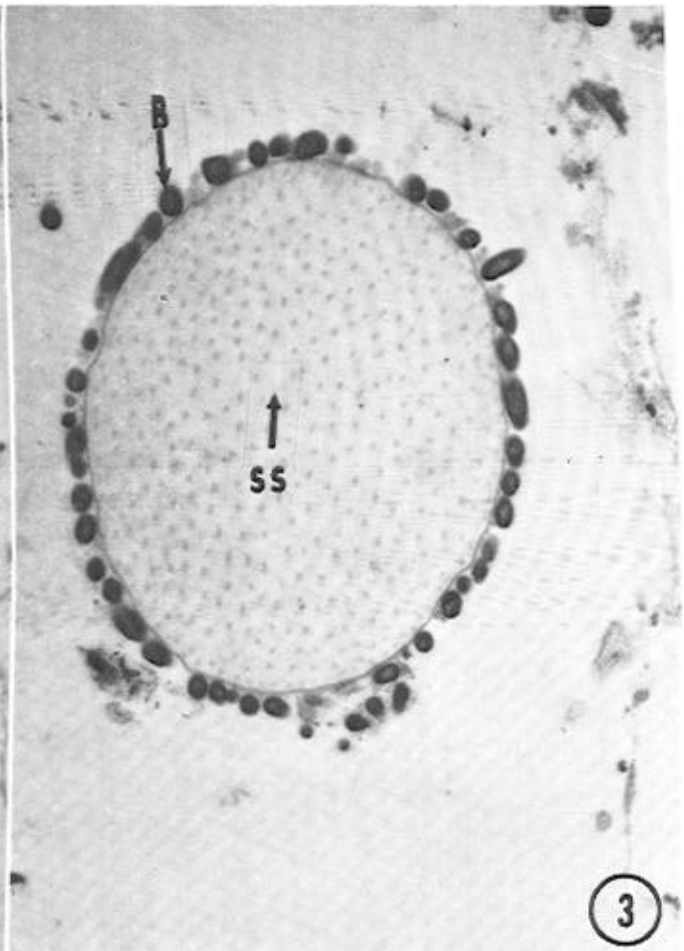
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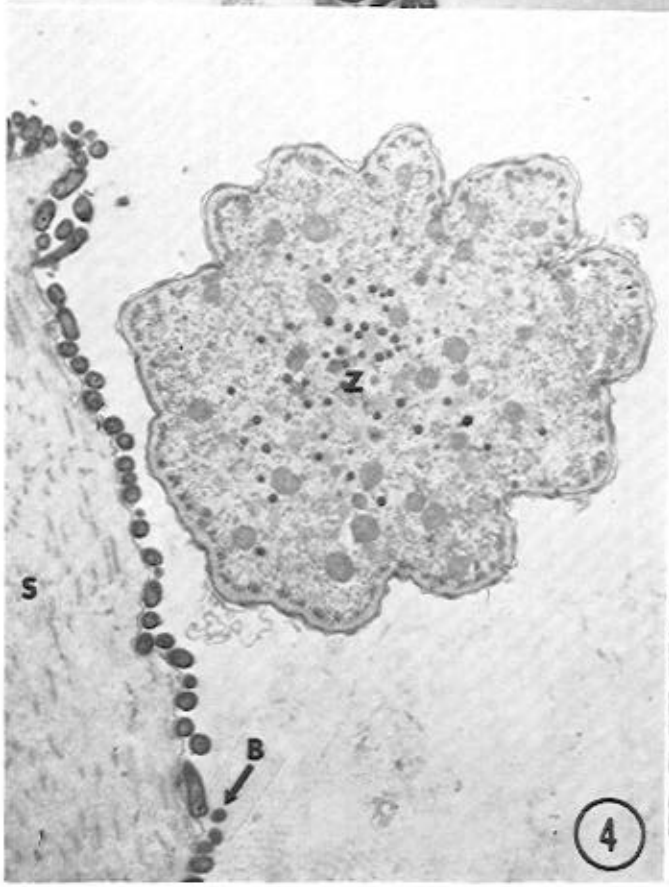
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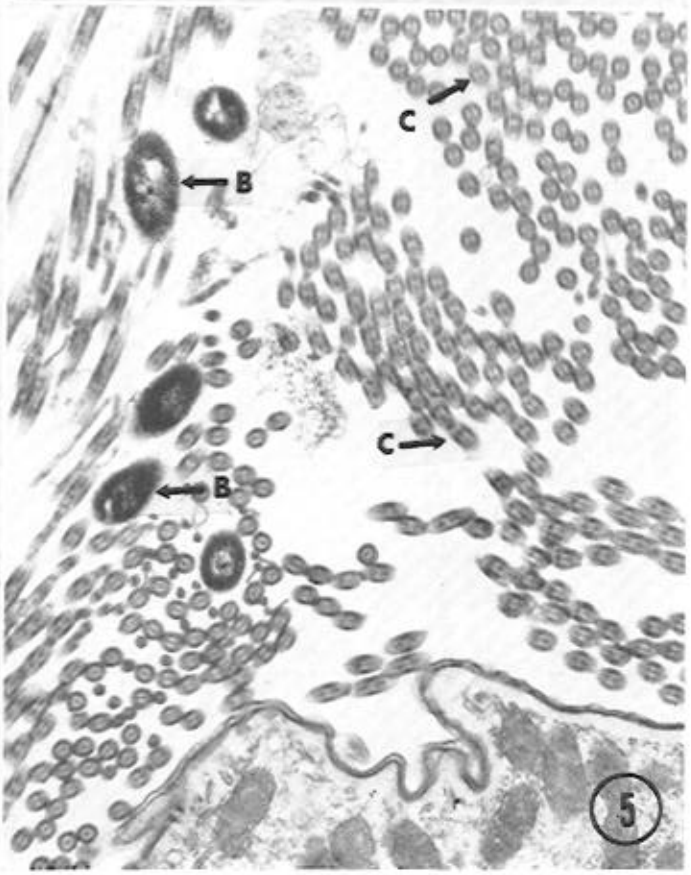
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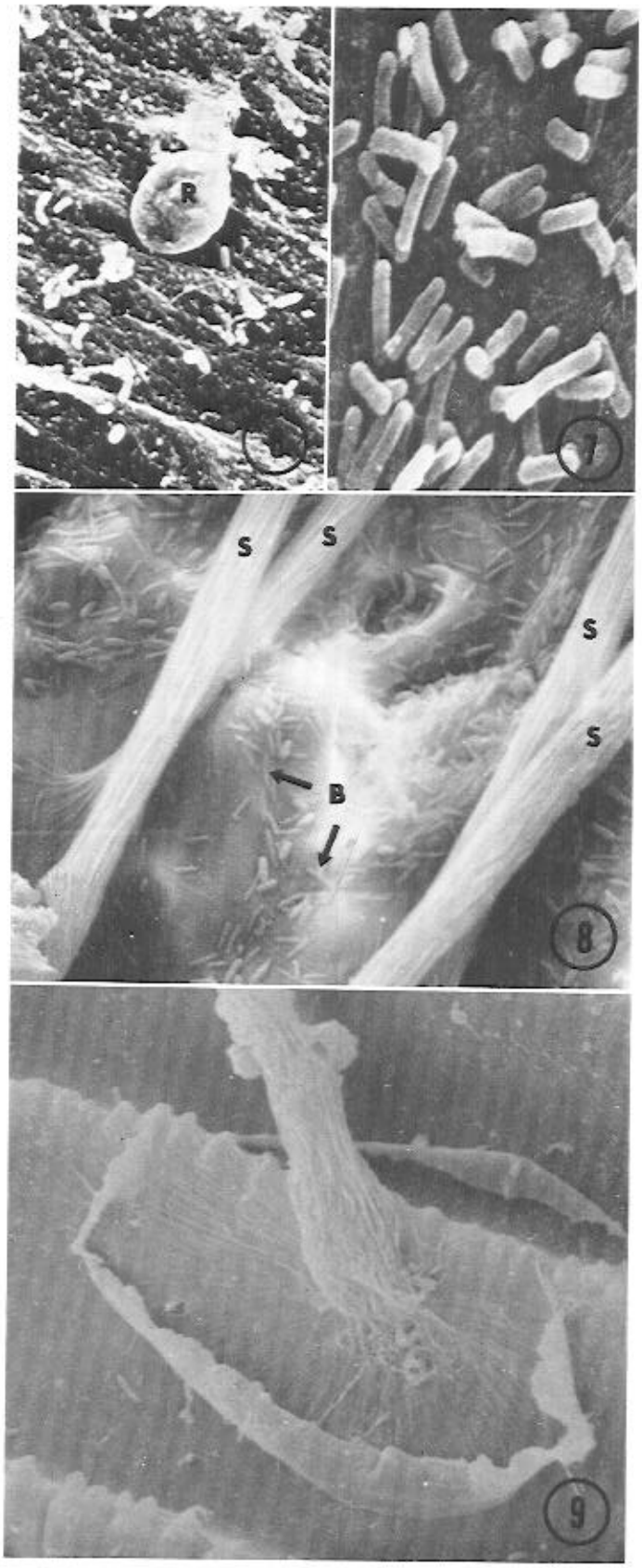


Figs. **6-9**. [Scanning electronmicrographs of largemouth bass surface lesions.] **6**. Largemouth bass epithelium within a lesion showing bacteria (B) and red blood cells (R). × 2,280. **7**. Higher magnification showing packing of rod-shaped bacteria. × 10,000. **8**. Surface of largemouth bass lesion near stalks (S) of *Epistylis* sp. showing accumulations of rod-shaped bacteria (B). × 1,000. **9**. Attachment point of *Epistylis* sp. stalk on scale. × 2,280.

upon capture, the fish were examined for lesions, weighed, and measured. If lesions were found, they were excised and fixed.

For scanning electron microscope studies, lesions were fixed in 4% (v/v) glutaraldehyde in 0.1 M Sorensen's phosphate buffer (8), pH 7.5, for 8 h at 4 C. The specimens were then washed in this buffer, dehydrated in an ethanol series, and critical-point dried using a Bomar SPC-900 (Tacoma, Wa.) critical point dryer with CO_2 as a solvent. All specimens were coated with 400 nm gold-palladium using a Technic Incorporated (Alexandria, Va.) Hummer II sputtering system and examined in a Super Mini-Sem (ISI) scanning electron microscope at accelerating voltages from 10 to 25kV.

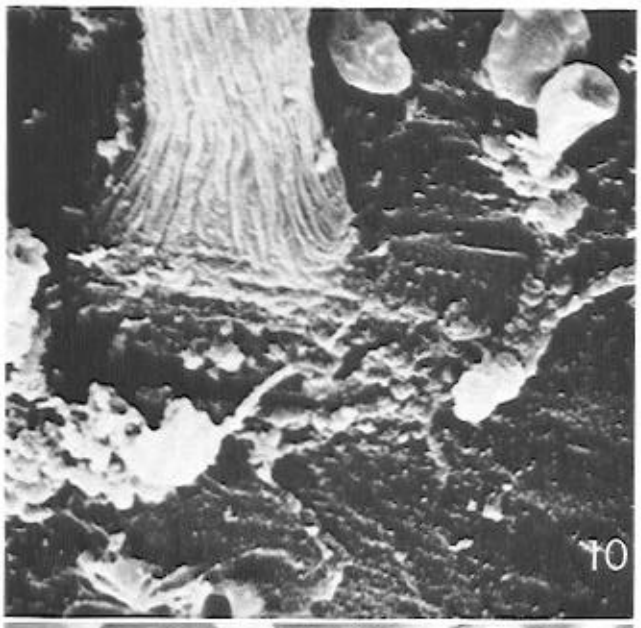
For transmission electron microscope studies, lesions were fixed in 4% (v/v) glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.5, for 2 h at 4 C. The specimens were then washed in this buffer and postfixed for 1 h at room temperature in 2% (w/v) OsO_4 in the same buffer. The specimens were then rinsed in 0.2 M Na cacodylate buffer, pH 7.5, and dehydrated in an ethanol series. Dehydrated specimens were infiltrated with propylene oxide and embedded in an Epon-Araldite plastic mixture. Embedded specimens were sectioned on a Porter Blum MT2-B ultramicrotome and double stained with 3% (w/v) uranyl acetate, followed by 0.2% (w/v) lead citrate (13). Stained sections were examined in a Zeiss EM9S-2 transmission electron microscope with an accelerating voltage of 60 kV.

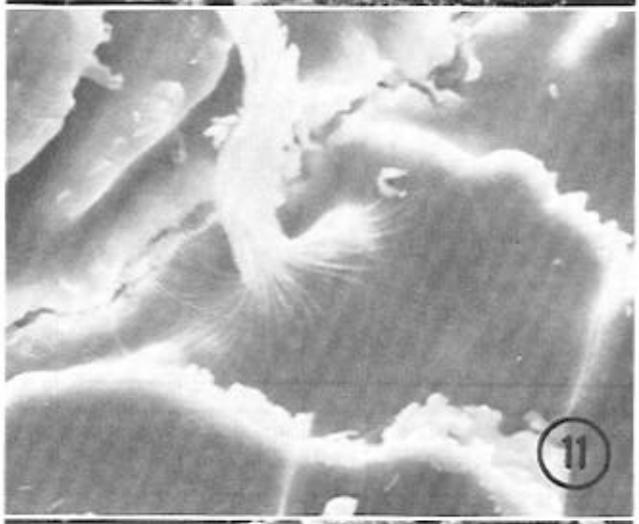
One hundred fourteen external body lesions on 114 largemouth bass were examined for the presence of *Epistylis* sp. and *A. hydrophila*. Scrapings of lesions were fixed in phosphate-buffered formalin and stained with Semichon's acetic-carmine (5) before being examined for *Epistylis* sp. These lesions also were swabbed with sterile loops which were then streaked on Rimler-Shotts (R-S) medium, known to be 94% efficient for growing *A. hydrophila* (11). The R-S medium plates were incubated at 35 C for 20 h and then examined for yellow colonies, characteristic for *A. hydrophila*. Colonies were selected and subjected to further verification using API 20 strips (Analytab, Inc.). Isolates also were tested for antibiotic sensitivity patterns, oxidase production, and gram stain reaction.

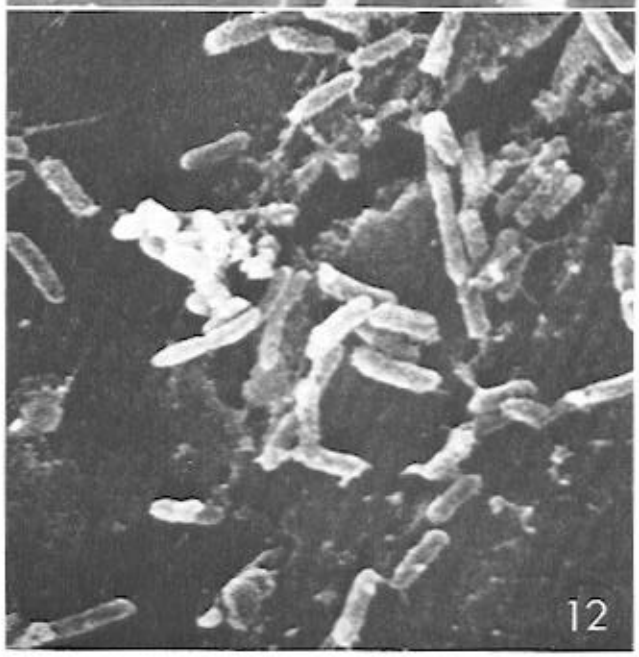
Specific anti-*A. hydrophila* antiserum was prepared by intravenous injection into rabbits of heat-killed, washed suspensions of *A. hydrophila* cells. Clonal cultures of *A. hydrophila* isolates from our lab and an isolate obtained from the American Type Culture Collection (ATCC #7966) were used as antigens. Rabbits were bled by cardiac puncture when the circulating antibody to the homologous antigen had an agglutinating titer greater than 1:2560. Antiserum was fractionated by ammonium sulfate precipitation and conjugated to fluorescein isothiocyanate. Impression slides of lesions were stained directly with fluorescent antibody. All FA technics followed the procedures of Fliermans et al. (4).

RESULTS

Cultures and stained scrapings of bass lesions from 114 fish revealed that 96% (110/114) contained cultivable *A. hydrophila*, while *Epistylis* sp. was observed in only 35% (40/114) of the same lesions. The quantities of *A. hydrophila* cultivated from the lesions also indicated that large numbers of viable bacteria were present. Impression slides of the lesions, stained with anti-







A. hydrophila conjugate were positive and further substantiated that this bacterium was present in large numbers and was not a spurious contaminant. Swabs from infected areas of both infected and uninfected fish yielded less than 1% positive R-S cultures and, when positive cultures were found, they indicated low densities of *A. hydrophila*.

Scanning electron-micrographs revealed large numbers of rod-shaped bacteria closely adhering to the stalk of *Epistylis* sp. (Fig. 1). Transmission electron-micrographs of longitudinal (Fig. 2) and transverse sections (Fig. 3) of the stalk revealed a close association of the bacteria and the ciliates. Furthermore, bacteria were never found on the zooid of *Epistylis* sp.; rather, they were present only on the stalk even when a stalk and a zooid were juxtaposed (Fig. 4). Bacteria were, however, found within the oral area (Fig. 5), which is not surprising since *Epistylis* sp. is a bacteriovore. Rod-shaped bacteria were also present in lesions that did not contain any *Epistylis* sp. (Fig. 6); however, the density of bacteria in these areas was reduced compared to that in lesions containing *Epistylis* sp. (Figs. 7, 8).

The attachment of *Epistylis* sp. to the surface of the fish did not appear to cause any pathologic changes. At no time was the stalk of *Epistylis* sp. observed to penetrate the epithelium covering the scales of bass. The attachment appeared as a spreading of stalk fibers over the substrate (Fig. 11); the striated stalk fibers (myonemes) can be seen in transverse and longitudinal sections (Figs. 2, 3). This type of attachment was observed on both soft (epithelium) and hard (scale) substrates (Figs. 9, 10). Close examination of the attachment revealed no penetration or pitting of fish tissue by *Epistylis* sp. (Figs. 9-11). The rod-shaped bacteria, however, were found to be associated with perforations in the tissue (Fig. 12). Sections of the stalk contained only stalk fibers and a few scattered mitochondria; no Golgi bodies or other organelles could be identified (Figs. 2, 3).

DISCUSSION

The basis for the intimate association between *A. hydrophila* and the stalk of *Epistylis* sp. is not readily explainable. The stalk has been reported to exude a mucilaginous material (7) which may act as either a nutrient source for the bacteria or may have "adherence" qualities lacking on other nearby surfaces. Both transverse and longitudinal sections of *Epistylis* sp. stalks, covered with bacteria, contain a fuzzy material which may represent the mucilaginous coat.

Aeromonas hydrophila was found associated with 96% of the red-sore lesions examined, while *Epistylis* sp. was observed in only 35% of these lesions. It was shown in other studies that high densities of *A. hydrophila* can induce lesions in bass held in laboratory aquaria (Hazen & Esch, unpublished observations), while *Epistylis* sp. alone will not produce lesions (7). *Aeromonas hydrophila* is known to produce a number of lytic enzymes (1, 6, 12), while *Epistylis* sp. is said to be incapable of producing such enzymes (7). Sections of the stalk did not reveal any organelles capable of producing enzymes, and the scarcity of mitochondria further suggested an inactive role for the stalk. It is reasonable to assume that if *Epistylis* sp. could induce lesions, the point of attachment of the protozoon to the

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Fig. 10. Attachment point of *Epistylis* sp. stalk on epithelium. × 2,280.
Fig. 11. Attachment of *Epistylis* sp. to a largemouth bass scale. × 1,000.
Fig. 12. Bacteria associated with perforation of largemouth bass epithelium. × 6,000.

fish surface would be the site of pathologic changes. Our evidence and that of Lom (7), however, suggest a benign relationship.

Contrary to the conclusions of Rogers (10), our results indicate that the etiologic agent of red-sore disease is not *Epistylis* sp. but the gram-negative bacterium, *A. hydrophila*. Thus, as the primary invader, *A. hydrophila* appears to induce the lesion and *Epistylis* sp. seems to be a secondary, but benign, ectocommensal; or, alternatively, a teletroch of *Epistylis* sp. attaches to the surface of a fish, develops into a colony, and then is subsequently colonized by *A. hydrophila*. Once the density of *A. hydrophila* reaches a threshold level on *Epistylis* stalks, the bacteria produce lytic enzymes in sufficient quantity to cause a lesion.

The frequency of occurrence of *A. hydrophila* in lesions free of *Epistylis* sp. and our observations that *A. hydrophila* alone can induce lesions in fish held in aquaria indicate that *A. hydrophila* is most likely the primary cause of red-sore disease in largemouth bass.

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