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The Bacterial Biota on Crustose (Nonarticulated) Coralline Algae from Tasmanian Waters

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Abstract. The bacterial biota associated with the cuticle surface of healthy benthic samples of crustose nonarticulated coralline algae from the east coast of Tasmania (Australia) was examined by bacteriological cultivation and electron microscopy. In 32 samples studied, the viable count on Zobell's marine agar (supplemented with vitamins) was 3.3×10^6 bacteria g^{-1} wet wt. (range 2.9×10^4–2.7×10^7). Of 732 strains isolated from 16 out of 32 samples and identified to genus level, *Moraxella* was the predominant genus (66%). In contrast, *Moraxella* comprised only 11% of 217 strains isolated from benthic seawater samples collected at the same time as coralline algae. In 22 out of 32 algal samples examined by scanning electron microscopy, the total count was 1.6×10^7 bacteria g^{-1} wet wt. (range 5.1×10^6–3.8×10^7); the major morphotype was cocco-bacilli (80%). Several environmental factors did not significantly influence the viable count or generic distribution, or the total count or morphotypic distribution of bacteria on the cuticle. These factors included geographical site, season, storage of samples in aquarium conditions, and the presence or absence of abalone from shells that the coralline algae encrusted. The microbiota, consisting mostly of the nonmotile bacterial genus *Moraxella,* appeared to be highly adapted to its calcerous plant host.

Introduction

Crustose (nonarticulated) coralline algae (Corallinaceae, Rhodophyta) are a benthic subtidal marine alga. They grow very slowly as a red encrusting (calcerous) layer on shells, rocks, and other surfaces in areas of low light intensity. Very little is known about the coralline algae. A limited number of studies have been concerned with morphological, physiological, and reproductive aspects. The first monograph on coralline algae did not appear until very recently [10]. To our knowledge, nothing has been reported about the bacteriology of the group, apart from a single transmission electron micrograph presented in a morphological study [1]. The micrograph shows several bacteria on the thallus surface of an articulated genus (*Corallina*).

In this report we characterize, for the first time, the microbiota associated with the cuticule surface of crustose (nonarticulated) coralline algae. Algae that encrusted the external shell surface of adult abalone in Tasmanian waters were

selected for study. Genera present included *Mesophyllum* and *Lithophyllum*. Samples of algae were examined bacteriologically to determine the viable count, and representative isolates were identified to genus level. Parallel algal samples were investigated by scanning (SEM) and transmission (TEM) electron microscopy to determine the total microbial count and to describe the distribution of morphotypes on the surface. For electron microscopy (EM), specimens were fixed and prepared using methods that optimize the preservation of surface-associated microorganisms [6, 7]. We also examined the influence of various environmental factors on the size and composition of the microbiota on coralline algae. This included obtaining samples from different geographical sites and during different seasons, storing samples in aquarium conditions over several weeks, and examining algae on abalone shells that housed a living animal or were empty.

Methods

Collection of Samples of Coralline Algae

Benthic abalone (*Haliotis ruber*) shells encrusted with crustose (nonarticulated) Corallinaceae were collected by SCUBA diving from Bicheno, Safety Cove, and Dunalley. These sites are located on the eastern coast of Tasmania between latitudes 42°S and 43°S, on longitude 148°E. The shells were immersed in 20 l fresh seawater at ambient temperature and transported to the laboratory in Hobart within 4 hours. Certain shells from Dunalley were stored for up to 15 weeks in a running seawater aquarium at the Tasmanian Fisheries Development Authority's research laboratory at Taroona (Hobart).

The algal samples were collected between September 30, 1982 and January 4, 1984 at intervals of 1–2 months. In certain instances, samples of benthic seawater were also collected over this period. They were examined in the laboratory in the same manner as algae.

Bacteriological Cultivation

Approximately 0.5 g. algae was aseptically excised from the external encrusted surface of the abalone shell with a sterile scalpel blade and vigorously washed three times in sterile seawater to remove debris. The segments were weighed and hand-homogenized in 4.5 ml seawater in a grinding tube (Corningware) to produce particles no greater than 1.5 μm in diameter, as determined by phase-contrast microscopy. Serial tenfold dilutions of the homogenate were inoculated (0.1 ml spread plate) onto duplicate plates of Zobell's 2216 marine agar (Difco) supplemented with thiamine HCl (0.5 $\mu g\ l^{-1}$), biotin (2.5 $ng\ l^{-1}$) and vitamin B12 (2.5 $ng\ l^{-1}$). (Trials indicated that the addition of these vitamins to marine agar greatly improved the subculture recovery rate, from 60% on marine agar to 80–95% on marine agar with vitamins, termed MAV medium.) The plates were incubated at 22° ± 2°C for 7–10 days and total viable counts were then determined. For samples requiring identification of the bacterial biota, approximately 50 colonies were randomly selected from suitable plates and subcultured onto MAV. Pure cultures were stored on MAV slopes at 10°C.

Bacterial Identification

Isolates were identified to genus level using the schemes described in references [9] and [15]. The taxonomic tests included colonial morphology, Gram's stain, catalase and oxidase reaction, sen-

sitivity to vibriostatic agent 0/129 and growth on TCBS medium (Oxoid), glucose utilization in marine oxidation-fermentation medium and glucose-peptone-seawater medium, and fluorescein production in King's B medium. *Alteromonas* was distinguished from *Pseudomonas* by DNase production [14]. For studies of motility and flagellar arrangement, mildly turbid suspensions prepared in seawater broth [2] were examined by phase contrast microscopy and then stained with 1% phosphotungstic acid pH 7.0 for 15 *s* and examined on parlodion-coated grids in a Hitachi H-300 transmission electron microscope at 87 kV.

Preparation of Specimens for Electron Microscopy (EM)

At the same time as algal specimens were prepared for bacteriology, parallel samples were processed for EM. Washed samples were placed on bronze mesh supports above 10% aqueous acrolein, and vapor-fixed for 1 hour, then immersed overnight in 3% formaldehyde-3% glutaraldehyde fixative containing 0.1 M cacodylate buffer, 0.05% ruthenium red and marine salts (1.6% NaCl, 0.6% $MgCl_2$, 0.1% $CaCl_2$), pH 7.4 [7]. Fixed tissues were washed in 0.1 M cacodylate, pH 7.4, buffers of decreasing salt concentration: buffer 1 contained the salts at fixative concentration; buffer 2 contained 1.0% NaCl, 0.4% $MgCl_2$, 0.07% $CaCl_2$; buffer 3 contained 0.5% NaCl, 0.2% $MgCl_2$, 0.03% $CaCl_2$. Samples were stored in buffer 3 at 4°C.

For SEM, fixed specimens were dehydrated in a graded ethanol-acetone series [7], critical point dried in CO_2 and sputter-coated with gold [6], and examined in a Philips 505 scanning electron microscope at 15 kV.

For TEM, fixed specimens were decalcified by immersion for 4 hours at room temperature in the aldehyde fixative described above to which 2.5% EDTA [1] had been added and from which the marine salts had been omitted. Samples were then stained with OsO_4 and uranyl acetate, dehydrated, and embedded in Spurr's low viscosity epoxy resin [7]. Ultrathin sections (LKB Ultratome 8800) were stained with 2% uranyl acetate in 50% aqueous ethanol for 20 min in the dark and then with Reynold's lead citrate for 10 min, and examined in a Philips 201 transmission electron microscope at 60 kV.

In situ Quantitation of Microorganisms on the Algal Surface by SEM

Areas on the algal surface were randomly selected at low magnification, then examined at a screen magnification of 7800×. The cuticle surface was focused and arbitrarily aligned normal to the viewing plane. At 7800× each field measured 250 μm^2 in area. All microorganisms within the field were counted, including cocco-bacilli (< 1 μm in diameter and < 1 μm long), rods and spiral-shaped bacteria (≤ 1 μm in diameter and 1–5 μm long), filamentous microorganisms (1–3 μm in diameter and 1–5 μm long, per cell), and diatoms. In fields of low microbial colonization, counts were made using the SEM's TV scanning mode. In densely colonized fields, the reduced raster viewing system was used in a 3 × 3 grid pattern; the total area of the 9 reduced raster fields corresponded to the area of one full field. For each algal sample, 36 full fields were enumerated.

The counts per unit surface area were calculated after adjustment for a 29% shrinkage factor (see below).

Weight and Surface Area of Algal Segments

Four fresh, excised algal segments were weighed. Their total surface area was measured by macrophotography. Analysis of the data (not shown) produced a correlation coefficient of 2.3 cm^2 surface area per g wet weight. The same segments were then fixed and prepared for SEM. After critical point drying, the surface area of the segments was again measured; the dried segments were found to have shrunk by 29%.

Table 1. Estimates (mean ± 1 SD unit) of the viable bacterial count and generic distribution of the bacterial biota on coralline alge

Sample source/ condition	Viable count		Generic distribution (%)[a]					
	No. of samples	Log_{10} nos. g^{-1} wet wt	No. of samples	M	F/C	P	V	A
Site								
Taroona	16	6.2 ± 1.5	2	72 ± 21	22 ± 24	4 ± 6	2 ± 2	—
Bicheno	7	6.8 ± 0.6	5	61 ± 26	13 ± 4	14 ± 7	11 ± 14	3 ± 3
Safety Cove	9	6.6 ± 0.9	9	69 ± 12	16 ± 16	13 ± 10	6 ± 7	2 ± 3
Season								
Spring	10	6.6 ± 0.5	4	67 ± 12	19 ± 8	19 ± 6	2 ± 4	1 ± 2
Summer	4	7.2 ± 0.1	4	68 ± 14	18 ± 20	10 ± 11	4 ± 5	3 ± 4
Autumn	11	6.3 ± 0.8	3	54 ± 36	20 ± 17	10 ± 12	13 ± 20	3 ± 5
Winter	7	6.6 ± 0.3	5	75 ± 7	8 ± 7	7 ± 4	7 ± 8	1 ± 1
Shell[b]								
+	19	6.7 ± 0.5	15	67 ± 18	14 ± 12	13 ± 9	7 ± 10	2 ± 2
−	13	6.2 ± 0.7	1	56	40	—	4	—
Storage								
Stored (1–15 wk)	19	6.4 ± 0.8	4	56 ± 30	19 ± 15	11 ± 10	11 ± 16	1 ± 1
Fresh	13	6.8 ± 0.4	12	70 ± 11	15 ± 14	12 ± 10	6 ± 6	2 ± 3
Mean of all samples	32	6.5 ± 0.6	16	66 ± 18	16 ± 14	13 ± 10	6 ± 9	2 ± 3

[a] M *Moraxella,* F/C *Flavobacterium/Cytophaga,* P *Pseudomonas,* V *Vibrio,* A *Alteromonas*
[b] Abalone shell; + with animal, − without animal (empty)

Statistical Analysis of Data

Analyses of variance, estimates of the mean and standard deviation, and Student's *t* test were computed as described elsewhere [6]. Regression analyses were computed as described in reference [3]. The accuracy of the in situ quantitative method was assessed as the ratio, standard error (mean): mean [5].

Results

Bacteriological Studies

Over a 16-month period, 32 samples of crustose (nonarticulated) coralline algae from Tasmanian waters were cultured to determine the number of plate-viable marine bacteria by which they were colonized. The mean estimate was 3.3×10^6 bacteria g^{-1} wet weight, range 2.9×10^4–2.7×10^7. The mean estimates (log_{10} values) gained from algal samples from different sites and seasons, from samples that were fresh or had been stored in aquarium conditions for up to 15 weeks, and from shells in which the host abalone was present or absent, are presented in Table 1. It can be seen that these environmental conditions did not greatly influence the size of the bacterial population on the algal surface.

Statistical analyses (variance ratio, Student's t tests) revealed no significant differences (0.05 level) either between or within the different parameter groups.

Seven hundred thirty-two strains of bacteria isolated from 16 out of 32 samples were identified to genus level. As shown in Table 1, 66% of the isolates belonged to the nonmotile genus *Moraxella*. Interestingly, the predominance of *Moraxella* colonization of coralline algae did not appear to be strongly influenced by the environmental factors of site, season, storage, and shell. Strains of the *Flavobacterium/Cytophaga* group, *Pseudomonas* group, *Vibrio* and *Alteromonas* were also associated with the algal surface but in much lower numbers than *Moraxella* (Table 1).

In some instances benthic seawater was sampled during collection of coralline algae. The plate-viable count in 12 samples had a mean estimate 2.3×10^3 bacteria ml^{-1} seawater, range 1.9×10^2–6.5×10^3. Two hundred seventeen representative isolates from 6 out of 12 seawater samples were identified to genus level and found to belong mostly to *Vibrio* (43%) and *Pseudomonas* (22%) groups. Only 11% of isolates were *Moraxella* spp. The significant difference between bacteria on coralline algae and those in the adjacent seawater was confirmed statistically (regression analysis).

EM Studies

The surface of coralline algae and the microbial biota associated with it was examined by EM. At low magnification, the topography was typically undulating with occasional conceptacles and protuberances (Fig. 1). The surface was free of epiphytic macroalgae and a single mollusc, probably a gastropod, was observed in only one sample. At high magnification, the cuticle was seen as a dense cover over the epithallium cells (Fig. 2). These cells secreted the finely granular cuticle as a layer up to 3 μm in thickness (Fig. 3).

The microbial biota on the cuticle was quantified in situ by SEM in 22 out of 32 samples of coralline algae. The mean estimate of the total microbial count was 1.6×10^7 g^{-1} wet weight (which represents 2.3 cm^{-2} surface area), range 5.1×10^6–3.8×10^7. The mean estimates ($\log_{10}$ values) of total counts on algal samples from different sites and seasons, from samples that were fresh or stored, and from shells in which the host abalone was present or absent, are presented in Table 2. It can be seen that these environmental factors did not greatly influence the total microbial count. No statistically significant differences (0.05 level) either between or within the factor groups were found by analysis of variance or Student's t test. It should be noted that the in situ counting technique used here was regarded as statistically accurate because the ratio, standard error of the mean : mean, was always <0.10.

The microbiota on the cuticle was composed of various morphotypes. As shown in Table 2, the frequency of the morphotypes was determined in 22 out of 32 samples of coralline algae. Cocco-bacilli predominated (Fig. 4, 6), whereas rods and spiral-shaped bacteria comprised 19% of the microbiota (Fig. 2, 5), filamentous organisms represented 3% (Fig. 5), and diatoms were seen very rarely (Fig. 4). The environmental factors (site, season, shell, storage) did not appear to greatly influence the morphotypic distribution (Table 2).

The different morphotypes were seen mostly on the cuticle, infrequently in

Table 2. Estimates (mean ± 1 SD unit) of the total microbial count and morphotypic distribution of the microbial biota on coralline alge

Sample source/condition	No. of samples	Log_{10} nos. g^{-1} wet wt (or 2.3 cm^{-2})[a]	Morphotypic distribution (%)[b]			
			Cocco-bacilli	Rods and spiral-shaped bacteria	Filamentous micro-organisms	Diatoms
Site						
Taroona	7	7.0 ± 0.2	66 ± 12	30 ± 11	5 ± 2	<1
Bicheno	6	7.2 ± 0.1	81 ± 13	17 ± 11	2 ± 2	<1
Safety Cove	9	7.4 ± 0.1	88 ± 4	13 ± 4	1 ± 1	<1
Season						
Spring	4	7.2 ± 0.1	92 ± 3	8 ± 3	1 ± 1	<1
Summer	4	7.4 ± 0.2	87 ± 5	14 ± 5	1 ± 1	<1
Autumn	8	7.0 ± 0.2	65 ± 11	30 ± 16	5 ± 2	<1
Winter	6	7.3 ± 0.1	83 ± 8	16 ± 8	1 ± 1	<1
Shell[c]						
+	17	7.3 ± 0.2	84 ± 9	15 ± 8	2 ± 2	<1
−	5	7.0 ± 0.2	63 ± 15	25 ± 11	2 ± 3	<1
Storage						
Stored (1–15 wk)	10	7.3 ± 0.2	72 ± 14	34 ± 15	6 ± 3	<1
Fresh	12	7.1 ± 0.2	88 ± 5	12 ± 4	1 ± 1	<1
Mean of all samples	22	7.2 ± 0.2	80 ± 13	19 ± 11	3 ± 2	<1

[a] For each sample, the counts were obtained in 36 randomly selected fields at 7,800×; the area of the field was adjusted in terms of the 29% shrinkage factor (see Methods section)
[b] Dimensions of the different morphotypes are given in the Methods section
[c] See Table 1

the cuticle (Fig. 7), and never in the epithallium cells. All morphotypes were very firmly associated with the cuticle. The simple bacteria appeared to attach by means of polymer bridges (Fig. 8), whereas certain filamentous bacteria (probably cyanobacteria) attached by means of holdfastlike structures (Fig. 9).

It was notable that the density of cuticle cover illustrated in Fig. 2 was seen in nearly all fields in the 22 coralline algae samples examined by SEM.

Discussion

Microorganisms colonize numerous biological surfaces in marine environments, including those of soft and stony corals [4] and macroalgae and phytoplankton (reviewed in reference 11). Thus it is not surprising that this first microbiological study of crustose (nonarticulated) coralline algae demonstrates microbial colonization of the secreted surface (cuticle). The microbiota was primarily bacterial, however, three aspects of the data are striking. The first concerns the relatively constant composition of the microbiota. The ability of a particular bacterial genus to consistently populate the cuticle of alga samples

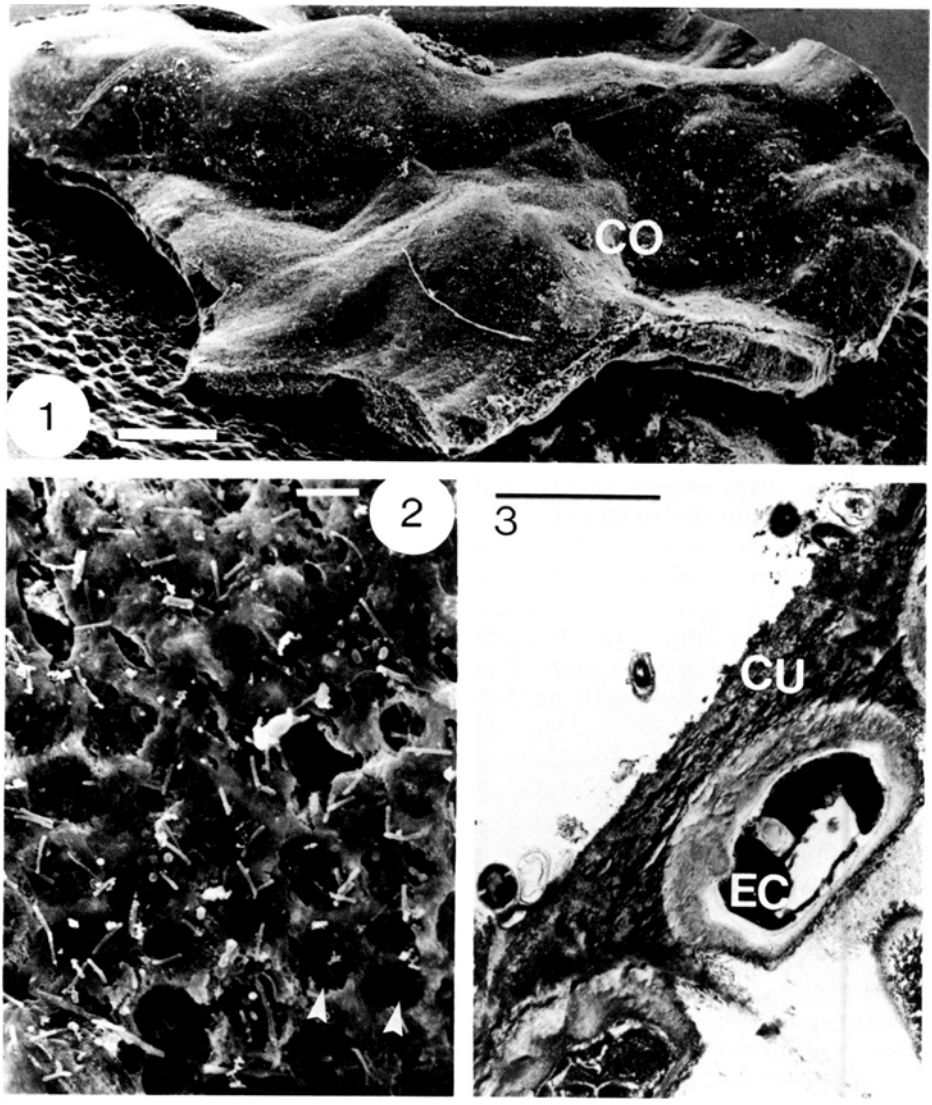

Fig. 1. The surface of crustose coralline algae is typically undulating, with occasional conceptacles (*CO*) (containing the gametes) and protuberances. The surface was free of epiphytic macroalgae. SEM × 40, bar = 0.5 mm.

Fig. 2. Typical cuticle cover on the surface of coralline algae; the epithallium cells (*arrowheads*) are outlined below. High numbers of cocco-bacilli and rods colonize the cuticle. SEM × 1,600, bar = 5 μm.

Fig. 3. Secretion of cuticle (*CU*), 0.5–3 μm thick, by epithallium cells (*EC*). Various bacteria, including dead cells (no cytoplasmic content), are attached to the cuticle. TEM × 8,500, bar = 2.5 μm.

obtained from different geographical sites and during different seasons indicates a stable association between specific microorganisms and the host. EM observations showed the microbiota to be firmly attached. In addition, the predominant bacterial genus tolerated a change in habitat, since encrusted shells taken

from the benthos and placed in an aquarium for up to 15 weeks remained colonized by the initial population. Furthermore, the association between the major bacterial genus and the coralline alga was not altered by removal of the living abalone within the encrusted shell. The capacity of the microbiota to tolerate changes in these environmental conditions indicates that it is highly adapted to its habitat.

The second striking aspect concerns the predominant bacterial genus *Moraxella*. Little is known about the general ecology of this nonmotile organism in marine environments [8, 16, 18]. Although it was the major organism on the cuticle, we found it as only a minor member of the population in the water column. This further supports the suggestion that *Moraxella* is specifically adapted to its habitat and is autochthonous to the secreted surface of crustose (nonarticulated) coralline algae.

The third interesting feature relates to the similarity between the total microbial count (1.6×10^7 g^{-1} wet weight) and the plate-viable bacterial count (3.3×10^6 g^{-1} wet weight). In several marine habitats, including seawater [12, 17], particulates in the water column and sediments [12], the total count can be 50–200× (and more) greater than the plate-viable count. Our finding of similar counts may reflect that a large proportion of the bacterial residents have nutritional needs provided for by the host, possibly in the form of photosynthate which may leak into the cuticle from the photosynthetically active epithallium cells below. The *Moraxella* population is not nutrient-deprived in situ. Accordingly, it can be recovered on Zobell's marine agar (supplemented with certain vitamins).

Laboratory cultures of *Moraxella* typically consist of cocco-bacilli [13]. It is possible then, that the major morphotype seen on the cuticle (viz., cocco-bacilli) correlates to the major genus isolated from it (viz., *Moraxella*). Clearly, further investigations are needed on the association between this poorly studied genus and its unusual calcerous host.

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Fig. 4. Cocco-bacilli (*arrowheads*) on the cuticle. A rarely seen diatom (*Skeletonema*) is also present. SEM × 2,600, bar = 2.5 μm.

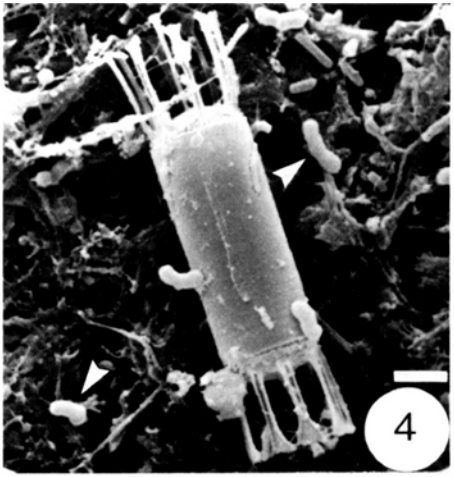
Fig. 5. Filamentous microorganisms (probably cyanobacteria) attached to the cuticle. Slender rod-shaped bacteria are also present (*arrowheads*). SEM × 2,600, bar = 2.5 μm.

Fig. 6. Typical cocco-bacilli in pairs and chains on the cuticle. SEM × 5,200, bar = 2.5 μm.

Fig. 7. Rarely seen bacteria within the cuticle (*CU*). TEM × 24,300, bar = 500 nm.

Fig. 8. Bacterium attached to the cuticle (*CU*) by means of a polymer bridge (*arrowheads*). TEM × 192,000, bar = 100 nm.

Fig. 9. A filamentous microorganism (possibly cyanobacteria) attached to the cuticle (*CU*) by means of a holdfastlike structure. TEM × 48,600, bar = 250 nm.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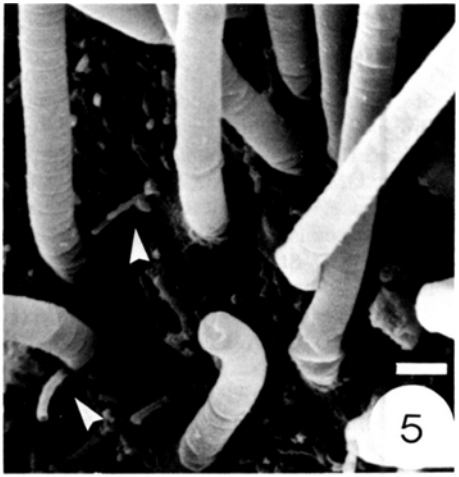
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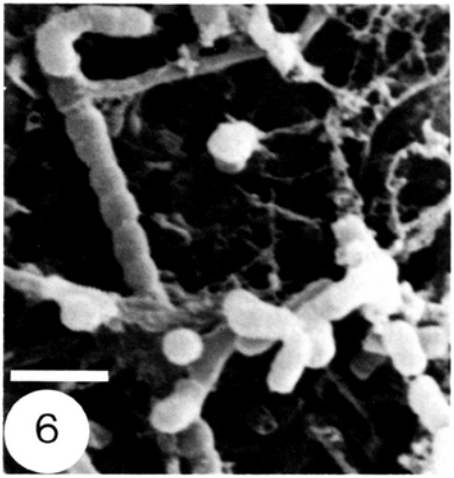
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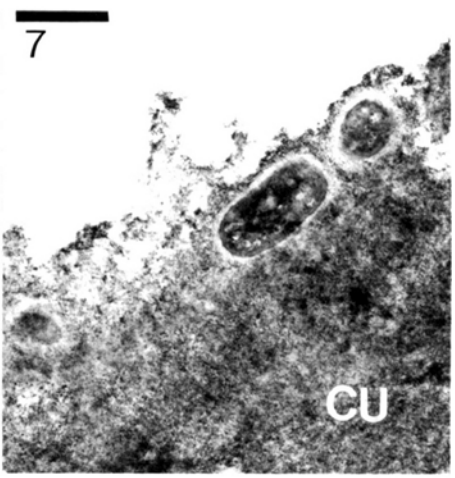
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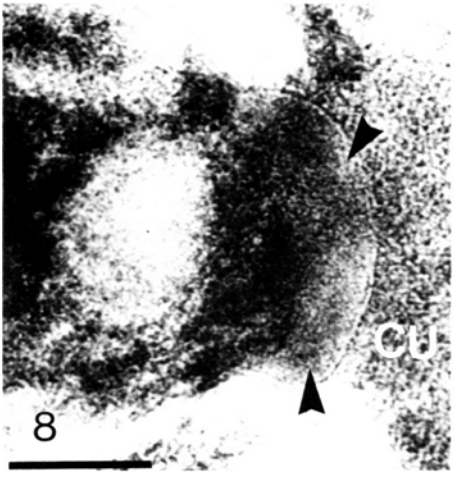
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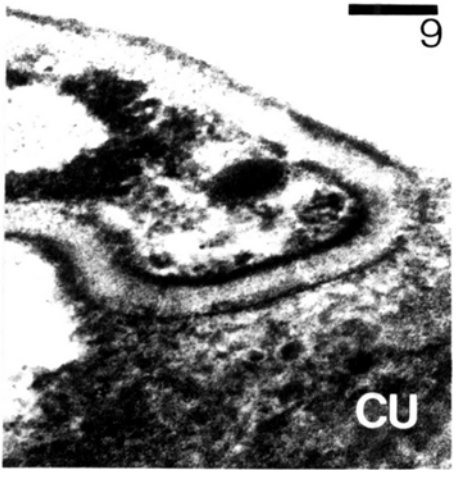
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CU

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