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Fine Structure and Composition of the Zoogloeal Matrix Surrounding *Zoogloea ramigera*

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The fingerlike projections, which have been considered to be characteristic of the genus *Zoogloea*, appear to consist of generally globular packets of cells, each surrounded by capsular matrix. The individual packets which surround *Z. ramigera* 115 cells appear to adhere one to another by intermeshed fibrils that measure 2 to 5 nm in diameter. The fibril polymer appears to be polyglucose that is susceptible to cellulase. The polymer resembles cellulose in several respects, with the exception that it is soluble in 1 N NaOH. Although *Z. ramigera* I-16-M does not possess an observable zoogloeal matrix when viewed under a light microscope, chemical data indicate that it does produce cellulase-susceptible polymer. Fibrils can be observed under the electron microscope, which are resistant to 1 N NaOH and may be cellulose. Less polymer fibrils are observed around isolate I-16-M cells than around isolate 115 cells; the inability to observe the I-16-M material as a zoogloeal matrix under light microscopy may be due to lack of sufficient amount of polymer.

Zoogloea ramigera is a gram-negative rod which appears to be a pseudomonad. It is characterized on the basis of its flocculent growth habit (3, 6, 8, 9, 21, 23) and a unique exocellular matrix which may be analogous to a capsule (8, 9, 21, 22). The isolate *Z. ramigera* 115 has been described as producing a zoogloeal or capsular matrix, whereas another isolate, *Z. ramigera* I-16-M, has been described as having no matrix. Comparisons between these two isolates regarding flocculation and biochemical characteristics have been reported, in addition to comparisons to several other floc-forming pseudomonads (9). The exocellular zoogloeal matrix has previously been examined for the purpose of elucidating its functional properties. The capacity of the exocellular polymer for concentrating metal ions from solution has been reported, and some of the ecological implications have been discussed (10).

This report describes the structure and composition of the zoogloeal matrix and considers it in relationship to metabolic activities of the organism, which has been reported to possess an extremely active oxidative metabolism in the natural habitat (3, 4, 23).

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MATERIALS AND METHODS

Cultures. Cells were grown in a modified version of Crabtree's (7) arginine salts medium which contained, per 100 ml: arginine·hydrochloride, 0.05 g; $MgSO_4 \cdot 7HOH$, 0.02 g; K_2HPO_4, 0.2 g; KH_2PO_4, 0.1 g; glucose, 0.5 g; B_{12}, 1.5×10^{-7} g. Cultures were shaken at 28 C for 72 hr.

Freeze-etching. The flocs were centrifuged into a pellet, and a portion of this was placed on a small copper disc (3 mm), previously scratched to insure greater adherence. The disc was immediately frozen in liquid Freon 22 and transferred to liquid nitrogen. The object was then placed on a precooled (−150 C) table in a Balzers apparatus (model BA 360 M; Balzers, Principality of Leichtenstein) and freeze-etched as described by Moor and Mühlethaler (15) and Moor (14). After freeze-etching, the discs containing the specimens were removed, warmed to room temperature, and dipped into distilled water to remove replicas from specimens. To remove cells which remained attached to replicas, treatments of 70% sulfuric acid (1 hr), distilled water rinse, Eau de Javell (a commercial bleaching agent containing 14% NaHOCl in NaOH), and final distilled water rinses were employed. After the replicas were picked up on Formvar-coated grids and allowed to dry, they were ready for examination in the electron microscope.

Negative staining. The cell pellets from centrifuged flocs were washed twice with sterile distilled water, and a portion was placed on a carbon-coated Formvar grid. This was stained with 0.5% phosphotungstic acid (*p*H 7.0) and was examined in an electron microscope (50 kv).

Thin-section preparation. Flocs were fixed in 1.0% OsO_4 in Veronal buffer overnight in a refrigerator and were stained in uranyl acetate for 2 hr. Dehydration of the flocs was performed with a linear gradient dehydration device (19) using ethyl alcohol. Each floc was embedded in Epon 812 monomer according to the method of Luft (13), and was polymerized by heat. Sections were cut on a Porter-Blum MT-1 Ultra Microtome (Ivan Sorvall, Inc., Norwalk, Conn.) with glass knives and were poststained with lead citrate.

Purification of exocellular polymer from culture 115 cells. The cell-floc matrix was separated from culture supernatant fluid by centrifugation at 10,000 × *g* for 20 min, and the supernatant fraction was discarded. The floc pellet was washed once with distilled water, centrifuged, and suspended in distilled water [1 g (wet weight) of cells/7 ml of water]. The suspension was exposed to ultrasound (Branson Sonifier, 20-kc output at 3.0 amp for 5 min).

The efficiency of matrix removal from cells was determined by examining the suspension periodically in a phase-contrast microscope using wet mounts containing cells suspended in 0.03% crystal violet. The ultrasound-treated suspension was centrifuged at 8,000 × *g* for 10 min to remove cells and debris. The viscous supernatant fraction was saved, added to five volumes of methyl alcohol, and stored at −20 C overnight. A white flocculent precipitate rose to the surface upon standing. It was removed with the aid of a watch glass and was air-dried to remove aqueous methyl alcohol. The precipitate was dissolved in distilled water at 60 C with agitation, and was precipitated in five volumes of methyl alcohol. The precipitate was recrystallized twice, using the above procedure, and was air-dried. The dry material was dissolved in 2 N NH_4OH (approximately 100 μg/5 ml), and was centrifuged at 40,000 × *g* for 3 hr to remove material insoluble in NH_4OH. The water-clear supernatant fraction containing dissolved polymer was added to an equal volume of 95% ethyl alcohol. As the polymer precipitated from the solution, it was removed by means of a glass rod and added directly to a carbon-coated grid for examination in the electron microscope.

Infrared spectroscopy of purified polymer. The purified polymer was also added directly to a NaCl crystal under constant exposure to a heat lamp to facilitate rapid solvent evaporation. The NaCl crystal which was coated with a thin film of the polymer, was examined with a Perkin Elmer Model 237B spectrophotometer.

Enzyme susceptibility of purified polymer. α-Amylase (*Bacillus subtilus*, type IIA, Sigma Chemical Co., St. Louis, Mo.) and β-amylase (barley, type IIB, Sigma Chemical Co.) were added separately to purified polymer, and hydrolytic activity was assayed according to the procedures described by Bernfeld (2).

A 1-ml amount of an α-amylase solution (0.03 mg/ml) was added to 1.0 ml of polymer solution [0.36 mg (per ml) of 0.02 M NaH_2PO_4 − 0.006 M NaCl at *p*H 6.9], and replicas were incubated at 20 C for time intervals up to 120 min.

A 1-ml amount of β-amylase (0.06 mg/ml) was added to 1.0 ml of polymer solution (0.36 mg of 0.016 M sodium acetate per ml, *p*H 4.8), and replicas were incubated at 20 C for time intervals up to 120 min.

A 1-ml amount of soluble starch (10 mg/ml) was added to both α- and β-amylase solutions instead of polymer to serve as a positive control.

The assays were repeated several times by using whole isolate 115 cell-flocs and I-16-M cell-flocs in place of purified polymer.

Cellulase (*Aspergillus niger*, type II, Sigma Chemical Co., 30 mg/15 ml of 0.05 M sodium citrate, *p*H 4.0) was added to whole isolate 115 cell-flocs or I-16-M cell-flocs and was incubated at 24 ± 1 C for periods up to 120 min. At desired time intervals, 1.0 ml of Nelson's alkaline copper reagent was added to each tube, and the solution was assayed colorimetrically for sugar as described by Nelson (17).

Carboxymethylcellulose (12.0 mg/ml) was added to buffered enzyme instead of cells, and it served as a positive control.

Bovine serum albumin for phase-contrast microscopy. A 42% solution of bovine serum albumin (Nutritional Biochemicals Corp., Cleveland, Ohio) was prepared and mixed with cell suspension (1:1) on a glass slide for the purpose of increasing the refractive index of the suspending fluid. The wet mount was examined by using phase-contrast microscopy (1). Cells were stained for light microscopy as previously reported (9).

Chromatography of polymer hydrolysate. Purified polymer was added to 2 N HCl (1 mg/ml), placed in a glass tube, and sealed. The tube was held at 100 C for 18 hr. The contents were removed and evaporated to dryness at 90 C under a stream of air. Water (2 ml) was added to the residue, and again was evaporated to dryness to remove HCl. The evaporation procedure was repeated three times. The residue was then suspended in 0.3 ml of distilled water, and the hydrolysate (0.02 ml) was spotted onto Whatman no. 4 paper for chromatography. An ascending two-dimensional technique was used [phenol-water (4:1, w/w) and isopropyl alcohol-water (4:1, v/v)]. Individual chromatograms were sprayed with the following reagents: aniline-diphenylamine (20), silver nitrate (20), benzidine (12), aniline-acid-oxalate (5), ninhydrin (20).

The hydrolysis procedure was repeated by using 2 N H_2SO_4 in place of HCl, and portions of the hydrolysate were chromatogramed in the same manner.

RESULTS

Microscopy. The zoogloeal matrix which has historically been described as a fingerlike projection (3, 8) can be observed in the photomicrographs of isolate 115 shown in Fig. 1, 2, and 3. Figure 4 is a phase-contrast photomicrograph of individual packets of isolate 115 cells. We have previously reported that the fingerlike projections appear to consist of individual packets of cells (9). The arrows in Fig. 3 point to interfaces between individual cell packets which have been stained by using dilute crystal violet.

Examination of the zoogloeal flocs in the native state using the freeze-etching technique and subsequent electron microscopy revealed the floc structure shown in Fig. 5. Matrix structure resembling or analogous to the individual packet structure shown in Fig. 4 can be observed; an interface (I) between two floc packets can also be observed. Cells embedded within the matrix are not readily observed in Fig. 5; however, Fig. 6 clearly shows the presence of a cell (C) embedded within the matrix. The matrix appears to consist of polymer which forms a network of fibrous strands (S) composed of two units of different diameter, one measuring 40 to 60 nm and the other 4 to 5 nm. Figure 7 illustrates exocellular strands adhering to cells of isolate 115.

The floc matrix was also examined after negative staining. This technique (Fig. 8) also reveals strands resembling those observed in frozen-etched specimens. The strands in this micrograph are 6 to 13 nm in diameter.

Figures 9 and 10 are thin sections of zoogloeal flocs which have been embedded and stained with ead citrate. The exocellular strands can be detected as branching fibrils with a diameter of 4 to 6 nm. Figure 10 shows void space (V) surrounding cells embedded in the matrix. This could result from motility within the floc, from lack of polymer synthesis at this location on the cell surface, or it may consist of substances undetectable by use of this technique. A characteristic gram-negative cell wall is present, and the cell appears to be packed with ribosomes.

Purified exocellular matrix material was also examined with the electron microscope (Fig. 11). Polymer strands resembling those shown in the native state (Fig. 6, 7, 8, 9, 10) can be observed, and they measure approximately 2 to 5 nm in diameter. An infrared absorption spectrum of the purified material shown in Fig. 11 is presented in Fig. 12. The following functional group assignments were made: OH, 2.95 μm; C—H, 3.38 to 3.44 μm; C=O of ionized carboxyl or aldehyde, 6.15 μm and 7.1 μm; weak C=O of aldehyde, 5.8 μm and 7.3 μm; tertiary CH—OH, 8.65 μm; band typical of cellulose or polysaccharide ring, 9.4 μm. The IR spectrum is identified as that of

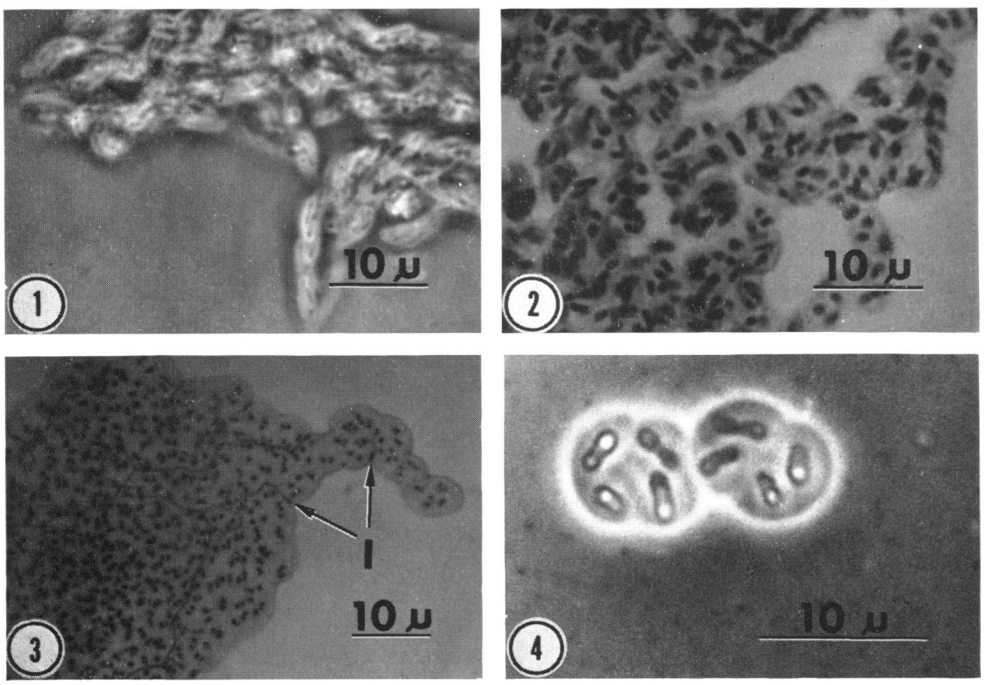
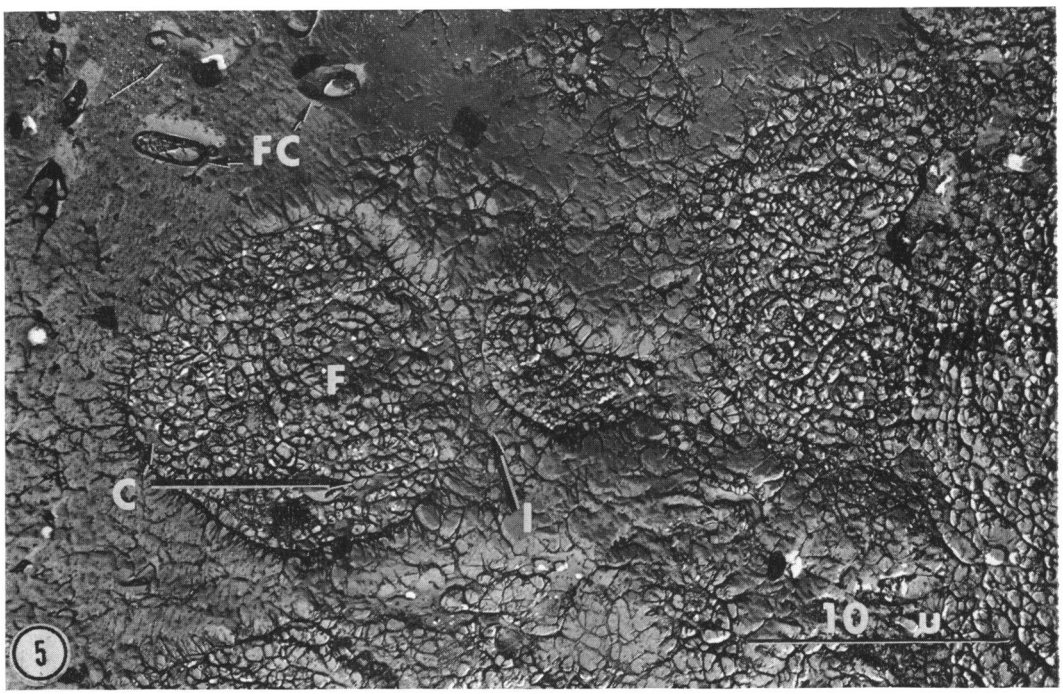


FIG. 1. *Z. ramigera 115 fingerlike projections composed of cells embedded within the zoogloeal matrix. Cells were suspended in 42% bovine serum albumin.*

FIG. 2. *Z. ramigera 115 flocs stained with 1.0% crystal violet.*

FIG. 3. *Z. ramigera 115 flocs stained with crystal violet, showing interfaces (I) between individual clumps of cells.*

FIG. 4. *Phase-contrast photomicrograph of Z. ramigera 115 cells stained by Maneval's method and showing individual packets of cells. Each packet is surrounded by capsular or zoogloeal matrix.*



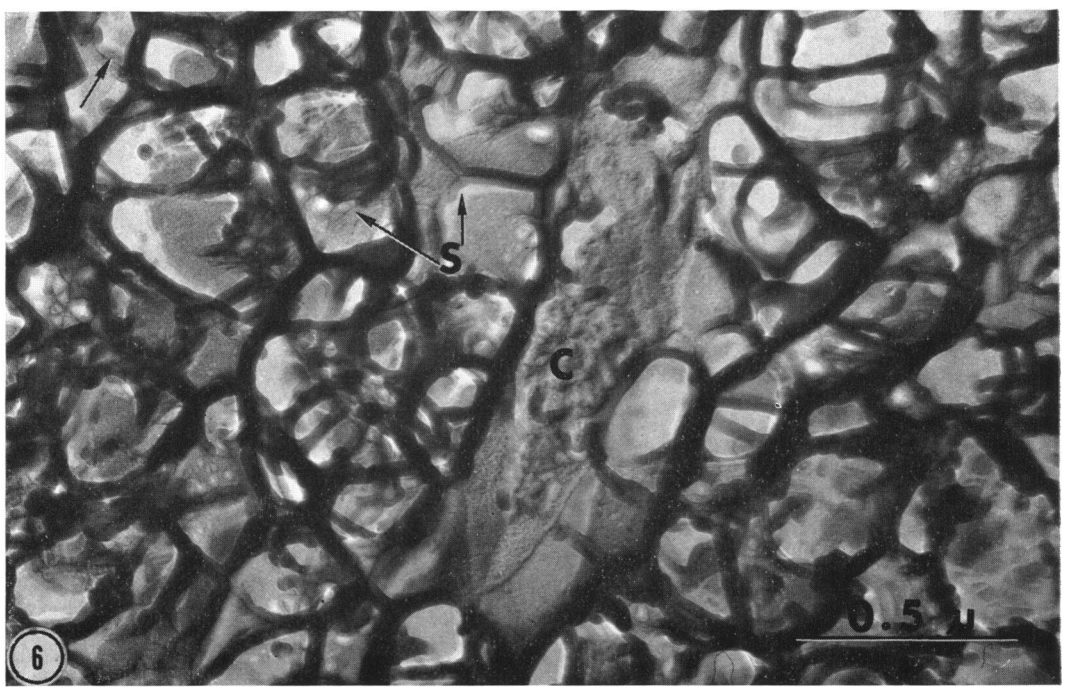


FIG. 5. *Electron micrograph of frozen-etched Z. ramigera 115 flocs. An interface (I) between individual packets of cell-flocs (F) can be observed. Individual cells (C) are present within the floc, and free cells (FC) are also present in the background.*

FIG. 6. *A cell (C) is clearly shown to be embedded within a matrix network composed of individual strands or fibrils (S). Strands appear to be of two different sizes, one measuring 40 to 60 nm and the other 2 to 5 nm.*

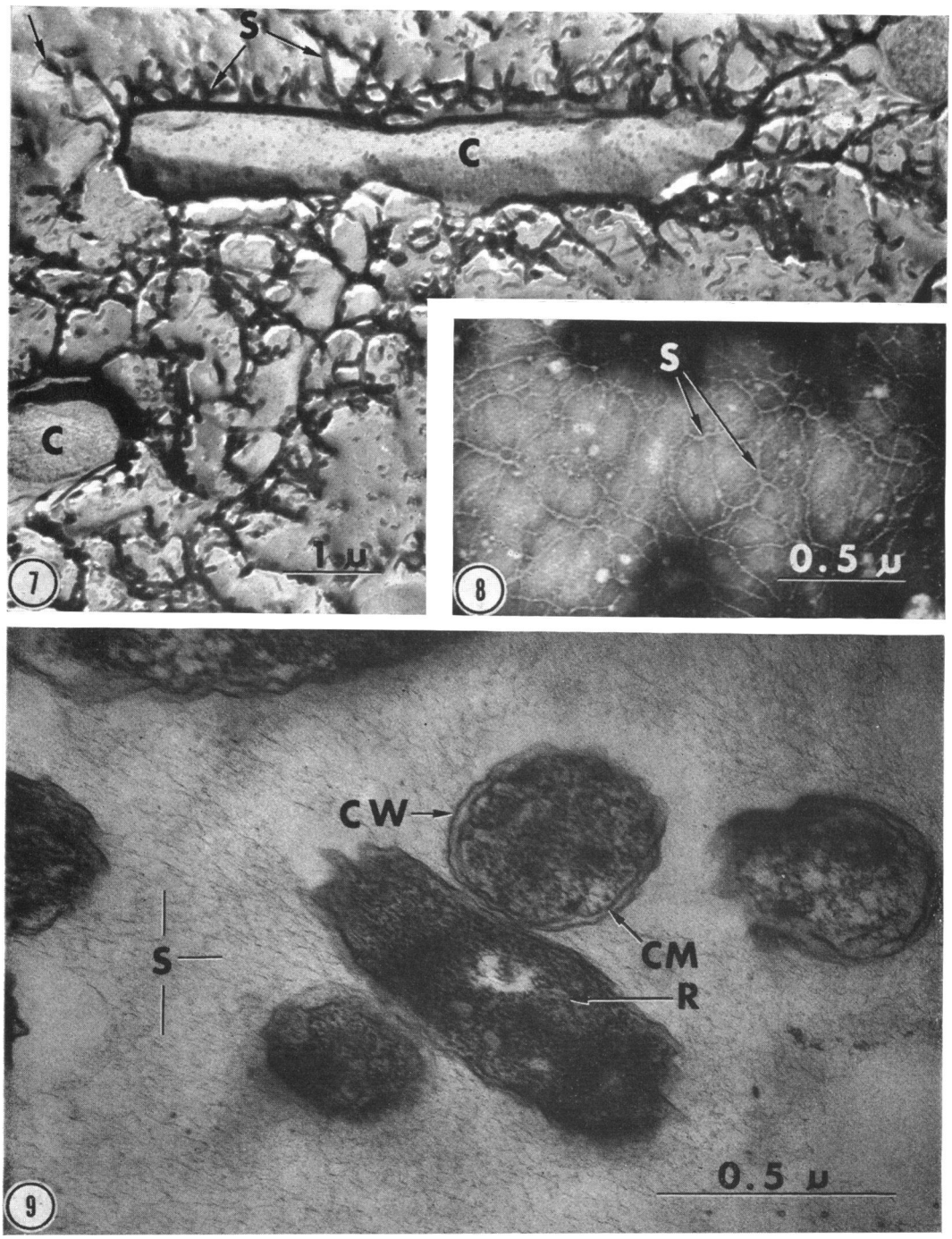
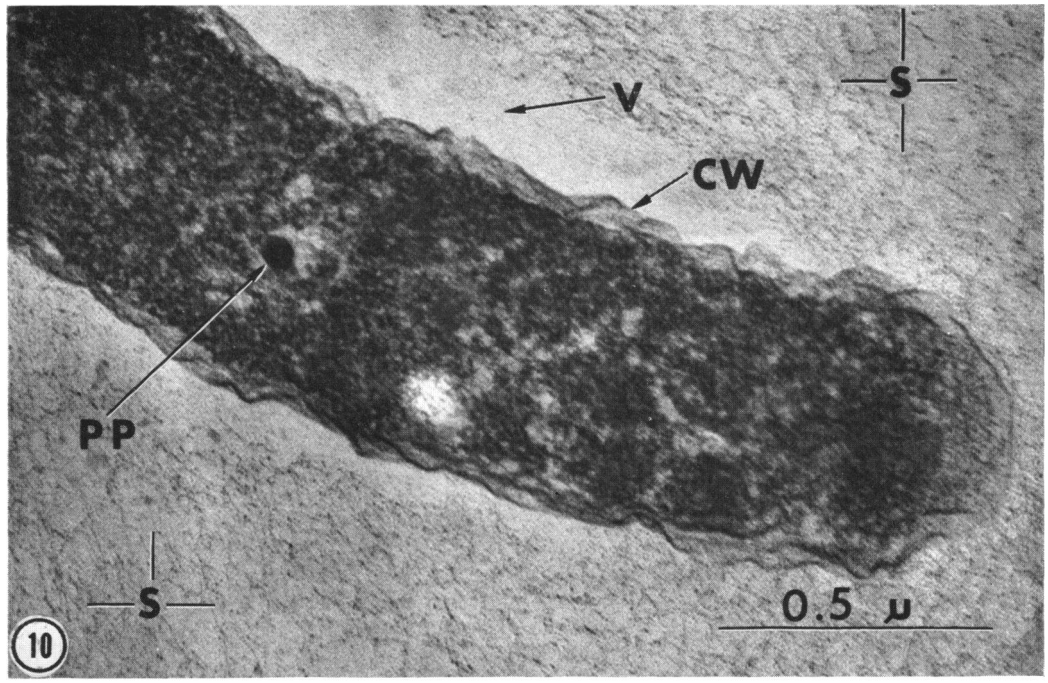


FIG. 7. *Frozen-etched Z. ramigera 115 cells showing polymer strands (S) adhering to individual cells (C).*

FIG. 8. *Negatively stained exocellular polymer strands (S) which measure 6 to 13 nm in diameter.*

FIG. 9. *Thin section of Zoogloea floc which contains cells within a matrix composed of polymer strands (S). The cells have a typical gram-negative cell wall (CW) and cytoplasmic membrane (CM). Ribosomes (R) can be observed within the cells.*



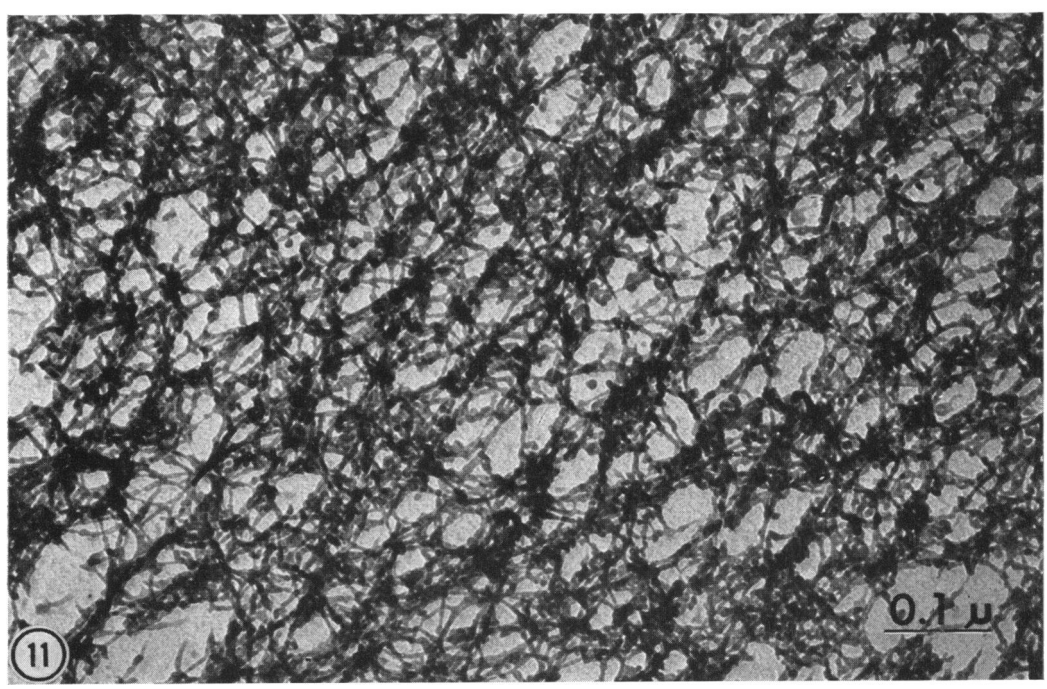


FIG. 10. *Thin section through the zoogloeal matrix. Strands measuring 4 to 6 nm are visible. A void area (V) between the cell wall (CW) and polymer strands is often observed. The inclusion granule (PP) is presumed to be polyphosphate.*

FIG. 11. *Purified strands of matrix polymer isolated from Z. ramigera 115 cells. The strands are 2 to 5 nm in diameter.*

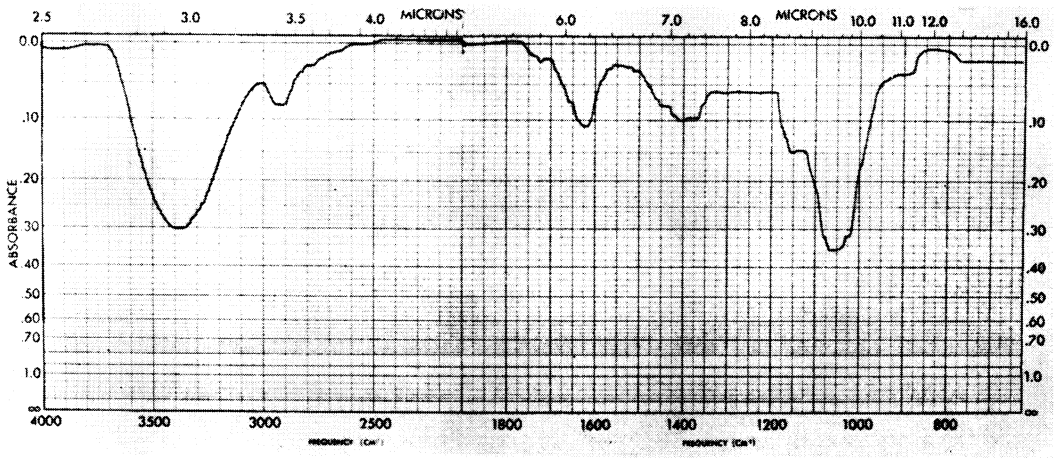


FIG. 12. *Infrared spectrum of purified polymer from Z. ramigera 115.*

a polysaccharide which may contain carboxyl functions or terminal aldehyde groups.

Chromatograms of polymer hydrolysate (HCl or H_2SO_4) revealed a single spot when sprayed with either aniline-diphenylamine or aniline-acid-oxalate. These reagents developed spots which had an R_F value identical to glucose in the solvent systems employed. The spot color was also identical to that of glucose. It is, therefore, concluded that glucose is the primary and possibly the only constituent of the polymer. Several ninhydrin-positive spots were present in crude polymer extracts but disappeared during the polymer purification procedure; this was considered evidence of polymer purity. Although the IR spectrum was interpreted as possibly containing carboxyl groups, no uronic or other organic acids could be demonstrated.

The polymer was not susceptible to hydrolysis by either α- or β-amylase under the described conditions. The polymer and whole 115 cell-flocs repeatedly gave significant positive responses to cellulase in comparison to carboxymethylcellulose controls.

The only other available *Z. ramigera* (isolate I-16-M from Crabtree and McCoy, 6, 7) culture has been examined for comparative purposes. Figure 13 is a shadow-cast preparation of cells which were treated with 1 N NaOH (18). An exocellular polymer is present which appears distinctly different from that produced by isolate 115 when treated in the same manner. No shadow-cast photographs of isolate 115 cells are presented because the 115 polymer is soluble in 1 N NaOH. Figure 14 is a frozen-etched preparation of the I-16-M isolate. Distinct exocellular strands can be observed attached to cells, but the polymer configuration is different from that shown for 115 cells in Fig. 5, 6, and 7.

DISCUSSION

The zoogloeal matrix which has been the primary basis for differentiating *Zoogloea* from other pseudomonads is composed of polymer strands. The fingerlike projections appear to be built up from generally globular packets of cells which adhere one to another. This is interpreted as being responsible for the flocculent habit of growth of this particular organism. The involvement of poly-β-hydroxybutyric acid (PHB) as reported by Crabtree et al. (7) has not been ruled out, but it does not appear to play a role. Both 115 and I-16-M isolates produce large amounts of PHB under certain growth conditions.

Two sizes of polymer strands are shown in Fig. 5, 6, and 7. The purified polymer (Fig. 11) has only one size strand that measures 2 to 6 nm in diameter. This suggests that the larger strand shown in Fig. 6 may have been induced by the formation of ice crystals during the freeze-etching procedure. The loss of pure water during sublimation could cause a concentration of the residual solution, leaving a eutectic mixture remaining between ice crystals. This mixture would tend to associate with the small strands causing them to appear larger.

The available chemical and spectroscopic data indicate that the polymer of isolate 115 is a polysaccharide; it is slightly soluble in water, and solubility increases in alkaline solutions (NH_4OH < NaOH). Glucose is the only polymer component obtained from acid hydrolysis, and susceptibility of the polymer to cellulase strongly suggests that the polymer resembles cellulose (i.e., contains

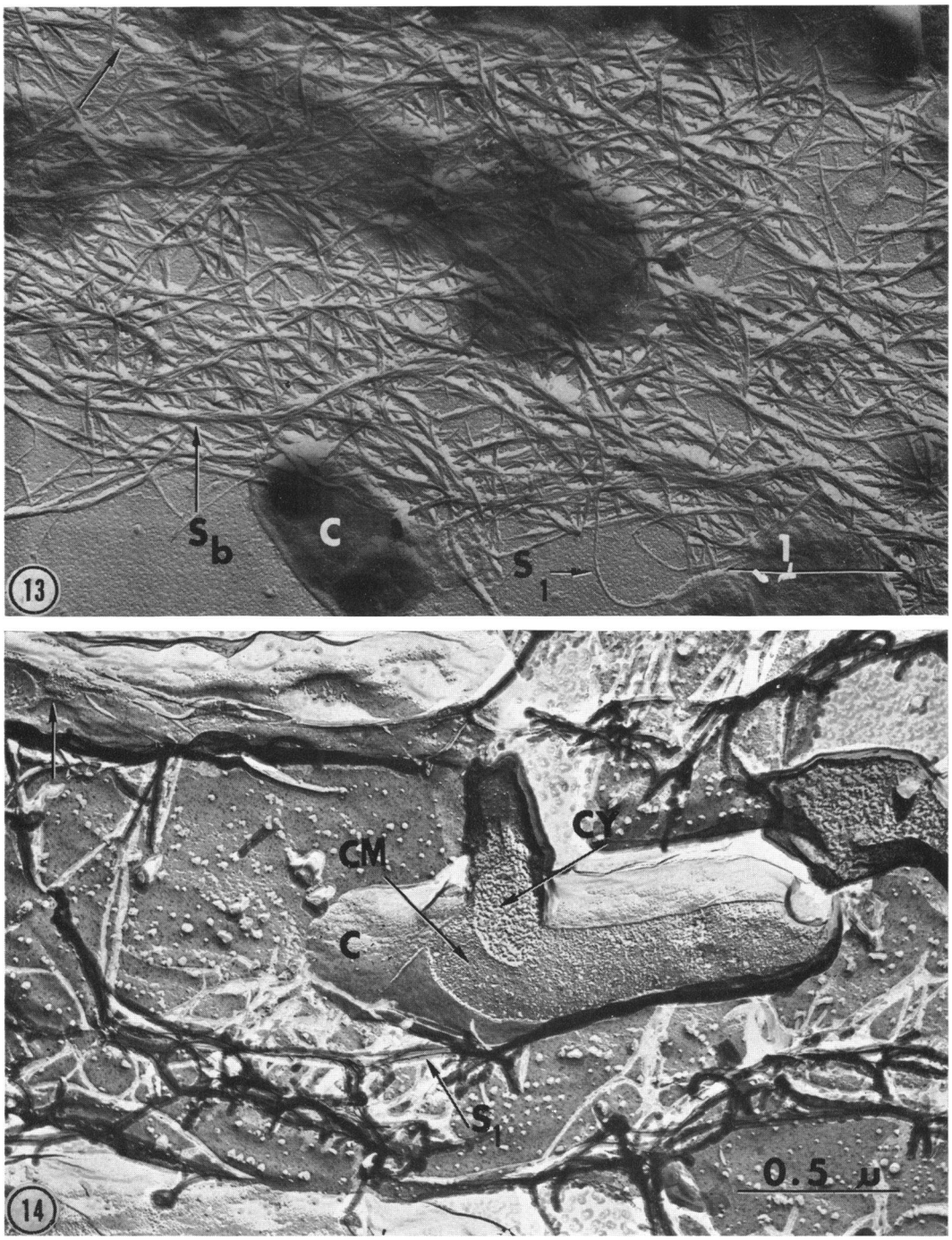


FIG. 13. *Shadow-cast preparation of Z. ramigera I-16-M cells (C) showing individual exocellular strands (S_1) or fibrils and bundles of strands or fibrils (S_b).*

FIG. 14. *Frozen-etched preparation of Z. ramigera I-16-M cells (C). Individual strands (S_1) can be observed adhering to a fractured cell. The cytoplasmic membrane (CM) and the cytoplasm (CY) are evident.*

1,4-β-glycosidic bonds). Identification of the polymer as a type of cellulose must be made with caution because the crude cellulase enzyme may contain an unknown activity on the polysaccharide substrate. The polymer does not appear to be identical to the bacterial cellulose described by Mühlethaler (15), but it has some resemblance to the cellulose fibrils shown by Gibson and Colvin (11). Further supporting evidence is being sought. Lack of susceptibility to either α- or β-amylase indicates an absence of 1,4-α-glycosidic bonds. A negative color response to iodine is also noted.

The exopolymer which surrounds the I-16-M isolate has not been isolated and purified so that comparisons to the polymer of isolate 115 must be made with caution. However, the difference in fine structure, as well as different solubility properties in basic solutions, suggests that the polymers are not identical, although the polymers from both 115 and I-16-M cells are susceptible to cellulase.

The polymer of isolate 115 appears to act as a polyelectrolyte, analogous to synthetic ion exchange resins or those used in gel filtration. It is probable that the matrix can serve a nutritional function for the cell by concentrating nutrients from solution. This could explain how organisms growing in extremely dilute nutritional aquatic habitats can accumulate essential nutrients. These same suggestions are applicable to the I-16-M isolate, which we conclude is a distinctly different organism than *Z. ramigera* isolate 115.

The possibility of cellulose synthesis by cells which do not produce acids raises some interesting taxonomic problems. If the generality of this result can be established, it may be a definitive criterion that will aid in identifying organisms in the genus *Zoogloea*.

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