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Structure of the Sulfur Inclusion Envelopes from Four Beggiatoas

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Abstract. The sulfur inclusions from four strains of *Beggiatoa alba* were observed by using a ruthenium red–glutaraldehyde technique and a modified Ryter-Kellenberger technique. Three of the four strains contained 4- to 5-nm-thick, single, electron-dense, layered sulfur inclusion envelopes. The fourth strain (B15LD) contained a complex pentalaminar sulfur inclusion envelope, 12–14 nm thick. The sulfur inclusions from all four strains were external to the cytoplasmic membrane but internal to the complex *Beggiatoa* cell walls. Freeze-etching of the *B. alba* strain B18LD trichomes revealed the unusual cross-fracture morphology of the sulfur in the inclusions. Fractures around the sulfur inclusions revealed a surface similar to that of the fractured cytoplasmic membrane.

Beggiatoas are colorless gliding bacteria capable of oxidizing hydrogen sulfide to sulfur, which they deposit in their cells [17]. Although several ultrastructural studies of various *Beggiatoa* strains have been done [3,7,8,16,17,18,20], the structure of the sulfur inclusions and their envelopes has not been fully determined.

In this study, we used two thin-section techniques that we have found to be successful for preserving the sulfur inclusion envelopes from several *Beggiatoa* strains. Moreover, we used freeze-etching to characterize further the sulfur inclusions of *B. alba* strain B18LD. In the companion paper to this study, Lawry, Jani, and Jensen [6] have used X-ray microprobe analysis to prove that the putative "sulfur inclusions" to which we refer in this paper do indeed contain sulfur.

Materials and Methods

Organisms and culture conditions. *Beggiatoa alba* B18LD has already been described [2]. *B. alba* B25RD was isolated from a ditch near Baton Rouge, Louisiana, and is similar in physiology to *B. alba* B18LD. *B. alba* strain B15LD has been described [16,19]; it is included in this paper for comparison. All three strains belonged to our group E strains [17]. *B. alba* strain L1401-13 was obtained from the Sammlung von Algenkulturen, Göttingen, Federal Republic of Germany.

All strains were grown mixotrophically for 48 h on MP05 solid medium (0.5% sodium acetate, 0.03% sodium sulfide, and modified Pringsheim's basal salts) [2] at 25°C before fixation for electron microscopy.

Electron microscopy. The trichomes were prepared for thin sections by using a modified Ryter-Kellenberger technique (RK) [17] or a ruthenium red–glutaraldehyde (RRG) fixation technique [16]. The fixed trichomes were dehydrated in a graded series of ethanol and were embedded in "ERL" plastic according to Spurr [14]. Thin sections were obtained with a Reichert II ultramicrotome and post-stained with uranyl acetate [21] and lead citrate [11].

B. alba B18LD was freeze-etched after 48 h growth on the MP05 mixotrophic growth medium [2]. The *B. alba* trichomes were scraped from the agar surface with a wooden applicator stick, placed into 20% glycerol for 1 h, pelleted by centrifugation, and then placed into complementary specimen holders. They were etched for 2 min at −98°C, shadowed at a 45° angle with platinum-carbon, and then carbon-coated. A Leybold Bio-Etch 2005 automatic freeze-etching apparatus (Leybold-Heraeus GMBH and Co., KG, Cologne, Federal Republic of Germany) was used for the freeze-etching preparations. All micrographs were taken using a Zeiss 10 electron microscope.

Results

Ruthenium red–glutaraldehyde fixation. Fig. 1 shows the RRG fixation results for *Beggiatoa alba* strains B15LD, L1401-13, B25RD, and B18LD. Three of the *B. alba* strains contained single, electron-dense, layered sulfur inclusion envelopes, approximately 4–5 nm thick (Fig. 1b–d). The fourth strain, B15LD, contained a densely stained, single, electron-dense, layered envelope, 40 nm thick (Fig. 1a). The sulfur inclusions from all four strains were surrounded by the cytoplasmic membrane and were

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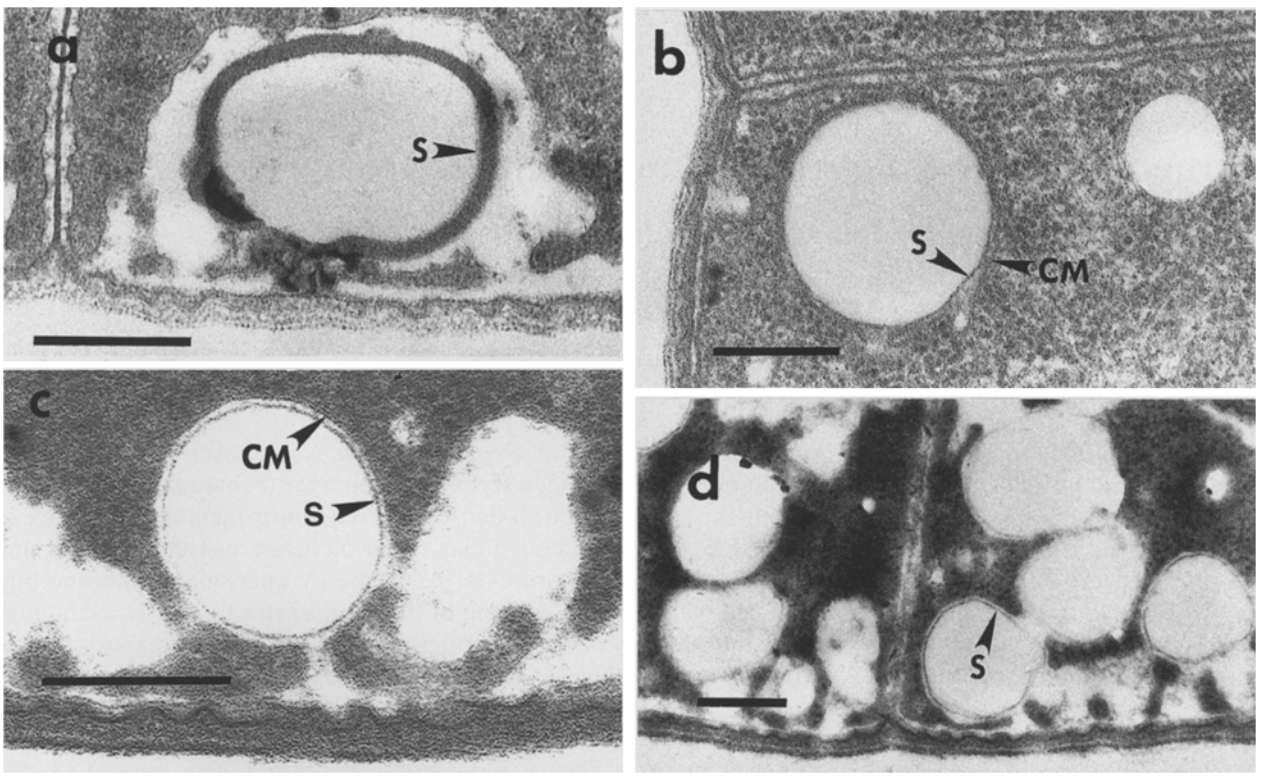

Fig. 1. The sulfur inclusion envelopes of the four strains of *Beggiatoa alba* as depicted by ruthenium red–glutaraldehyde fixation. (a) The unresolved sulfur inclusion envelope of *B. alba* strain B15LD. (b) The sulfur inclusion and the closely associated cytoplasmic membrane in strain L1401-13. (c) The sulfur inclusion of strain B25RD, enclosed within the invagination of the cytoplasmic membrane. (d) Multiple sulfur inclusions in strain B18LD; in some, the sulfur inclusion envelopes have not been preserved through fixation and sectioning. Symbols: sulfur inclusion envelope (S); cytoplasmic membrane (CM). Bars represent 250 nm.

periplasmic in their location. In Figs. 1a and 1c, the invaginations of the cytoplasmic membranes that surround the sulfur inclusions are apparent.

Modified Ryter-Kellenberger fixation. Fig. 2 shows the sulfur inclusion envelopes of three of the *B. alba* strains when modified Ryter-Kellenberger fixation was used. Fig. 2a shows the pentalaminar-type sulfur inclusion envelope of strain B15LD [17, 18,19].

Figs. 2b and 2c show serial sections of the sulfur inclusions from strain L1401-13. The sulfur inclusion envelopes consisted of single electron-dense layer, 4–5 nm thick. The serial sections show how important the plane of sectioning was for the observation of the cytoplasmic membrane invagination that surrounded the sulfur inclusions. Furthermore, the apparent integrity and clarity of the sulfur inclusion envelopes was dictated by the plane of section (compare Fig. 2b with 2c). Fig. 2d shows the single, electron-dense, layered, sulfur inclusion envelope of strain B18LD. The invaginations of the cytoplasmic membrane from both the side of the cell and the septal region around the sulfur inclusion can be observed (Fig. 2d). When the modified Ryter-Kellenberger technique was used, strain B25RD was very similar to strain B18LD (data not shown).

Freeze-etch morphology. Fig. 3a shows the surface morphology of a freeze-etched sulfur inclusion and a poly-β-hydroxybutyric acid (PHB) inclusion from strain B18LD. The PHB inclusion contained linear surface fibers approximately 10 nm in diameter [4]. These were consistently observed in cells grown under heterotrophic conditions (data not shown). The surface of the sulfur inclusion was ornamented with several 8- to 10-nm studs and circular 40- to 70-nm particle-free patches (Fig. 3a). Moreover, the sulfur inclusions were often directly associated with the cell envelope (Fig. 3a,c). Fig. 3b shows the freeze-etched protoplasmic half of the cytoplasmic membrane of strain B18LD, which also exposed a surface containing 8- to 10-nm studs and 40- to 70-nm circular particle-free patches. Fig. 3c shows the morphology of the surface of the sulfur inclusion

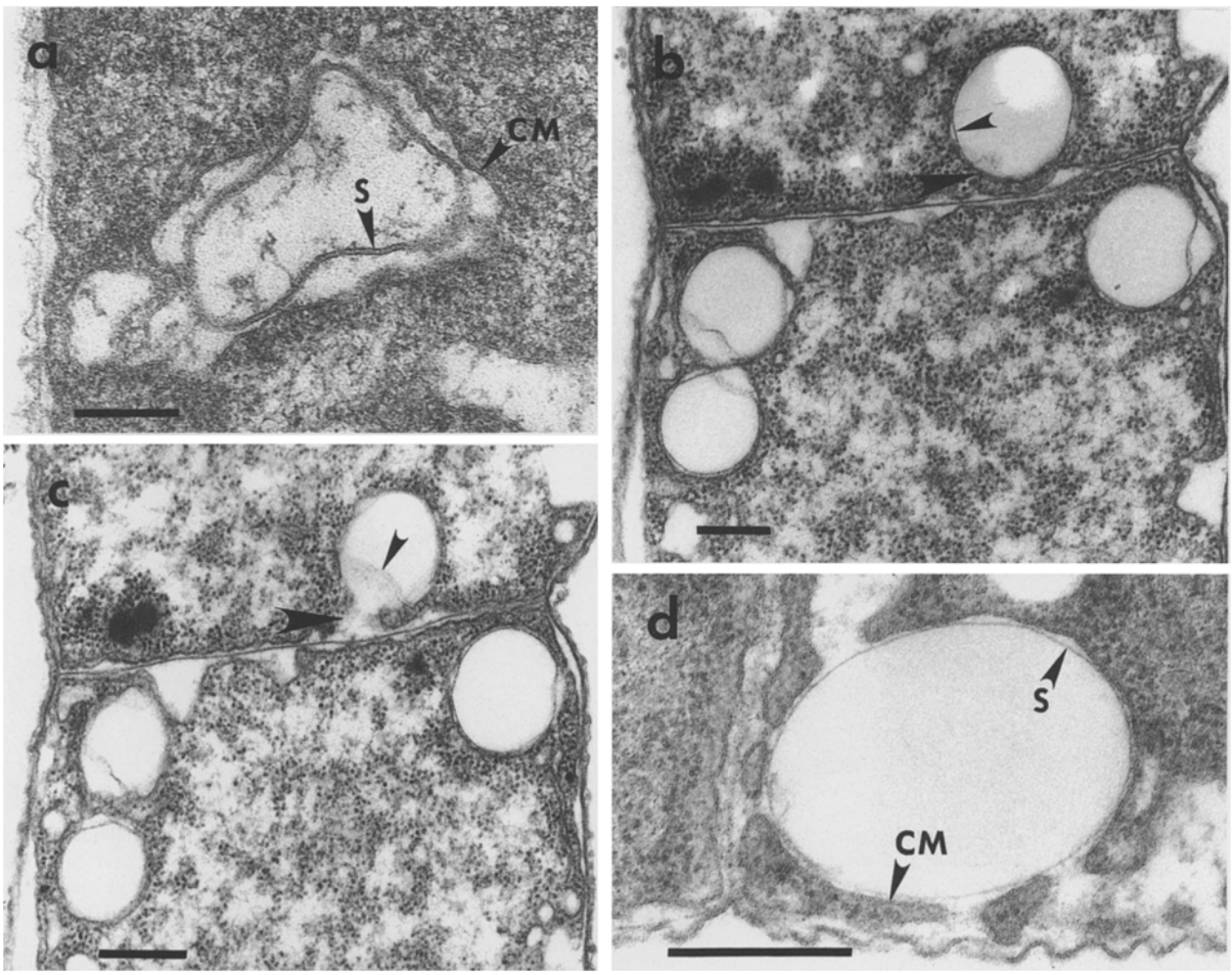

Fig. 2. The sulfur inclusion envelopes of strains of *Beggiatoa alba* after fixation by the modified Ryter-Kellenberger technique. (a) The pentalaminar sulfur inclusion envelope of strain B15LD. (b and c) Serial sections of the sulfur inclusions and the septal region in strain L1401-13. Note that the plane of sectioning affects the observation of the cytoplasmic membrane invagination (large arrows), as well as the integrity of the sulfur inclusion envelopes (small arrows). (d) Sulfur inclusion of strain B18LD, enclosed by the invagination of the cytoplasmic membrane from both the septal region and the side of the cell. Symbols are the same as in Fig. 1. Bars represent 250 nm.

envelopes of strain B18LD as compared with the unusual morphology of the cross-fractured sulfur inclusions in the same trichome. Fig. 3d shows two sulfur inclusions in strain B18LD that have been partially fractured away, revealing both the surface and the unusual internal sulfur morphology.

Discussion

The sulfur inclusion envelopes observed in *Beggiatoa alba* strain B15LD were different from the sulfur inclusions observed in *Chromatium* [9], *Thiothrix* [1], *Thioploca* [7], *Thiovulum* [22], and all other *Beggiatoa* strains studied thus far [3,7,8,20] (strains B18LD, B25RD, and L1401-13 of this paper). We were unable to observe the layered nature of the sulfur inclusion envelopes in poorly fixed or sectioned Ryter-Kellenberger thin sections of strain B15LD. Moreover, in several other fixation techniques, the B15LD sulfur inclusion envelopes were either unresolved (see Fig. 1a) or destroyed (unpublished results). The use of phosphate-buffered glutaraldehyde and osmium postfixation resulted in washed-out spaces very similar in appearance to those in the literature [3,7,8,20].

The sulfur inclusion envelopes of our other three *B. alba* strains were morphologically different from those of strain B15LD, although in all cases

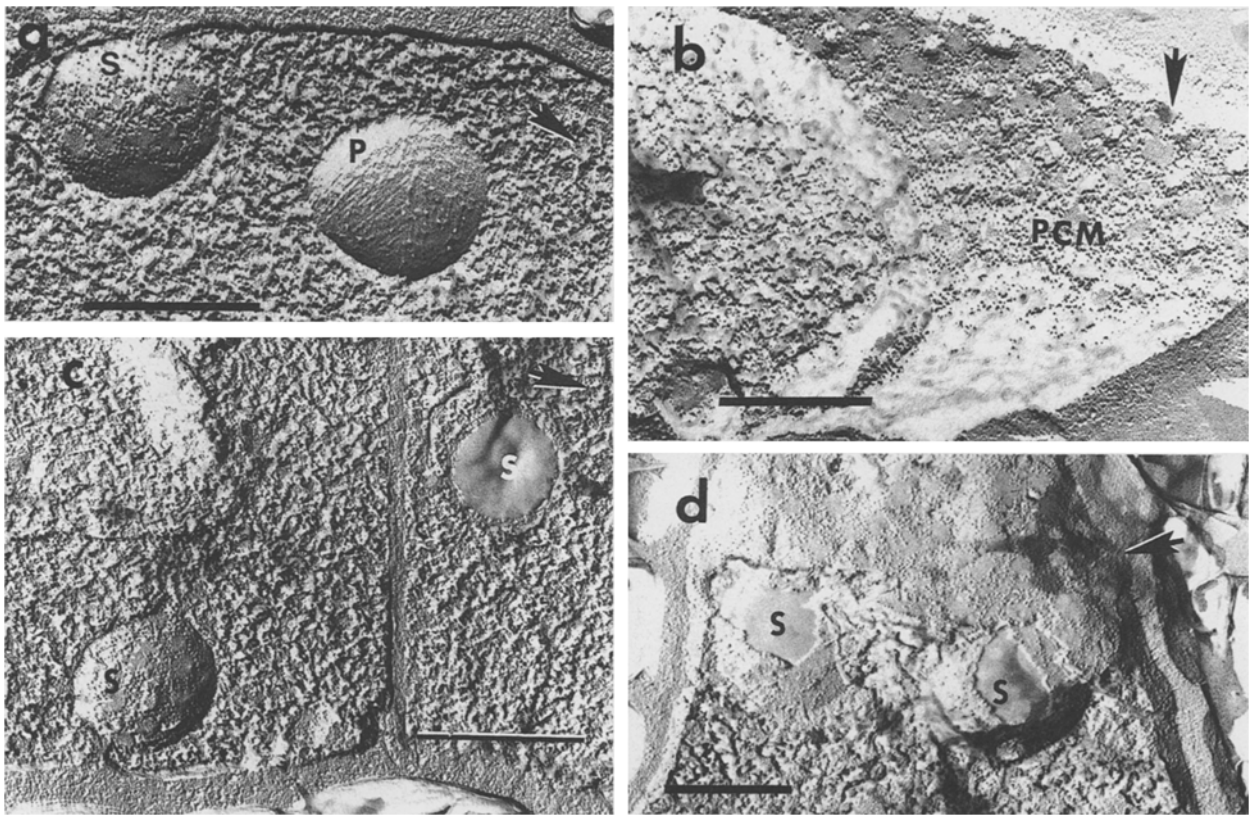

Fig. 3. The sulfur inclusions of *Beggiatoa alba* strain B18LD as observed after glycerol-cryoprotected freeze-etching. (a) A comparison of the fracture faces of the surfaces of poly-β-hydroxybutyric acid inclusions and of the sulfur inclusion envelope–cytoplasmic membrane complex that surrounds the sulfur inclusions. (b) The protoplasmic fracture face of the cytoplasmic membrane. Compare the morphology of the sulfur inclusion fracture face surfaces in Fig. 3a and 3c with this surface. (c) Comparison of the unusual morphology of the sulfur in the cross-fractured sulfur inclusion with the morphology of the sulfur inclusion envelope–cytoplasmic membrane complex that surrounds the sulfur inclusions. Note the attachment of the sulfur inclusion to the side of the cell, which probably corresponds to the invaginations of the cytoplasmic membrane as seen in thin sections. (d) Sulfur inclusions in which part of the overlying layers were partially fractured away, exposing the unusual fracture face of the sulfur. Symbols: sulfur inclusions (S); protoplasmic face of the cytoplasmic membrane (PCM); poly-β-hydroxybutyric acid inclusions (p). Arrows indicate the direction of shadow (white shadows). Bars represent 250 nm.

they were external to the cytoplasmic membrane. The sulfur inclusions of other *Beggiatoa* strains studied have appeared as washed-out areas bounded by the cytoplasmic membrane; none contained the morphologically distinct sulfur inclusion envelopes that we have observed in our *B. alba* strains [3,7,8]. There are two possible explanations for these differences. First, the use of different fixation techniques by various investigators—such as fixation for 2 h with neutral-pH, veronal acetate–buffered OsO_4 [8], methacrylate [3], or glutaraldehyde-acrolein and OsO_4 postfixative [J. V. Tredway, Ph.D. dissertation, 1977, Brigham Young University]—may have led to different observations. This is supported by the apparent fragility of the B15LD sulfur inclusion envelopes in fixatives other than the modified Ryter-Kellenberger fixative that we have employed here. However, Maier and Murray [7] used the Ryter-Kellenberger technique and did not observe the unique sulfur inclusion envelopes. Thus, the second explanation is that the morphology of the sulfur inclusion envelopes from different strains of *Beggiatoa* may be quite diverse. This explanation is supported by the obvious differences between the B15LD sulfur inclusions and those of the other three *B. alba* strains after identical fixation procedures.

The sulfur inclusion envelope of *Chromatium* was described as a proteinaceous, single electron-dense layer 2.5–3.0 nm thick [9,12]. The sulfur inclusions in *Thiovulum* were enclosed by a "non-unit membrane", but they were not external to the cytoplasmic membrane [22]. Thus, the sulfur inclusions described for those nonfilamentous, nonglid-

ing, sulfur-depositing bacteria were different from the sulfur inclusion envelope–cytoplasmic membrane complexes observed in these beggiatoas.

The freeze-etch morphology of the surface of the sulfur inclusions of strain B18LD closely resembled the morphology of the protoplasmic fracture face of the cytoplasmic membrane. Moreover, the single electron-dense layer of the strain B18LD sulfur inclusion envelope suggests that the envelope may be composed only of protein like a proteinaceous "cage" [12]. If so, the morphology of the fractured sulfur inclusion envelope would be expected to consist entirely of 8- to 10-nm particles associated with membrane proteins [15]. At this point it is difficult to correctly ascertain the cleavage plane in the sulfur inclusion–cytoplasmic membrane complex, but the morphological similarity to the cytoplasmic membrane (Fig. 2b) suggests that it is through the surrounding cytoplasmic membrane portion of the complex.

The morphology of the sulfur in the cross-fractured sulfur inclusions of strains B18LD and B15LD (unpublished results) was similar to that obtained with *Chromatium* [10]. Although the physical state of the sulfur in the beggiatoas was not investigated, the similarity in the freeze-etch morphologies suggests that the sulfur is in the liquid state, as it is in *Chromatium* [5]. The observation that the sulfur inclusions in thin sections were "empty", i.e., that they contained no electron-dense sulfur, is consistent with the washing out of the sulfur by 100% ethanol during dehydration [1,13].

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Literature Cited

1. **Bland, J. A., Staley, J. T.** 1978. Observations on the biology of *Thiothrix*. Archives of Microbiology **117**:79–87.
2. **Cannon, G. C., Strohl, W. R., Larkin, J. M., Shively, J. M.** 1979. Cytochromes in *Beggiatoa alba*. Current Microbiology **2**:263–266.
3. **Drawert, H., Metzner-Küster, I.** 1958. Fluorescenz und elektronmikroskopische Untersuchung an *Beggiatoa alba* und *Thiothrix nivea*. VI. Mitteilung der Reihe: Zellmorphologische und zellphysiologische Studien an Cyanophyceen. Archiv für Mikrobiologie **31**:422–434.
4. **Dunlop, W. F., Robards, A. W.** 1973. Ultrastructural study of poly-β-hydroxybutyrate granules from *Bacillus cereus*. Journal of Bacteriology **114**:1271–1280.
5. **Hageage, G. J., Jr., Eanes, E. D., Gherna, R. L.** 1970. X-ray diffraction studies of the sulfur globules accumulated by *Chromatium* species. Journal of Bacteriology **101**:464–469.
6. **Lawry, N. H., Jani, V., Jensen, T. E.** 1981. Identification of the sulfur inclusion body in *Beggiatoa alba* B18LD by X-ray energy-dispersive microanalysis. Current Microbiology **6**:71–74.
7. **Maier, S., Murray, R. G. E.** 1965. The fine structure of *Thioploca ingrica* and a comparison with *Beggiatoa*. Canadian Journal of Microbiology **11**:645–656.
8. **Morita, R. Y., Stave, P. W.** 1963. Electron micrograph of an ultrathin section of *Beggiatoa*. Journal of Bacteriology **85**:940–942.
9. **Nicolson, G. L., Schmidt, G. L.** 1971. Structure of the *Chromatium* sulfur particle and its protein membrane. Journal of Bacteriology **105**:1142–1148.
10. **Remsen, C. C.** 1978. Comparative subcellular architecture of photosynthetic bacteria, pp. 31–60. In: Clayton, R. K., Sistrom, W. R. (eds.), The photosynthetic bacteria. New York: Plenum Press.
11. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. Journal of Cell Biology **17**:208–212.
12. **Schmidt, G. L., Nicolson, G. L., Kamen, M. D.** 1971. Composition of the sulfur particle of *Chromatium vinosum* strain D. Journal of Bacteriology **105**:1137–1141.
13. **Shively, J. M.** 1974. Inclusion bodies in prokaryotes. Annual Reviews of Microbiology **28**:167–187.
14. **Spurr, A. R.** 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructural Research **26**:31–43.
15. **Strohl, W. R.** 1979. Ultrastructure of *Cytophaga johnsonae* and *C. aquatilis* by freeze-etching. Journal of General Microbiology **112**:261–268.
16. **Strohl, W. R., Larkin, J. M.** 1978. Cell division and trichome breakage in *Beggiatoa*. Current Microbiology **1**:151–155.
17. **Strohl, W. R., Larkin, J. M.** 1978. Enumeration, isolation, and characterization of *Beggiatoa* from freshwater sediments. Applied and Environmental Microbiology **36**:755–770.
18. **Strohl, W. R., Larkin, J. M., Howard, K. S.** 1978. Characterization and ultrastructure of *Beggiatoa* sulfur inclusions. Abstracts of the Annual Meeting of the American Society for Microbiology **1978**:77.
19. **Strohl, W. R., Howard, K. S., Larkin, J. M.** 1981. Ultrastructure of *Beggiatoa alba* strain B15LD. Journal of General Microbiology, in press.
20. **Tredway, J. V., Lee, J. D., Burton, S. D.** 1977. Morphological evaluation of the ultrastructure of marine and freshwater *Beggiatoa* species. Abstracts of the Annual Meeting of the American Society for Microbiology **1977**:240.
21. **Watson, M. L.** 1958. Staining of tissue sections for electron microscopy with heavy metals. II. Applications of solutions containing lead and barium. Journal of Biochemical and Biophysical Cytology **4**:475–479.
22. **Wirsen, C. O., Jannasch, H. W.** 1978. Physiological and morphological observations on *Thiovulum* sp. Journal of Bacteriology **136**:765–774.