INFECTION AND IMMUNITY, May 1980, p. 546-556
0019-9567/80/05-0546/11$02.00/0

Vol. 28, No. 2

Production of Mucoid Microcolonies by *Pseudomonas aeruginosa* Within Infected Lungs in Cystic Fibrosis

J. LAM, R. CHAN, K. LAM, AND J. W. COSTERTON*

Department of Biology, University of Calgary, Calgary, Alberta T2N 1N4, Canada

Direct electron microscopic examination of postmortem lung material from cystic fibrosis patients infected with *Pseudomonas aeruginosa* has shown that these bacterial cells form distinct fiber-enclosed microcolonies in the infected alveoli. Similar examination of bronchoscopy material from infected cystic fibrosis patients showed that the fibers of the enveloping matrix are definitely associated with the bacterial cells. The fibers of the extracellular matrix stain with ruthenium red and are therefore presumed to be polyanionic. When mucoid strains of *P. aeruginosa* were recovered from cystic fibrosis patients and grown in a suitable liquid medium, they were found to produce large microcolonies whose component cells were embedded in a very extensive matrix of polyanionic fibers that could be stabilized by reaction with antibodies to prevent collapse during the dehydration steps of preparation for electron microscopy. When these mucoid strains of *P. aeruginosa* were used to produce pulmonary infections of rats by the agar bead method, the infected alveoli contained large fiber-enclosed bacterial microcolonies. We conclude that the cells of *P. aeruginosa* that infect cystic fibrosis patients form microcolonies that are enveloped in a fibrous anionic matrix and that these microcolonies can be duplicated in in vitro cultures and in animal model systems.

As early as 1964, Doggett et al. (7) noted that the majority of *Pseudomonas aeruginosa* isolates from cystic fibrosis (CF) patients with chronic pulmonary infections grew as slimy mucoid colonies on agar. This observation has been confirmed many times, and Thomassen et al. (M. J. Thomassen, B. Boxerbaum, and C. A. Demko, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, C126, p. 56) have described two different mucoid colony types from CF patients ("mucoid" and "gelatinous") in addition to four "nonmucoid" colony types. The types that were mucoid on agar generally lost their mucoid colony morphology upon several subcultures (8), but Govan has shown that by growing these mucoid strains in the presence of deoxycholate (10) or carbenicillin (11) their mucoid nature can be maintained. *P. aeruginosa* has been found to colonize the lungs of from 50% (13) to 90% (14) of all CF patients, with the severity of the lung infection being correlated to the presence of mucoid strains (12), which appear to arise in conjunction with antibiotic therapy (13).

We have examined the four colony types of *P. aeruginosa* described by Thomassen et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, C126, p. 56) as nonmucoid and have found that all four exhibit a mucoid type of growth in our modification of the chemically defined liquid medium E described by Vogel and Bonner (20). Our findings indicate that most *P. aeruginosa* isolates from CF patients with chronic lung infections are capable of mucoid growth if the correct medium is used. This raises the possibility that growth within the CF patient's lungs may select for variants that can produce large amounts of exopolysaccharide.

To determine whether *P. aeruginosa* grows in a mucoid form in the lungs of CF patients, various pulmonary samples from infected CF patients were examined in this study.

MATERIALS AND METHODS

Postmortem material was recovered between 1 and 3 h after death from the lungs of two CF patients who had died after prolonged pulmonary infections in which *P. aeruginosa* was consistently the predominant pathogen. Bronchial mucus was recovered by bronchoscopy from the lung of a 13-year-old female patient (Cleveland, Ohio, September, 1977) with a 3-year history of infection with mucoid strains of *P. aeruginosa*. Cubes (ca. 3 cm^3) were removed from the parenchyma tissue of the left lower lobe of the lungs at postmortem, viscous masses of approximately the same dimensions were collected from the bronchoscopy material, and these specimens were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 0.15% ruthenium red at room temperature for 2 h. These specimens were then washed three times with the buffer containing 0.05% ruthenium red and shipped to Calgary for examination. Immediately upon receipt, the material was cut into smaller cubes (ca. 1 cm^3) and washed twice in the buffer with 0.05% ruthenium red, postfixed for 2 h at 20°C in 2% OsO_4 in

0.1 M cacodylate buffer (pH 7.2) containing 0.05% ruthenium red, and washed five times in the buffer with 0.05% ruthenium red. The material was dehydrated in an acetone series in which acetone concentrations less than 50% were made up with the buffer containing ruthenium red, whereas acetone concentrations above 50% were made up with distilled water because of difficulties with the solubility of the stain, and exposure of the material to these solutions was minimized because the stain was progressively lost. After further dehydration in propylene oxide (two changes in 100% propylene oxide), the material was embedded in Vestopal, sectioned on a LKB type 8800 ultramicrotome, stained with uranyl acetate and lead citrate (19), stabilized with evaporated carbon, and examined using an AEI model 801 electron microscope operating at an accelerating voltage of 60 kV.

Chronic pulmonary infections of young male Sprague Dawley rats (ca. 200 g) were induced by the method of Cash et al. (2) in which cells of the dwarf strain of *P. aeruginosa* (Thomassen et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, C126, p. 56) were incorporated into agar "beads" and introduced into the lungs of the rats via a tracheotomy. These animals developed chronic pulmonary infections from which very large numbers (10^4 to 10^6 colony-forming units per ml) of cells of the infecting strain could be recovered on homogenization and plating of the lung tissue. Animals that had been infected by this method were sacrificed at regular intervals of 7 days up to 35 days (135 animals in all), their lungs were removed and fixed by positive pressure perfusion (2) with 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 0.15% ruthenium red, and these specimens were prepared for transmission electron microscopy (TEM) by the method detailed above for postmortem and bronchoscopy specimens. Specimens from the same areas of these infected lungs were prepared for scanning electron microscopy by fixation by positive-pressure perfusion for 2 h by using 5% glutaraldehyde in 0.1 M cacodylate buffer, washing five times in buffer after removal from the body, postfixing in 2% OsO_4 in 0.1 M cacodylate buffer (pH 7.2), treating with thiocarbohydrazide (16), drying by the critical point method (3) with freon 13, and sputter-coating with gold palladium (Au-Pd).

Cultures of the six colony types most commonly isolated from CF patients at the Rainbow Hospital, Cleveland, were kindly supplied by Mary Jane Thomassen. These strains were grown in brain heart infusion agar and in our modification (MVBM) of Vogel and Bonner's medium (20) with and without the addition of agar. MVBM was made up as follows: $MgSO_4 \cdot 7H_2O$, 0.2 g; citric acid (anhydrous), 2.0 g; $NaNH_4HPO_4$, 3.5 g; K_2HPO_4, 10.0 g; dissolved in order in glass-distilled water making a final volume of 919 ml with a final pH of 7.2. After autoclaving, 1 ml of $CaCl_2 \cdot 2H_2O$ from a stock of 36 g/100 ml and 80 ml of a 25% solution of D-gluconate were added; both of these were filter sterilized, and the latter was added as a supplementary carbon source. Cells of the dwarf strain grown in liquid MVBM were incubated for 30 min at 20°C in physiological saline with 0.1 M phosphate buffer (pH 7.2) with serum containing antibodies against Formalin-killed cells of the same strain to stabilize their exopolysaccharide (1, 15) during the dehydration necessary for preparation for TEM. These antibody-stabilized cells were prepared for TEM by the methods detailed above for postmortem and bronchoscopy specimens.

RESULTS

Preservation of human postmortem material is not optimal, because of degenerative changes after death, but alveolar spaces could be easily identified in the lungs of CF patients who had died of pulmonary infections with *P. aeruginosa*. These spaces contained many masses of bacteria enclosed in fibrous matrices that stained heavily with ruthenium red (Fig. 1). Closer examination reveals that the bacteria within these discrete fiber-enclosed packages have an ultrastructure that indicates that they are gram negative (4). We know that exopolysaccharide fibers are radically condensed during dehydration for TEM and that they condense down onto the surface to which they are attached (15), and we therefore assume that these condensed fibrous masses comprised a much more extensive matrix before dehydration. Bacteria were often seen in immediate juxtaposition to the alveolar surface (Fig. 2), but in this location, perhaps because of multiple attachment points, the fibers are less condensed and their relationship to the bacteria is more difficult to define because there are many fibrous polymers of host origin in the lung.

The bacterial origin of the ruthenium red-staining fibrous material was perhaps best illustrated in the coherent masses recovered by bronchoscopy from the airways of a CF patient infected with *P. aeruginosa*. Within these coherent masses the gram-negative bacteria were widely separated and the bulk of the electron-dense fibrous material was concentrated immediately around each of the bacterial cells (Fig. 3). Because the slimy masses were coherent, we can assume that the regions between bacteria were filled with the ruthenium red-staining fibers before they condensed towards their attachment points on the bacterial cell during dehydration. These attachment points will then have been broken to produce a separation of the bacterial cells from their attached fibers during the shrinkage of the cells due to the polymerization of the embedding plastic. The relationship of the extracellular fibers to the bacterial surface, the extent of the fibrous matrix, and the gram-negative nature of the cell walls of these bacteria are more clearly seen at higher magnification (Fig. 4).

When cells of the dwarf strain (Thomassen et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, C126, p. 56) were used to produce chronic infections in the lungs of rats by Cash's method (2), bacteria were found in the alveolar spaces (Fig.

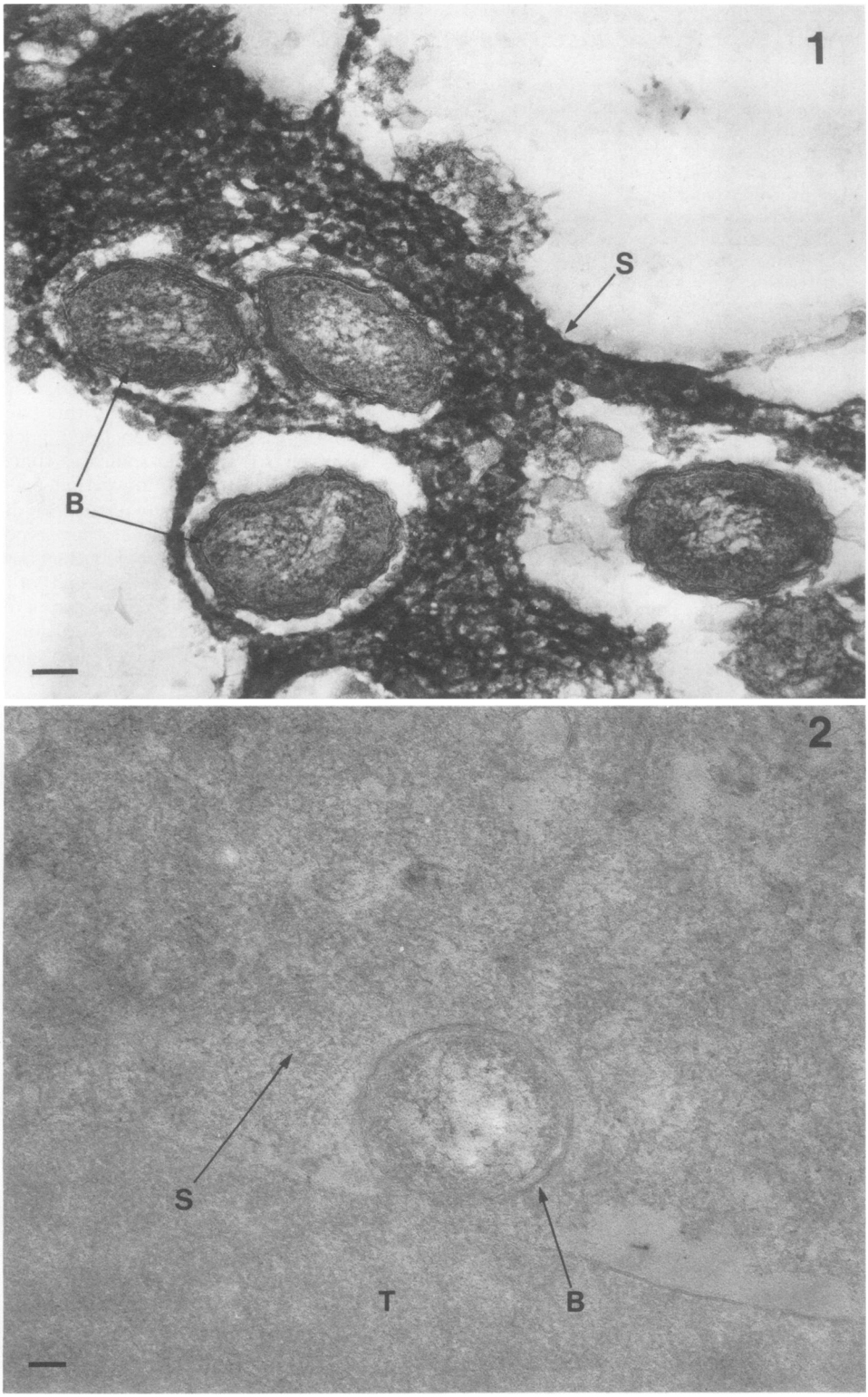
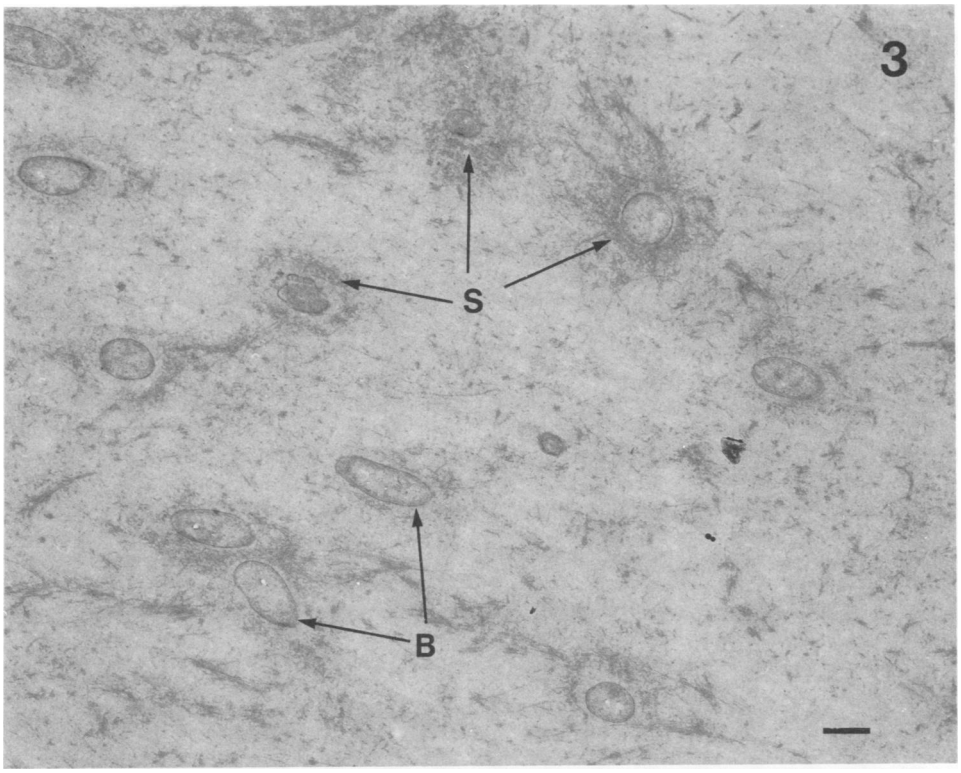


FIG. 1. *Electron micrograph of a thin section of ruthenium red-stained alveolar material obtained by postmortem excision from a P. aeruginosa-infected CF patient. Note the very thick fibrous mass of bacterial exopolysaccharide (S) surrounding the gram-negative bacterial cells (B) in the alveolar spaces. The bar indicates 0.1 μm.*

FIG. 2. *Electron micrographs of similar postmortem material from a P. aeruginosa-infected CF patient, showing a bacterium (B) at the alveolar tissue surface (T), which was surrounded by a very large mass of fibrous slime (S) of undetermined origin. The bar indicates 0.1 μm.*



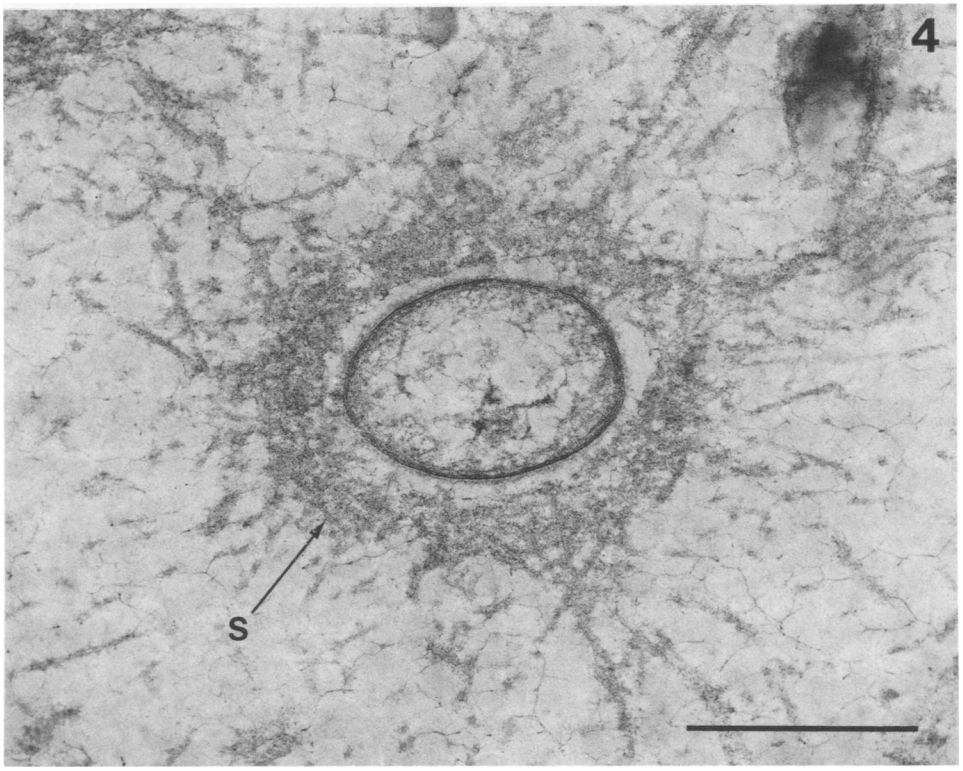


FIG. 3. *Electron micrograph of a thin section of ruthenium red-stained material obtained by bronchoscopy from the airway of an infected CF patient showing gram-negative bacteria (B) that are enclosed by slime (S) material whose fibrous elements are very clearly associated with the individual bacterial cells. The bar indicates 0.5 μm.*

FIG. 4. *Electron micrograph of a thin section of similar bronchoscopy material from a CF patient. At this higher magnification the slime material (S) extending from the cell surface and the gram-negative nature of the bacterial cell wall are more clearly seen. The bar indicates 0.5 μm.*

5). The improved preservation was possible because animal specimens can be processed under optimal conditions, and we note that the intact gram-negative bacteria are enclosed, with cell fragments, in microcolonies (Fig. 5 and 6) held together by a matrix of fine less-condensed extracellular fibers. These bacteria were introduced into the rat lungs in agar beads, but their subsequent widespread distribution throughout the alveoli indicates that they grew out of their initial artificial protective matrix and produced their own extracellular fibers which are clearly seen at high magnification (Fig. 7, S). Scanning electron microscopy revealed intense alveolar congestion in these chronically infected rat lungs (Fig. 8), which contrasted sharply with the open state of normal rat lungs (Fig. 9), and detailed examination of the congested alveoli revealed rod-shaped bacteria (Fig. 10, B) embedded to various degrees in an amorphous, condensed matrix at the alveolar surface.

To assess the extent of the collapse of the exopolysaccharide fibers of mucoid strains of *P. aeruginosa* during the dehydration steps in preparation for TEM, we examined pure cultures of the dwarf strain that had been grown in a slime-promoting medium, with and without stabilization by antibodies (1, 15). When cells of the dwarf colony type from patently mucoid cultures in MVBM were fixed and prepared for TEM in the presence of ruthenium red, their exopolysaccharide fibers formed coarse condensed masses on the bacterial cell surface (Fig. 11, S). When the condensation of exopolysaccharide fibers was prevented by stabilization of this matrix with antibodies, the same cells were seen to be surrounded by a very extensive mass of fine fibers (Fig. 12, S) that surrounded each cell and extended as much as 0.5 μm away from the bacterial cell surface.

Thus, we have shown that a CF nonmucoid strain of *P. aeruginosa* that produces extensive exopolysaccharide in liquid media of suitable chemical composition produced large fiber-enclosed microcolonies when introduced, in an initially protected state, into the lungs of rats. These fiber-enclosed microcolonies are very similar, morphologically, to those found in the bronchial mucus of a CF patient infected with a mixture of mucoid and nonmucoid strains and to those found postmortem in the alveoli of CF patients who had died of a pulmonary infection with a mixture of mucoid and nonmucoid strains. The relationship of the extracellular fibers to the bacterial cell surface indicates that they are a bacterial product, their staining reaction indicates that they are an anionic polymer, and a comparison with the antibody-stabilized exopolysaccharide of cells of a CF strain in pure culture indicates that they form a very extensive enveloping matrix at the bacterial cell surface.

DISCUSSION

The demonstration of the extracellular fibrous polysaccharide matrix formed by many bacteria has been very difficult for the electron microscopist because his techniques require dehydration and the matrix is a very highly hydrated structure (1). In the absence of any stabilizing influences the fibers of the matrix simply collapse back onto the bacterial cell surface, during dehydration, and ruthenium red staining shows them as coarse electron-dense aggregates (Fig. 11). We can cross-link, and thus stabilize, this fine fibrous matrix by reacting it with an excess of antibodies (1, 15) or lectins so that it does not collapse during dehydration, and ruthenium red staining shows an extended system of fine fibers (Fig. 12). The specimen taken from an infected CF patient by bronchoscopy would, almost certainly, contain antibodies against the infecting bacteria (12), and these antibodies may have partially stabilized the fibrillar extracellular matrix seen in Fig. 3 and 4. Similarly, we have shown that rats infected by the agar bead method (2) also produce high levels of antibodies against the infecting strain (unpublished observations), and the fine extracellular fibers surrounding the bacteria in Fig. 7 may also be partially stabilized by these antibodies. Another factor that can stabilize extracellular fibers during dehydration is their connection to more than one point, as when they connect bacterial cells, or when they connect a bacterial cell to a tissue surface and to alveolar detritus (Fig. 2). Thus, the ruthenium red-positive fibrous exopolysaccharide of bacteria presents a whole spectrum of appearances in electron micrographs because it may be tightly condensed into coarse electron-dense aggregates by dehydration (Fig. 1 and 11), it may be partly stabilized by multiple attachment or by antibodies to produce a fibrous matrix after dehydration (Fig. 2, 3, 4, 6 and 7), or it may be fully stabilized by an excess of cross-linking antibodies to maintain a very delicate and extended fibrous matrix (Fig. 12).

The use of electron microscopy, and of stains specific for anionic polymers, in the examination of a large number of natural environments (e.g., soil, water, plant roots, etc.) has shown that bacteria live in adherent fiber-enclosed microcolonies in these natural environments (5) and that these adherent bacterial populations are numerically dominant in many systems (9). Direct examination of the surfaces of many animal organs has also revealed very extensive adherent populations of symbiotic and saprophytic bacteria enmeshed in a fibrous matrix (18). We have

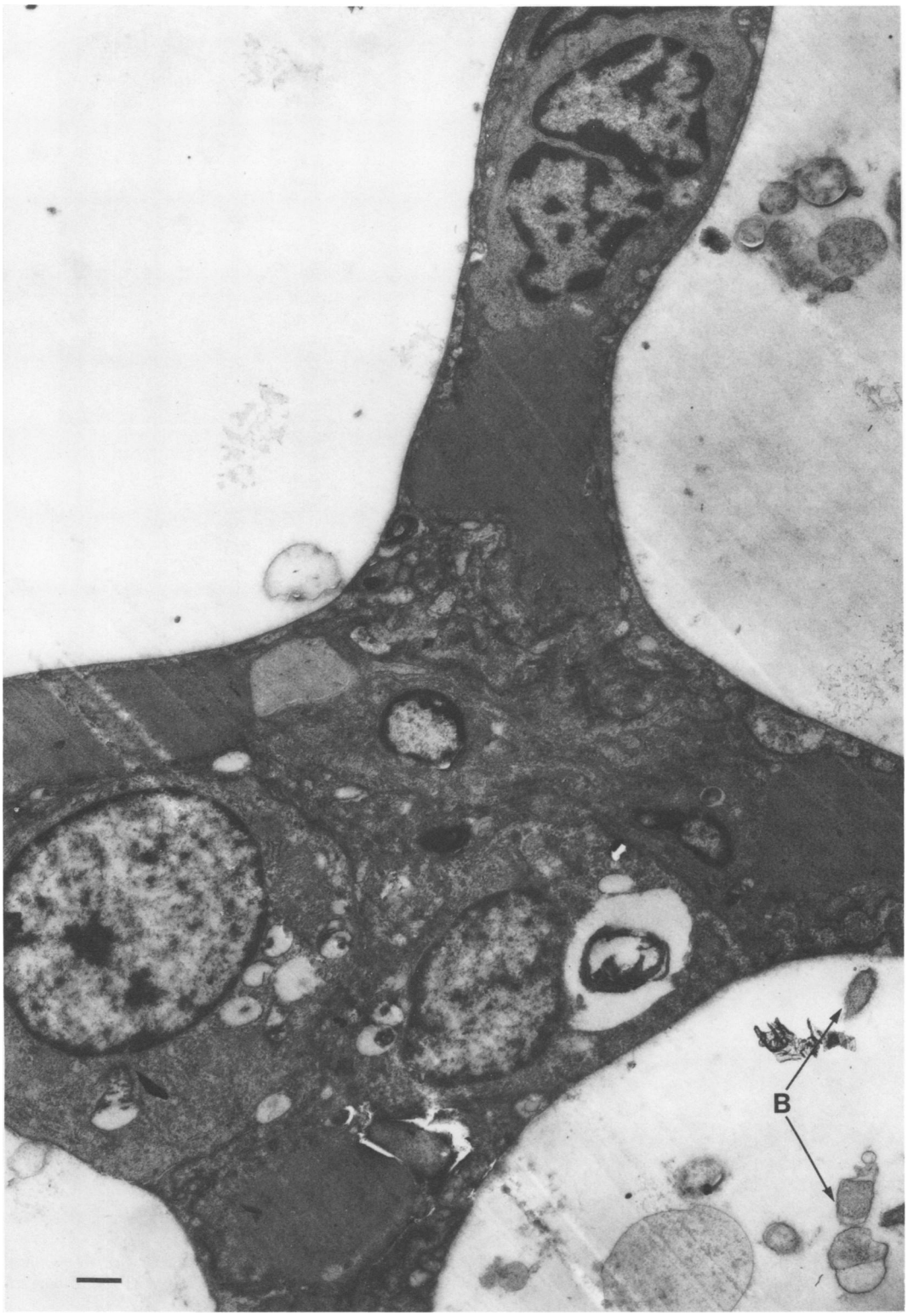
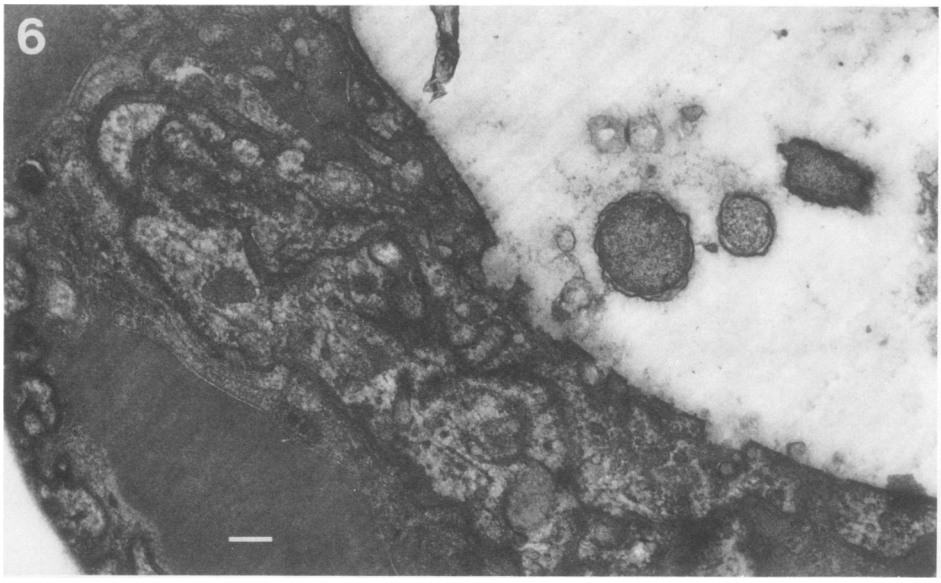


FIG. 5. *Electron micrograph of a thin section of ruthenium red-stained lung parenchyma obtained from a chronically infected rat showing the presence of bacteria (B) in the alveolar spaces. The bar indicates 1 μm.*



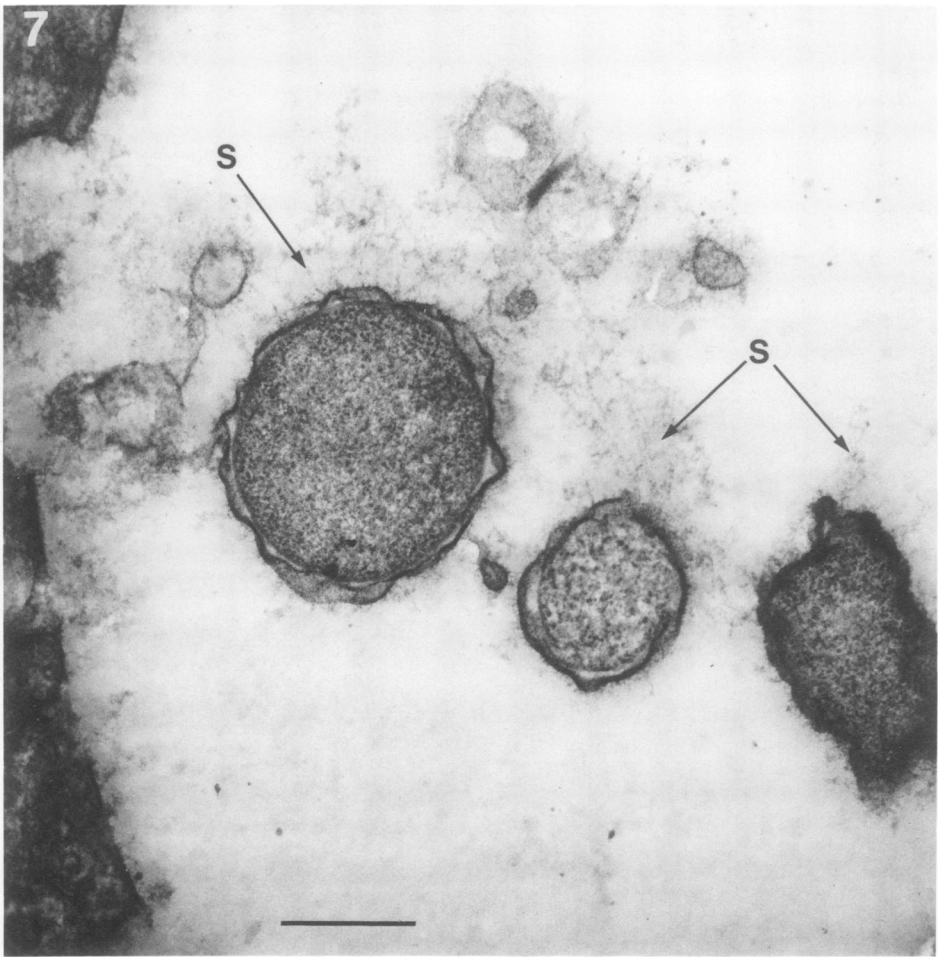


FIG. 6. *Electron micrograph of a thin section of ruthenium red-stained lung tissue obtained from a chronically infected rat showing the presence of a bacterial microcolony in close proximity to the damaged alveolar surface. The bar indicates 0.5 μm.*

FIG. 7. *Higher magnification of the bacterial microcolony seen in Fig. 6 showing the extracellular slime material (S) that binds the microcolony together and the gram-negative nature of the bacterial cell walls. The bar indicates 0.5 μm.*

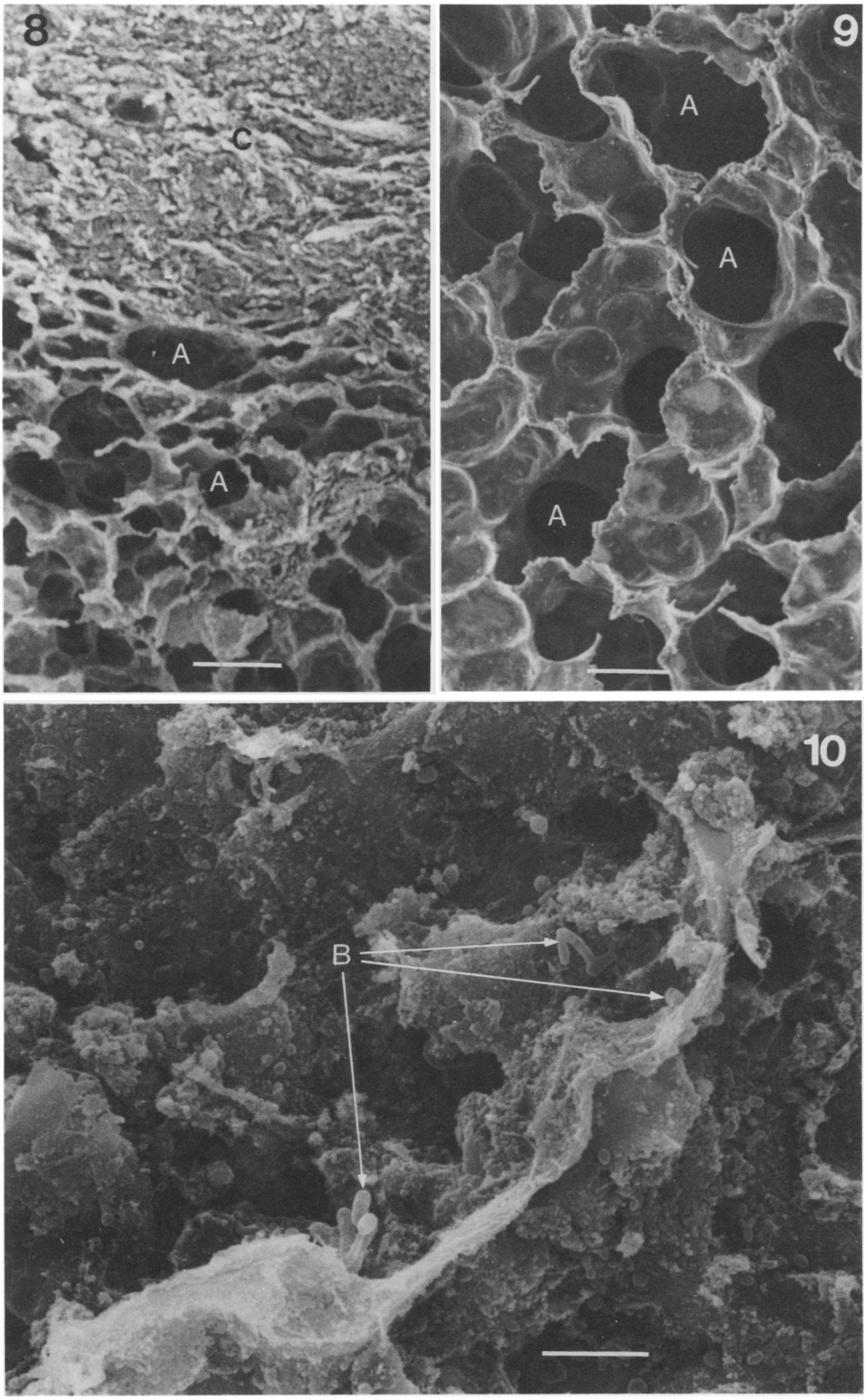
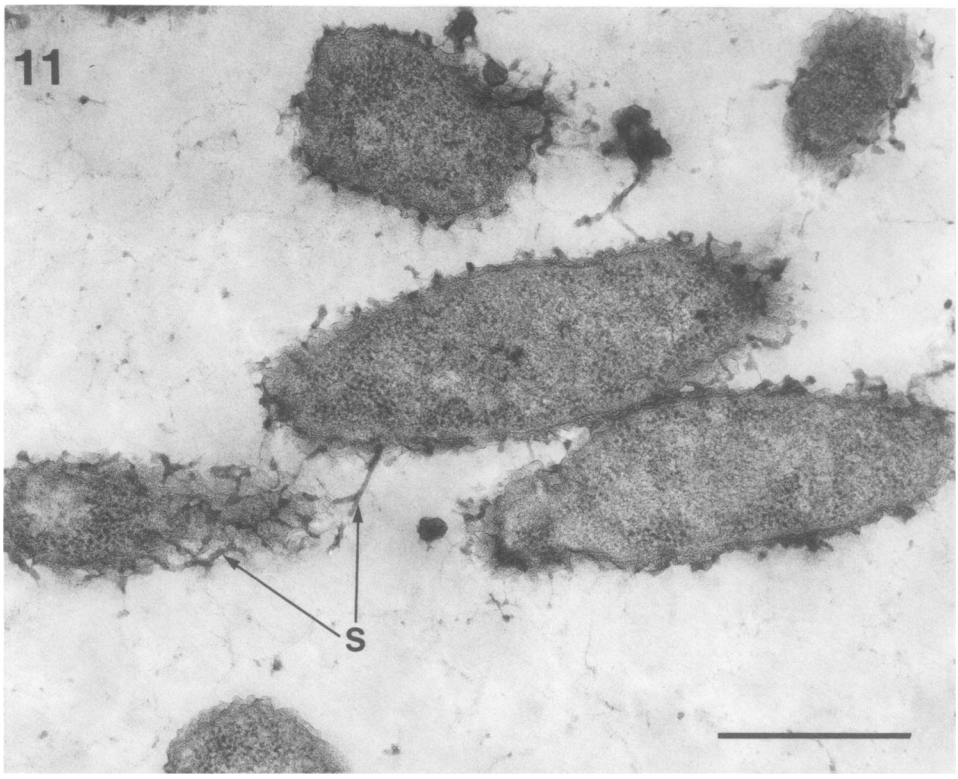


FIG. 8. *Scanning electron micrograph of lung tissue from a chronically infected rat showing the alveolar spaces (A) and the massive congestion (C) caused by the bacterial infection. The bar indicates 100 μm.*

FIG. 9. *Scanning electron micrograph of normal rat lung tissue showing the clear alveolar spaces (A). The bar indicates 100 μm.*

FIG. 10. *Higher magnification scanning electron micrograph of the surface of an alveolus from the lung tissue of a chronically infected rat showing rod-shaped bacterial cells (B) embedded to various degrees in an amorphous mass of slime and exudate. The bar indicates 5 μm.*



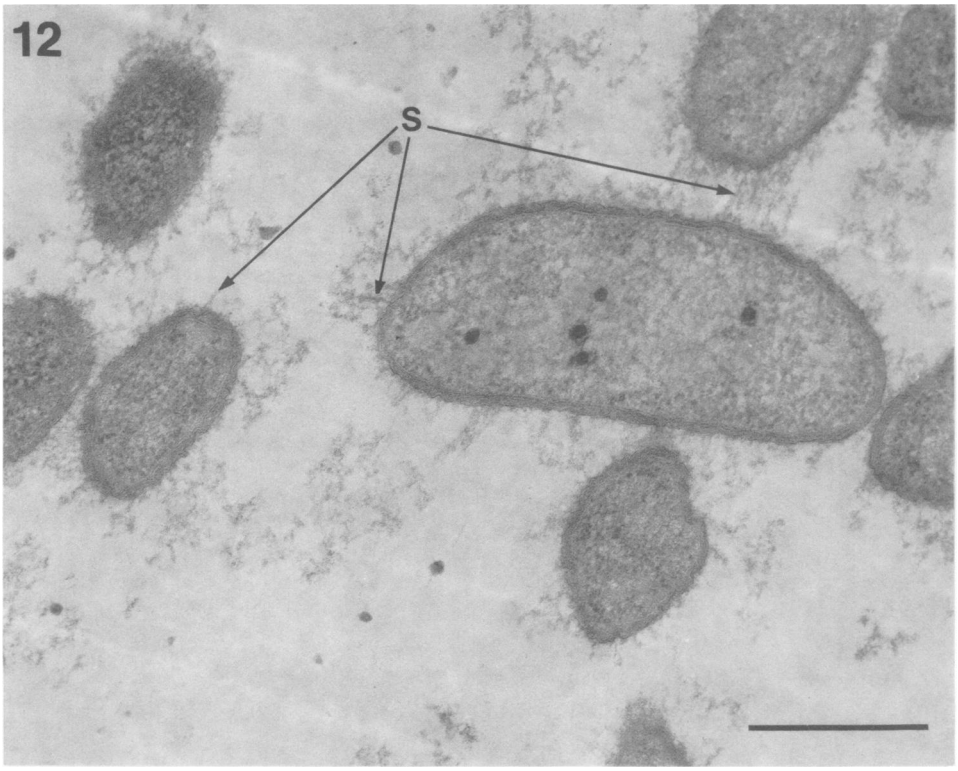


FIG. 11. *Electron micrograph of a thin section of ruthenium red-stained cells of P. aeruginosa (dwarf colony type) grown in MVBM showing coarse aggregates of condensed slime material (S) on the bacterial cell surface. The bar indicates 0.5 μm.*

FIG. 12. *Electron micrograph of a thin section of ruthenium red-stained cells of dwarf colony type of P. aeruginosa treated with antibodies. Here fine fibers of antibody-stabilized slime material (S) are seen to extend from the cell rather than collapsing back onto the cell surface. The bar indicates 0.5 μm.*

previously examined the bacteria in sediments of urines from catheterized patients with urinary tract infections caused by *P. aeruginosa* (17) and found that they were present as large fiber-enclosed microcolonies that were often attached to sloughed epithelial cells. These strains were not mucoid in that they did not produce a slimy colony on brain heart infusion or blood agar, but they produced very large amounts of exopolysaccharide when grown in liquid MVBM and produced so much fibrous material when growing in the urinary tract that the cells were not antibody-coated even though the kidneys were infected and anti-*Pseudomonas* antibodies were present in the urine (17).

The importance of mucoid strains in the development of pulmonary *P. aeruginosa* infections of CF patients has been recognized since 1964 (7), and our recent finding that even nonmucoid CF strains produce large amounts of exopolysaccharide in a suitable medium (unpublished observations) has reinforced the long-standing suggestion that mucoidy is advantageous to the bacteria in this pathological process. We have suggested that mucoidy is selected for because fiber-enclosed microcolonies have increased resistance to phagocytic attack (6) and Govan has shown that pure cultures of mucoid strains have an increased resistance to surfactants (10) and to carbenicillin (11). Indirect evidence for this suggestion is provided by the success of the method of Cash et al. (2) in inducing chronic pulmonary infections in normal rats by coating cells of a normally nonpathogenic strain of *P. aeruginosa* with an artificial polysaccharide matrix (agar) that protects them from host defense mechanisms until they can grow and produce their own protective exopolysaccharides. Once the bacteria are removed from the lung and grown in vitro these selective pressures no longer apply and mucoid CF strains quickly revert to the nonmucoid type (8). We suggest that this reversion may involve the emergence and selection of faster-growing nonmucoid variants (6).

The growth of this opportunistic pathogen, in the form of exopolysaccharide-enclosed microcolonies in the CF lung, may begin because of unusually high levels of Mg^{2+} and Na^{+} in the lung secretions (6) of CF patients, which appear to enhance exopolysaccharide production by *P. aeruginosa* (unpublished data), and this colonization may proceed quickly to overt disease or may persist for several years without serious pathological sequelae.

Perhaps the most important consequence of this perception that the pathogens in this bacterial disease of the CF lung are enclosed in a fibrous exopolysaccharide matrix, which mediates their formation of microcolonies, is that we must assess the efficacy of normal defense mechanisms (phagocytosis, antibodies, surfactants, etc.) and of therapeutic agents (antibiotics) against these bacteria in the same exopolysaccharide matrix-enclosed state that is found in the infected CF lung. We cannot presume that the exopolysaccharide matrix will always be protective of the bacteria (as it is against macrophages, antibodies, surfactants, and carbenicillin) but the simple expedient of using bacterial cells enclosed in exopolysaccharide as the subject of challenge experiments will give results that can be applied to the control of the progressive colonization of the lungs of CF patients by mucoid strains of *P. aeruginosa*.

ACKNOWLEDGMENTS

This work was supported by grants from the Canadian Cystic Fibrosis Foundation, The Thrasher Research Fund (Salt Lake City), and the National Science and Engineering Research Council of Canada.

We thank Mary Jane Thomassen for her advice, for the gift of *P. aeruginosa* cultures, and for processing the material obtained by bronchoscopy. We are most grateful for the skilled technical assistance of Dale Cooper and Beverly Bartuccio and for the photographic work of Sheila Costerton.

LITERATURE CITED

1. **Bayer, M. E., and H. Thurow.** 1977. Polysaccharide capsule of *Escherichia coli*: microscope study of its size, structure, and sites of synthesis. J. Bacteriol. **130**:911-936.
2. **Cash, H. A., D. E. Woods, B. McCullough, W. G. Johanson, Jr., and J. A. Bass.** 1979. A rat model of chronic respiratory infection with *Pseudomonas aeruginosa*. Am. Rev. Respir. Dis. **119**:453-459.
3. **Cohen, A. L., D. P. Marlow, and G. E. Garner.** 1968. A rapid critical point method using fluorocarbons ("Freons") as intermediate and transitional fluids. J. Microsc. (Paris) **7**:331-342.
4. **Costerton, J. W., J. M. Ingram, and K.-J. Cheng.** 1974. Structure and function of the cell envelope of gram-negative bacteria. Bacteriol. Rev. **38**:87-110.
5. **Costerton, J. W., G. G. Geesey, and K.-J. Cheng.** 1978. How bacteria stick. Sci. Am. **238**:86-95.
6. **Costerton, J. W., M. R. W. Brown, and J. M. Sturgess.** 1979. The cell envelope: its role in infection in *Pseudomonas aeruginosa*, p. 41-62. *In* R. G. Doggett (ed.), Clinical manifestations of infection and current therapy. Academic Press Inc., New York.
7. **Doggett, R. G., G. M. Harrison, and E. S. Wallis.** 1964. Comparison of some properties of *Pseudomonas aeruginosa* isolated from infections of persons with and without cystic fibrosis. J. Bacteriol. **87**:427-431.
8. **Doggett, R. G., G. M. Harrison, and R. E. Carter, Jr.** 1971. Mucoid *Pseudomonas aeruginosa* in patients with chronic illness. Lancet **i**:236-237.
9. **Geesey, G. G., R. Mutch, J. W. Costerton, and R. B. Green.** 1978. Sessile bacteria: an important component of the microbial population in small mountain streams. Limnol. Oceanogr. **23**:1214-1223.
10. **Govan, J. R. W.** 1975. Mucoid strains of *Pseudomonas aeruginosa*: the influence of culture medium on the stability of mucus production. J. Med. Microbiol. **8**: 513-522.
11. **Govan, J. R. W., and J. A. M. Fyfe.** 1978. Mucoid *Pseudomonas aeruginosa* and cystic fibrosis: resistance

of the mucoid form to carbenicillin, flucloxacillin, and tobramycin and the isolation of mucoid variants *in vitro*. J. Antimicrob. Chemother. **4**:233–240.

12. **Høiby, N.** 1974. *Pseudomonas aeruginosa* infections in cystic fibrosis: relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral immune response. Acta Pathol. Microbiol. Scand. **82**:551–558.
13. **Kulczycki, L. L., T. M. Murphy, and J. A. Bellanti.** 1978. *Pseudomonas* colonization in cystic fibrosis: a study of 160 patients. J. Am. Med. Assoc. **240**:30–34.
14. **Luray-Cuasay, L. R., K. R. Kundy, and N. H. Huang.** 1976. *Pseudomonas* carrier rates of patients with cystic fibrosis and of members of their families. J. Pediatr. **89**: 23–26.
15. **Mackie, E. B., K. N. Brown, J. Lam, and J. W. Costerton.** 1979. Morphological stabilization of the capsules of group B streptococci, types Ia, Ib, II and III with specific antibody. J. Bacteriol. **138**:609–617.
16. **Malick, L. E., and R. B. Wilson.** 1975. Modified thiocarbohydrazide procedure for scanning electron microscopy: routine use for normal, pathological or experimental tissues. Stain Technol. **50**:265–269.
17. **Marrie, T. J., G. K. M. Harding, A. R. Ronald, J. Dikkema, J. Lam, S. Hoban, and J. W. Costerton.** 1979. Influence of mucoidy on antibody coating of *Pseudomonas aeruginosa*. J. Infect. Dis. **139**:357–361.
18. **McCowan, R. P., K.-J. Cheng, C. B. M. Bailey, and J. W. Costerton.** 1978. Adhesion of bacteria to epithelial cell surfaces within the reticulorumen of cattle. Appl. Environ. Microbiol. **35**:149–155.
19. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17**:208–212.
20. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **219**:97–106.