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Morphological Examination of the Glycocalyces of *Staphylococcus aureus* Strains Wiley and Smith

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The glycocalyces of gram-positive bacteria have only been studied to a limited extent, with most studies being directed at the elucidation of capsules. With modern methods of electron microscopy, it has been shown that an extensive, diffuse polyanionic matrix surrounds *Staphylococcus aureus* cells of the Smith and Wiley strains, both in vivo and in modified staphylococcus 110 media. This slime layer was extracapsular in the case of the Smith strain, yet appeared to be the only layer peripheral to the teichoic acid in the Wiley strain. It is proposed that these glycocalyces serve a protective function and that their production is induced not only by excess nutrients in the growth medium but also by metabolic stress.

Staphylococcal capsules and glycocalyces have been studied since the discovery of variant colonies in long-term broth cultures by Bigger et al. (2). Several major papers have appeared on this subject (21, 23, 32), with the first being a definitive review. Since that time, the intensive study of staphylococcal capsules as a virulence factor has proceeded at a rapid rate. The study of staphylococcal slime, however, has had little direct investigation and has all but been undifferentiated from the study of capsules. Studies on staphylococcal cell envelopes have been concerned with the detection of capsules by the use of fluorescent antibodies (34), serum-soft agar diffuse growth (8, 20, 30, 33), and phage nontypability (24, 29), as well as India ink halos, quellung reactions, and isolation of capsular materials with associated serological examinations. Visualization of capsular materials by electron microscopy has been accomplished to some extent (15, 17), with the best preservation of slime layers being presented by Cagle (3).

It has been the finding of this laboratory that slime layers play an important role in pathogenesis (5, 7, 7a, 12, 14). This finding, coupled with improved techniques for the visualization of slime layers (1, 6, 13), prompted the current investigation into the occurrence of *Staphylococcus aureus* slime and its role in pathogenesis.

MATERIALS AND METHODS

Bacterial strains and growth media. *S. aureus* Smith strain, isolated from the sputum of a patient with cystic fibrosis, was the kind gift of K. N. Brown, University of Calgary, Calgary, Alberta. *S. aureus* Wiley strain was kindly provided by B. B. Wiley, University of Utah, Salt Lake City. A battery of freeze-dried preparations and agar slants of each cell type were made after initial growth in both tryptic soy (TS) broth (Difco Laboratories) and modified staphylococcus 110 (mS110) broth (31), additionally modified as follows: 2.5 g of yeast extract (Difco) and 10 g of peptone (Difco) in 50 ml of 3.0% NaCl–0.05 M phosphate buffer (pH 7.4) was dialyzed against 950 ml of the same buffer for 48 h at 4°C. The dialysate was autoclaved and supplemented with filter-sterilized mannitol and lactose (in a 10×-concentrated solution of the same dialysate) to 1.0 and 0.2% final concentrations, respectively. TS agar (Difco) and addition of agar to the mS110 broth to a final concentration of 1.5% were used when solid media were preferred. Lyophilized cells were stored at −20°C and agar slants were stored at −70°C after rapid freezing in liquid nitrogen.

Preparation of bacterial immunogenes. Both bacterial strains were grown in mS110 broth for 8 h at 37°C and 120 rpm in a New Brunswick shaker-incubator and formalinized to a concentration of 0.5% for 48 h. The cells were then centrifuged at 4,000 × *g* for 15 min, washed three times with sterile phosphate-buffered saline (PBS), and finally resuspended in 1/10 of the original volume. Killing was checked by plating a 0.2-ml sample of this final suspension. The immunogens were quick-frozen in liquid nitrogen and stored at −70°C in 1-ml samples.

Preparation of slime immunogens. Slime was prepared by a gentle procedure to retain as much original immunogenic character as possible. Both bacterial strains were grown in mS110 broth for 8 h at 37°C and 120 rpm and centrifuged at 10,000 × *g* for 30 min at 4°C. The 8-h growth, under the conditions described for the preparation of both bacterial and slime immunogens, was chosen due to previous studies (unpublished data) which showed the absence of cell lysis at this point in the growth curve as determined by lack of glucose 6-phosphate dehydrogenase, NADH oxidase, succinate dehydrogenase (11), and β-galactosidase activities in the supernatant of growing cultures. The supernatant was then dialyzed against glass-distilled water for 24 to 36 h at 4°C followed by concentration

by dialysis against 20,000-molecular-weight polyethylene glycol (10% solution) overnight. The concentrate was then recentrifuged under the original conditions and allowed to reach 37°C in an incubator. The concentrate was sterile as checked by plating a 0.2-ml sample at this point. RNase and DNase (bovine, pancreatic; Sigma Chemical Co.) were added to a concentration of 0.5 mg/ml and allowed to incubate for 3 h. Pronase (Calbiochem-Behring) was then added to a concentration of 0.1% and allowed to incubate for 5 to 6 h. The concentrate was then dialyzed against glass-distilled water for 24 to 36 h at 4°C and subsequently lyophilized and stored at −10°C. Slime was reconstituted in PBS to a concentration of 1 mg/ml before inoculation.

Preparation of antisera. New Zealand white rabbits were immunized by intramuscular inoculation on days 1, 3, 8, and 10 with 0.5 ml of a 1:1 (vol/vol) mixture of immunogen with Freund incomplete adjuvant (Difco). They were then inoculated weekly for 15 weeks and subsequently given booster injections every 2 weeks: 3 days before marginal ear vein bleeding, which routinely yielded 10 to 15 ml of whole blood from which the serum was separated and quickly frozen in liquid nitrogen before storage in 1-ml samples at −70°C. Antisera were heat inactivated at 56°C for 0.5 h before use. Response of the rabbits was determined by both bacterial agglutination tests (9) and counterimmunoelectrophoresis, as described previously (13), except that Gel Bond film (Marine Colloids) was used as the support film and barbituric acid-sodium barbital buffer was used (ionic strength, 0.0375 M). Preimmune sera were checked for response to both bacterial strains as well as to slimes by these two methods and were found to be nonreactive. Antiserum was used when antibody titers of 1/5,120 or higher were found by bacterial agglutination tests or with positive banding in counterimmunoelectrophoresis with a 1/20 serum dilution run against whole-cell antigen as well as slime. Antisera were absorbed with the partially purified slime from the parent strain by addition of slime to a 5-ml volume of heat-inactivated serum to a final concentration of 10 mg/ml. This mixture was then incubated at 37°C for 0.5 h followed by centrifugation at 40,000 × *g* for 0.5 h.

Plate passage. Cells were grown on mS110 and TS agars for a minimum of five serial passages followed by growth on the other medium as well as on brain heart infusion agar for eight serial passages, with embeddings being performed on cells from each of these latter passages.

Animal passage. The method of infection used was essentially that of Cash et al. (4), although agar beads were not utilized in this study. In short, young male Sprague-Dawley rats (ca. 250 g) were tracheotomized under ether, and a bacterial suspension of about 10^5 colony-forming units in 0.075 ml of PBS was deposited in the lower lobe of the left lung. Infected animals were sacrificed at intervals, and bacteria were recovered by removing whole lungs under sterile conditions and blending them in PBS in sterile Waring blenders, followed by dilution and plating on TS agar.

Preparation for electron microscopy. (i) Thin section. Cells were grown in either TS or mS110 broth or on similar plates and were harvested by centrifugation at 4,000 × *g* for 15 min or by scraping off the plates and suspending cells in PBS. Cells were washed twice in PBS followed by a final centrifugation. Antibody stabilization, when performed, was accomplished by addition of 3 to 5 ml of heat-inactivated antisera to 1 ml of sedimented cells followed by frequent mixing for 1 h. The cells were then washed twice in PBS and subjected to prefixation, fixation, dehydration, and embedding as outlined previously (13), with the exception of the bacteria isolated after animal passage. These cells were processed in 0.1 M Sym-Collidine buffer (Polysciences Inc., Warrington, Pa.), pH 7.4.

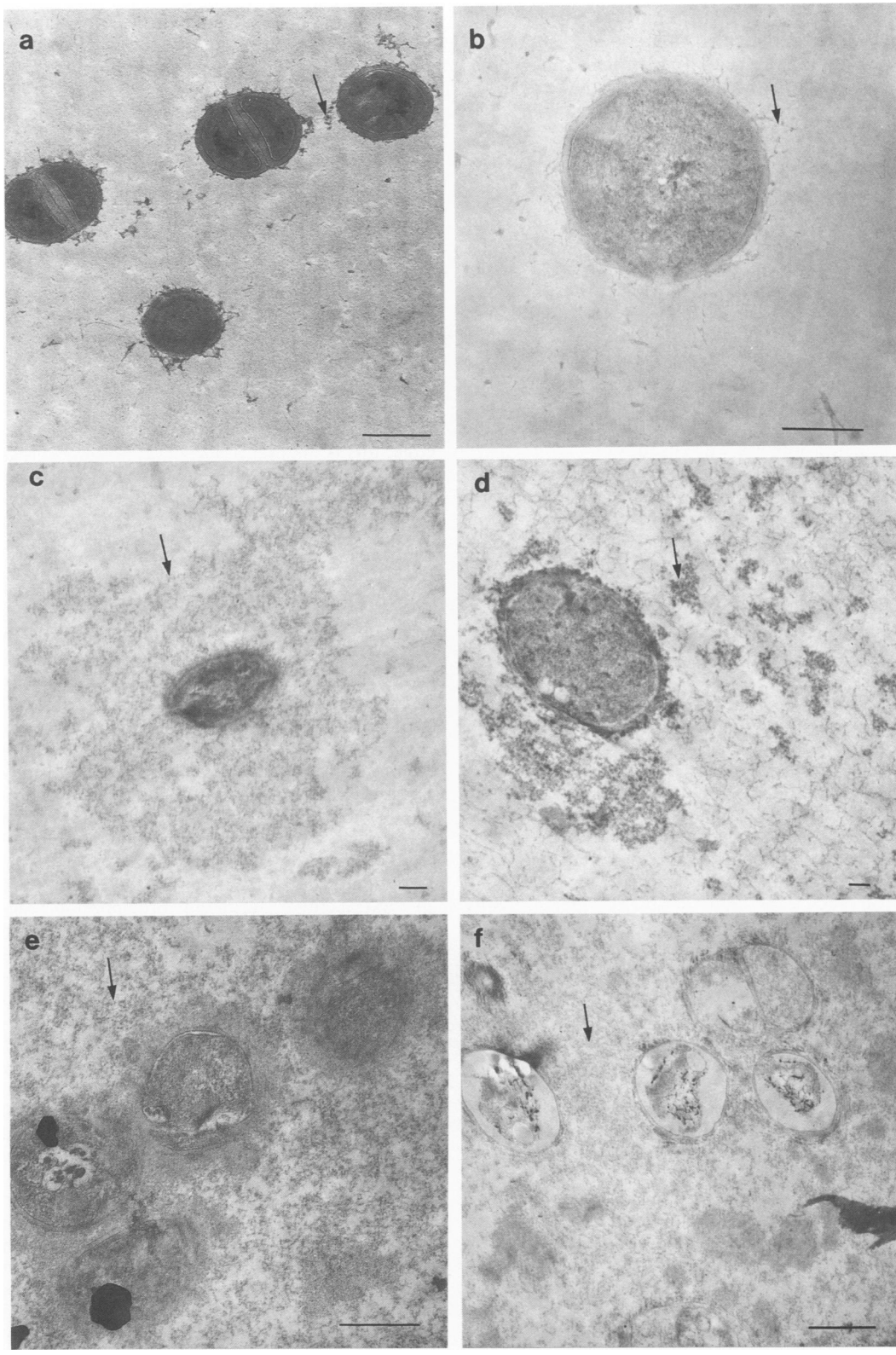
(ii) Critical point drying. For critical point drying, cells were grown and washed as for thin sectioning. Antibody stabilization was not performed. The procedure followed was that described previously (13) of dehydration, drying, and shadow casting.

RESULTS

The routine techniques used in the fixation of cells for thin-section examination, when performed carefully, allowed for the preservation of some extracellular slime (Fig. 1a and b). The remainder of the glycocalyx, however, is obviously torn away, as well as, in some circumstances, collapsed onto the cell surface (13). The partial integrity of the glycocalyx of the Smith strain (Fig. 1a) was much more readily preserved than that of the Wiley strain. This indicates that the part of the glycocalyx of the Smith strain closest to the cell wall is denser and more stable than that of the Wiley strain. Thus, the rigid, integral capsule of the Smith strain can be contrasted to the loose, peripheral slime of the Wiley strain (Fig. 1f) and its own, outermost slime layer (Fig. 1e).

The studies on medium-dependent slime production indicate that the Wiley strain produces extensive slime more readily and continues slime production for a greater number of transfers than does the Smith strain. That is, the Smith strain, once induced to produce slime on mS110 agar, lost most slime production within two transfers on TS agar plates and, in the converse experiment, gained significant slime production upon two to three subcultures on mS110 plates. The loss of slime production upon subculture to brain heart infusion agar plates was similar to that found for TS agar plates, although slime production was lost even more readily, i.e., within a single transfer for the Smith strain and within five transfers for the Wiley strain.

FIG. 1. Electron micrographs of thin sections of *S. aureus* cells grown in mS110 media. (a) Smith and (b) Wiley strain cells unstabilized; (c) Smith and (d) Wiley strain cells stabilized with whole-cell homologous antiserum; (e) Smith and (f) Wiley strain cells stabilized with slime-specific homologous antiserum. Arrows show glycocalyces. Bars, 0.5 μm.


a
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The antibody stabilization of both the Smith and Wiley strains shows a complex glycocalyx surrounding the cells (Fig. 1c and d) as well as much loose, although coherent, material in the space between cells. Stabilization with slime-specific antisera showed cells to be associated with one another by this complex, anionic matrix (Fig. 1e and f). In fact, the glycocalyx was so extensive that areas where this material was interrupted by embedding resin had to be searched for to show that the slime was not a part of the embedding material or a similar artifact. Cells stabilized with antiserum which had been absorbed by homologous slime showed much less stabilization of the extensive slime, although some stabilization of cell wall layers close to the cells was observed. The flocculation observed upon addition of both whole-cell and slime-specific antisera was not found when the absorbed antisera were used.

Collapse of the glycocalyx onto the cell surface was not readily observed with the Smith strain regardless of antibody concentrations used in stabilization (Fig. 1a versus 1c or e). Collapse and aggregation of the slime layer of the Wiley strain (Fig. 2e) was found to be due to a less than optimal antibody concentration in a particular stabilization mixture. Due to the extensive slime production by the Wiley strain, much high-titered serum had to be used to effectively stabilize the glycocalyx. These results coincide with those of Bayer and Thurow (1) in the study of *Escherichia coli* capsule stabilization.

Slime induced by in vivo passage of both staphylococcal strains appeared similar to that induced by mS110 medium (Fig. 2a and b). The Wiley strain produced more material and a looser, more amorphous slime (Fig. 2), whereas that produced by the Smith strain, although probably less in amount, was tighter and more adherent to the cell (Fig. 2b). Visualization of the glycocalyces by thin section was not optimal because of a lack of specific antiserum production by the rats, as judged by counterimmunoelectrophoresis. The slime was thus unable to be stabilized and only fortuitously residual slime could be seen, much as in Fig. 1a and b.

In an effort to envisage unstabilized slime, the technique of critical point drying was used. The technique involves much less cell manipulation as well as minimal dehydration and drying stress. The three-dimensional nature of the slime is somewhat recreated by shadow casting, although its extensive nature is diminished by slime fibers collapsing upon one another during dehydration. It is clearly seen by a comparison of Fig. 3 and 4 that slime production is morphologically very similar whether induced in vivo or by specialized media in vitro.

During the in vivo and in vitro slime induction experiments it was found that rod-shaped, gram-positive cells were present in samples from the Wiley strain (Fig. 2c and d). These atypical cell types were never found on brain heart infusion or TS agar or in similar broths but rather only from animal passage (single TS agar culture) or on mS110 medium. We have resolved that it is not a contaminant but rather a cell type whose presence coincides with slime production. Dimensions for these cells were typically 0.75 to 1.0 μm by 10 to 20 μm. They resemble cells of *S. aureus* which have been subjected to subinhibitory levels of β-lactam antibiotics such that those penicillin-binding proteins associated with septum formation and related cell wall production are inhibited in their actions (V. Lorian and B. Davis, personal communication), thus leading to elongate cell formation. To our knowledge, this is the first report of atypical *S. aureus* cells of the Wiley strain shown to grow on acceptable laboratory media without antibiotics present.

DISCUSSION

S. aureus capsules have previously been envisioned by negative staining-particle exclusion methods as well as by quellung reactions. A number of inconsistencies and controversies have arisen out of these studies which can now be resolved due to a better understanding of cell walls and glycocalyces. Mudd and DeCourcy (16) found the polysaccharide antigen from the Smith strain to be chemically and serologically different from that of the Wiley strain and proposed that the viscid colony strains (such as the Wiley strain) are not encapsulated. They proposed calling the abnormal, collapsing quellung reaction noted as occurring with the Wiley strain an "extracellular peripheral precipitation reaction." This group found and the Wiley group proved that the Smith strain is truly encapsulated and is the prototype of encapsulated coagulase-positive staphylococci. The Wiley group defended and upheld their finding of an abnormal but reproducible capsular swelling in the Wiley strain (21). Wiley also forwarded the proposition that pseudocapsules (those extensive "capsules" found after growth in mS110 medium) were purely a laboratory artifact and unimportant for studies pertaining to natural systems.

We have found that, although the remnant capsule of the Smith strain is able to exclude particles and exhibit capsular swelling, in an in vivo system as well as in mS110 medium, an extracapsular or at least a more extensive diffuse slime layer is produced. This slime layer, which could not be examined previously, is shown by electron microscopy to be gradually lost upon

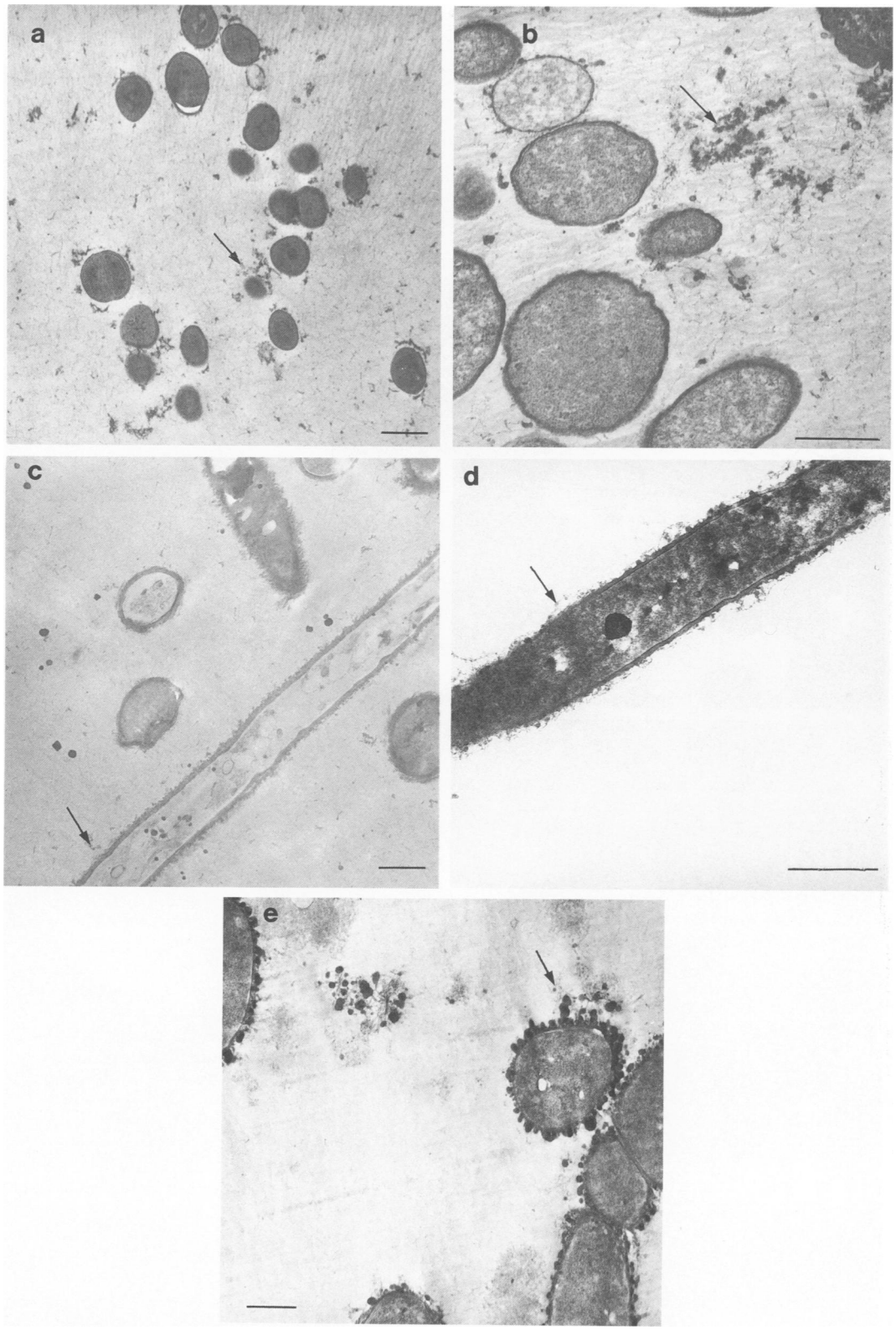

FIG. 2. Electron micrographs of thin sections of *S. aureus* cells. (a) Smith and (b) Wiley strain cells isolated from rat lungs; (c) Wiley strain atypical cell isolated from rat lungs; (d) Wiley strain atypical cell after growth in mS110 media; (e) Wiley strain cells stabilized 1:1 (vol/vol) with homologous whole-cell antiserum. Arrows show slime layers. Bars, 0.5 μm.

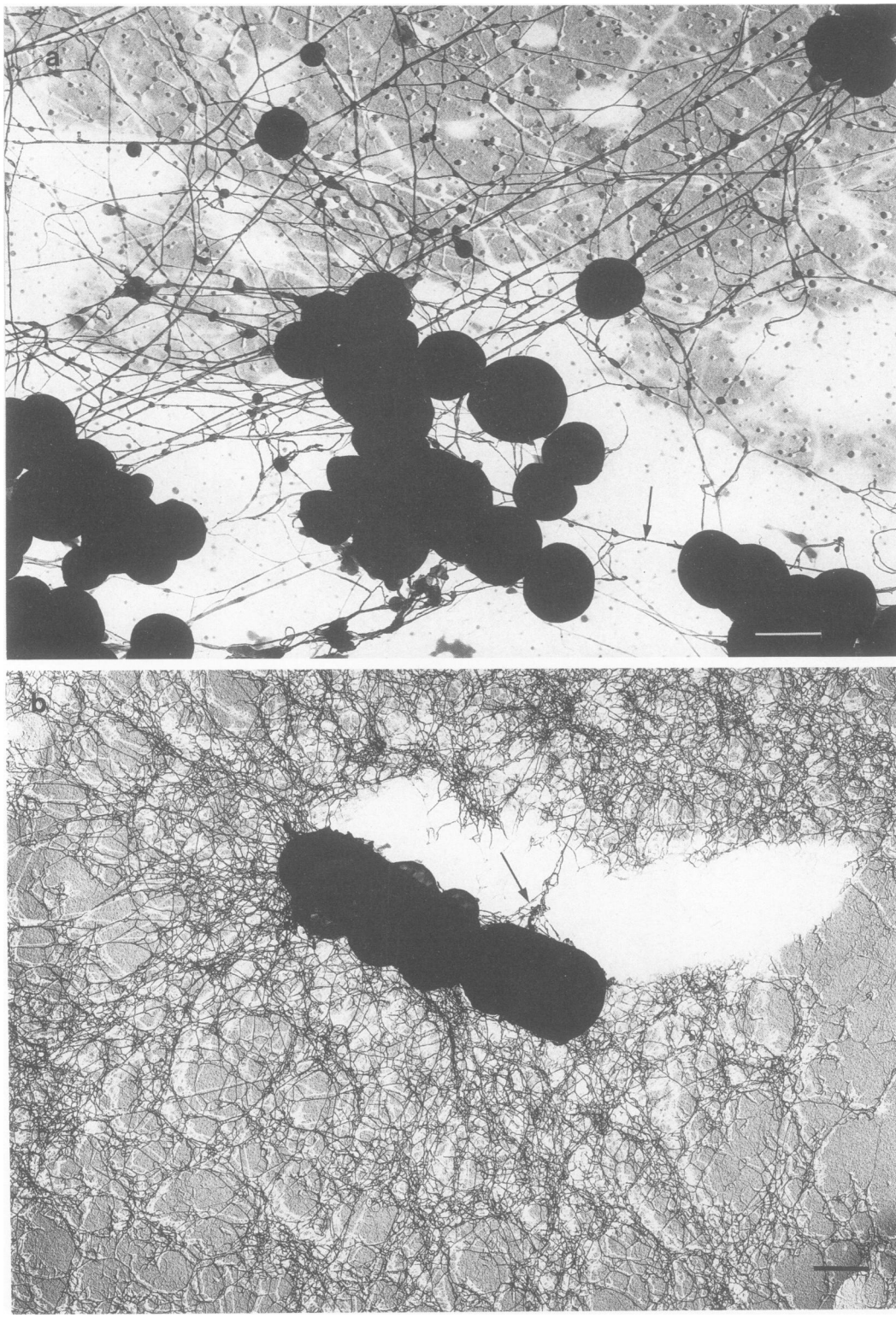

FIG. 3. Electron micrographs of critical point-dried preparations of *S. aureus* cells grown in mS110 media. (a) Smith strain; (b) Wiley strain. Arrows show slime layers. Bars, 0.5 μm.

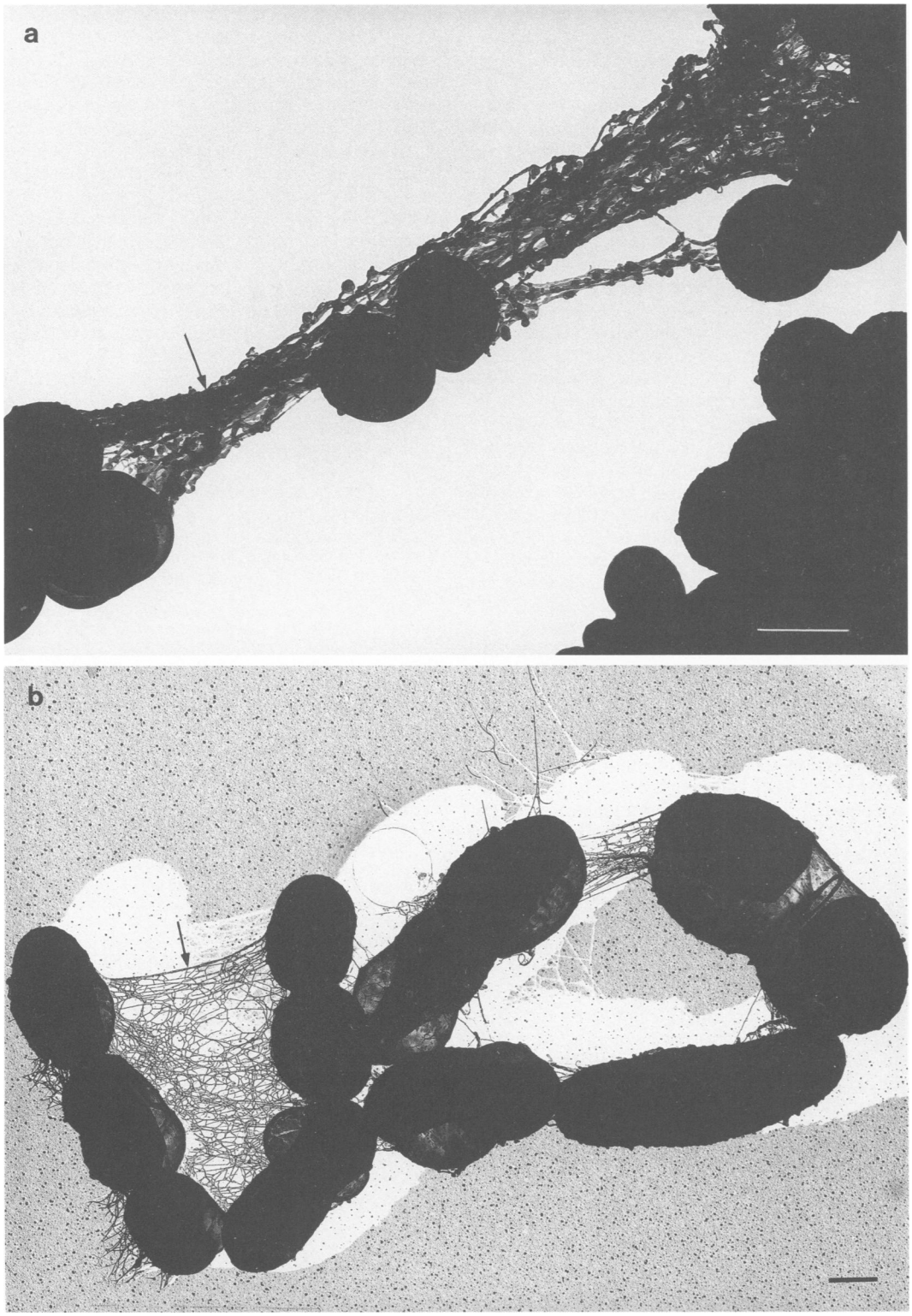

FIG. 4. Electron micrographs of critical point-dried preparations of *S. aureus* cells isolated from rat lungs. (a) Smith strain; (b) Wiley strain. Arrows show slime layers. Bars, 0.5 μm.

subculture in nonslime-inducing media, leaving the remnant capsule about Smith strain cells. The Wiley strain exhibits a very similar slime layer; however, there is no evidence for a rigid capsule about these cells, although slime production continues for extended subculture periods. The atypical capsular swelling noted previously with this strain is probably due to the looser nature of this slime layer in comparison with the remnant capsule of the Smith strain. The slime layer is inefficient at particle exclusion and has a tendency to collapse when antibody concentration is insufficient for complete stabilization (Fig. 2e). It is noteworthy that the antibody concentration in specific antisera may be high when tested against whole cells, yet slime stabilization may not be found. This is due to, first, the very large concentration of immunoglobulins necessary for cross-linking of the extensive fibrous matrix and, second, the relatively low concentration of slime-specific immunoglobulins in antisera raised against whole cells irrespective of the vast amounts of slime about the whole-cell immunogens.

These findings indicate that the slime layer serves a function similar to that previously ascribed to the "classic" capsule. That is, first, antibody is less readily raised to the polysaccharide matrix than to other cell wall components. Second, the slime may interfere with phagocytosis by polymorphonuclear leukocytes by masking the peptidoglycan (18, 19), enveloping the teichoic acid which has been shown to enhance opsonization (26), and consuming complement (25) as well as covering and possibly altering the configuration of bound complement, namely, the C3b fragment (27, 28). Third, phagocytosis by macrophages as well as by polymorphonuclear leukocytes would be inhibited by the large size of an aggregated clump of staphylococci joined by a complex matrix of slime fibers. This formation of microcolonies is analogous to that found with *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis (12).

The formation of microcolonies by the gram-positive *S. aureus* may be even more important for bacterial survival than this phenomenon is to the gram-negative bacteria due to the purely extracellular nature of many of the gram-positive bacteria's life processes. Due to the lack of a periplasmic space, the gram-positive bacterium must "export" a great number of enzymes into the external medium. It would obviously be of great metabolic value to be able to sequester these proteins near the cells producing them to be able to recover usable metabolites with minimal metabolic expenditure. This may also explain, to some extent, not only the production of slime when a large amount of nutrient is present, but also the need for some sort of metabolic "stress" on the cells. This stress may be caused by in vivo growth or some factor in the mS110 medium. It is known that cells grown at elevated temperatures in high salt concentrations clump, show irregular septation, and produce capsules (10). The atypical cell types of the Wiley strain grown in vivo (Fig. 2c) and in mS110 medium (Fig. 2d) may be indications of the metabolic stress placed upon these cells in these environments. The protection afforded the cell by a slime layer may not be as complete as that given by a tight capsule, and this may be the reason for the atypical cell types seen only with the Wiley strain. The slime layer, however, must afford additional protection to cells over that of a tight capsule, since extracapsular slime is produced by the Smith strain when cells are subjected to difficult growth conditions. Studies are under way to define the nature of the protection afforded by the glycocalyces of the staphylococci.

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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. **Bigger, J., C. Boland, and R. O'Meara.** 1927. Variant colonies of *S. aureus*. J. Pathol. **30**:261–269.
3. **Cagle, G.** 1975. Fine structure and distribution of extracellular polymer surrounding selected aerobic bacteria. Can. J. Microbiol. **21**:395–408.
4. **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.
5. **Cheng, K.-J., and J. W. Costerton.** 1980. The formation of microcolonies by rumen bacteria. Can. J. Microbiol. **26**:1104–1113.
6. **Costerton, J. W.** 1980. Some techniques involved in study of adsorption of microorganisms to surfaces, p. 403–423. *In* G. Bitton and K. C. Marshall (ed.), Adsorption of microorganisms to surfaces. John Wiley & Sons, Inc.
7. **Costerton, J. W., G. G. Geesey, and K.-J. Cheng.** 1978. How bacteria stick. Sci. Am. **238**:86–95.

7a. **Costerton, J. W., R. T. Irvin, and K.-J. Cheng.** 1981. The role of bacterial surface structures in pathogenesis. Crit. Rev. Microbiol **8**:303–338.

8. **Finkelstein, R., and S. Sulkin.** 1958. Characteristics of coagulase-positive and coagulase-negative staphylococci in serum-soft agar. J. Bacteriol. **75**:339–344.
9. **Garvey, J. S., N. E. Cremer, and D. H. Sussdorf (ed.).** 1977. Methods in immunology, 3rd ed., p. 149–152. W. A. Benjamin Inc., Reading, Mass.
10. **Hurst, A., A. Hughes, and R. Pontefract.** 1980. Mechanisms of the temperature protective effect of salts on

Staphylococcus aureus. Can J. Microbiol. **26**:511–517.
11. **Khouw, B, and H. McCurdy.** 1969. Tricarboxylic acid cycle enzymes and morphogenesis in *Blastocladiella emersonii*. J. Bacteriol. **99**:197–205.
12. **Lam, J., R. Chan, K. Lam, and J. W. Costerton.** 1980. Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. Infect. Immun. **28**:546–556.
13. **Mackie, E. B., K. N. Brown, J. Lam, and J. Costerton.** 1979. Morphological stabilization of capsules of group B streptococci, types Ia, Ib, II, and III, with specific antibody. J. Bacteriol. **138**:609–617.
14. **Marrie, T. J., J. Lam, and J. W. Costerton.** 1980. Bacterial adhesion to uroepithelial cells: a morphologic study. J. Infect. Dis. **142**:239–246.
15. **Melly, M., L. Duke, D. Liau, and J. Hash.** 1974. Biological properties of the encapsulated *Staphylococcus aureus* M. Infect. Immun. **10**:389–397.
16. **Mudd, S., and S. DeCourcy.** 1965. Interaction of viscid material of *Staphylococcus aureus* with specific immune serum. J. Bacteriol. **89**:874–879.
17. **Ohtomo, T., and K. Yoshida.** 1978. Encapsulation by transformation of strains of *S. aureus* determined by the serum-soft agar technique. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **242**:436–445.
18. **Peterson, P., Y. Kim, B. Wilkinson, D. Schmeling, A. Michael, and P. Quie.** 1978. Dichotomy between opsonization and serum complement activation by encapsulated staphylococci. Infect. Immun. **20**:770–775.
19. **Peterson, P., B. Wilkinson, Y. Kim, D. Schmeling, and P. Quie.** 1978. Influence of encapsulation on staphylococcal opsonization and phagocytosis by human polymorphonuclear leukocytes. Infect. Immun. **19**:943–949.
20. **Schimazu, T., B. W. Griffiths, and L. Greenberg.** 1963. Serological and immunological studies with Staphylococcal strains using the soft agar technique. Can. J. Microbiol. **9**:93–101.
21. **Wiley, B.** 1972. Capsules and pseudocapsules of *Staphylococcus aureus*, p. 41–63. *In* J. Cohen (ed.), The staphylococci. John Wiley & Sons, Inc., New York.
22. **Wiley, B., and N. Maverakis.** 1968. Virulent and avirulent encapsulated variants of *Staphylococcus aureus*. J. Bacteriol. **95**:1003–1010.
23. **Wiley, B. B., and N. H. Maverakis.** 1974. Capsule production and virulence among strains of *Staphylococcus aureus*. Ann. N.Y. Acad. Sci. **236**:221–232.
24. **Wilkinson, B., and K. Holmes.** 1979. *Staphylococcus aureus* cell surface: capsule as a barrier to bacteriophage adsorption. Infect. Immun. **23**:549–552.
25. **Wilkinson, B. J., Y. Kim, and P. K. Peterson.** 1981. Factors affecting complement activation by *Staphylococcus aureus* cell walls, their components, and mutants altered in teichoic acid. Infect. Immun. **32**:216–224.
26. **Wilkinson, B. J., Y. Kim, P. K. Peterson, P. G. Quie, and A. F. Michael.** 1978. Activation of complement by cell surface components of *Staphylococcus aureus*. Infect. Immun. **20**:388–392.
27. **Wilkinson, B., P. Peterson, and P. Quie.** 1979. Cryptic peptidoglycan and the antiphagocytic effect of the *Staphylococcus aureus* capsule: model for the antiphagocytic effect of bacterial cell surface polymers. Infect. Immun. **23**:502–508.
28. **Wilkinson, B., S. Sisson, Y. Kim, and P. Peterson.** 1979. Localization of the third component of complement on the cell wall of encapsulated *Staphylococcus aureus* M: implications for the mechanism of resistance to phagocytosis. Infect. Immun. **26**:1159–1163.
29. **Witte, W.** 1975. Capsule formation in *S. aureus* as a reason for nontypability by phages. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **223**:447–451.
30. **Yoshida, K.** 1971. Demonstration of serologically different capsular types among strains of *Staphylococcus aureus* by the serum-soft agar technique. Infect. Immun. **3**:535–539.
31. **Yoshida, K., and R. Ekstedt.** 1968. Relation of mucoid growth of *Staphylococcus aureus* to clumping factor reaction, morphology in serum-soft agar, and virulence. J. Bacteriol. **96**:909–915.
32. **Yoshida, K., and Y. Minegishi.** 1975. Capsular substance production in unencapsulated strains of *S. aureus*, p. 359–376. *In* J. Jeljaszewicz (ed.), Staphylococci and staphylococcal diseases. Springer-Verlag, New York.
33. **Yoshida, K., T. Ohtomo, and Y. Minegishi.** 1977. Mechanism of compact-colony formation by strains of *Staphylococcus aureus* in serum soft agar. J. Gen. Microbiol. **98**:67–75.
34. **Yoshida, K., M. Takahashi, T. Ohtomo, Y. Minegishi, Y. Ichiman, K. Haga, E. Kono, and C. San Clemente.** 1979. Application of fluorescent antibody for detecting capsular substances in *S. aureus*. J. Appl. Bacteriol. **46**:147–152.