INFECTION AND IMMUNITY, July 1981, p. 304-308
0019-9567/81/070304-05$02.00/0

Vol. 33, No. 1

NOTES

Encapsulated, Coagulase-Negative Strain of *Staphylococcus simulans*

J. C. ANDERSON[1]* AND C. D. WILSON[2]

Institute for Research on Animal Diseases, Agricultural Research Council, Compton, Newbury, England,[1] *and Central Veterinary Laboratory, Ministry of Agriculture, Fisheries and Food, Weybridge, Surrey, England*[2]

Received 31 March 1981/Accepted 16 April 1981

An encapsulated strain of *Staphylococcus simulans* (strain 76) isolated from a clinical case of bovine mastitis was demonstrated by the India ink technique, using both a light microscope and an electron microscope. The strain failed to kill mice after intraperitoneal inoculation but resisted ingestion by peritoneal phagocytic cells. The behavior of the strain helps to delineate the antiphagocytic role of the capsule.

Few staphylococci are encapsulated (14), and only one encapsulated, coagulase-negative strain of *Staphylococcus* has been described (15). Previously, Yoshida et al. (16) described a strain of *S. epidermidis* (strain 1142) which killed 90 to 100% of mice when it was inoculated, in saline (10^9 colony-forming units) or 5% mucin (10^7 colony-forming units), into the peritoneal cavity. A surface substance was obtained from this organism which induced resistance against intraperitoneal challenge, and this resistance was found to be related to neutralization of an antiphagocytic mechanism in the organism. No report has ever been made of a capsule on this strain of *S. epidermidis*; during storage, its biological properties altered, and further work was discontinued. However, Yoshida and Minegishi (15) subsequently discovered another strain (strain SMU-76) which was equally lethal for mice. The presence of a capsule was demonstrated by staining with antiserum conjugated with ferritin and examination in an electron microscope.

Our strain, designated 76, was isolated in pure culture from a clinical case of bovine mastitis. It was originally suspected of being capsulate because of the mucoid appearance and sticky texture of the colonies. The strain is a gram-positive coccus which produces catalase and is sensitive to lysostaphin (200 μg/ml) (10). It fails to produce free (tube test) or bound (slide test) coagulase in rabbit plasma. Strain 76 is weakly hemolytic on 5% ox blood agar. Under aerobic conditions, it produces acid from sucrose, trehalose, fructose, lactose, and maltose but fails to produce acid from xylose, arabinose, cellobiose, mannose, and mannitol. The strain does not produce phosphatase and grows in serum-soft agar (3) as diffuse colonies. This information allows the strain to be classified by the scheme of either Kloos and Schleifer (5) or Devriese (2) as *Staphylococcus simulans*.

The presence of a capsule was investigated by the India ink method (1), using methylene blue as a counterstain. Cells of strain 76 were found to be surrounded by an unstained halo, whereas control cells (*S. aureus* strain M60 and *S. epidermidis* strain 25) did not exhibit unstained haloes. The capsule on strain 76 was also demonstrated in an electron microscope by the India ink method (8) (Fig. 1).

The virulence of *S. simulans* strain 76 was assessed by intraperitoneal inoculation of Compton white mice (25-g average weight). For inoculation, strain 76 was grown on ox blood agar for 18 h at 37°C, harvested in sterile isotonic saline, and washed twice in saline; finally, with the aid of a nephelometer and calibration curves, the concentration of organisms in saline was adjusted to the required number. Viable cell numbers were determined by counting the number of colonies in four 20-μl drops of the appropriate 10-fold dilution of the suspension after incubation on blood agar. Sixty mice were divided into 10 equal groups and were inoculated intraperitoneally with 0.1 ml of a suspension of strain 76 in saline (5×10^9 cells per ml) and of 10-fold dilutions of the suspension. Twenty-four hours after inoculation of the highest concentration of strain 76 the mice were rough coated and had arched backs; the mice inoculated with 10^7 or-

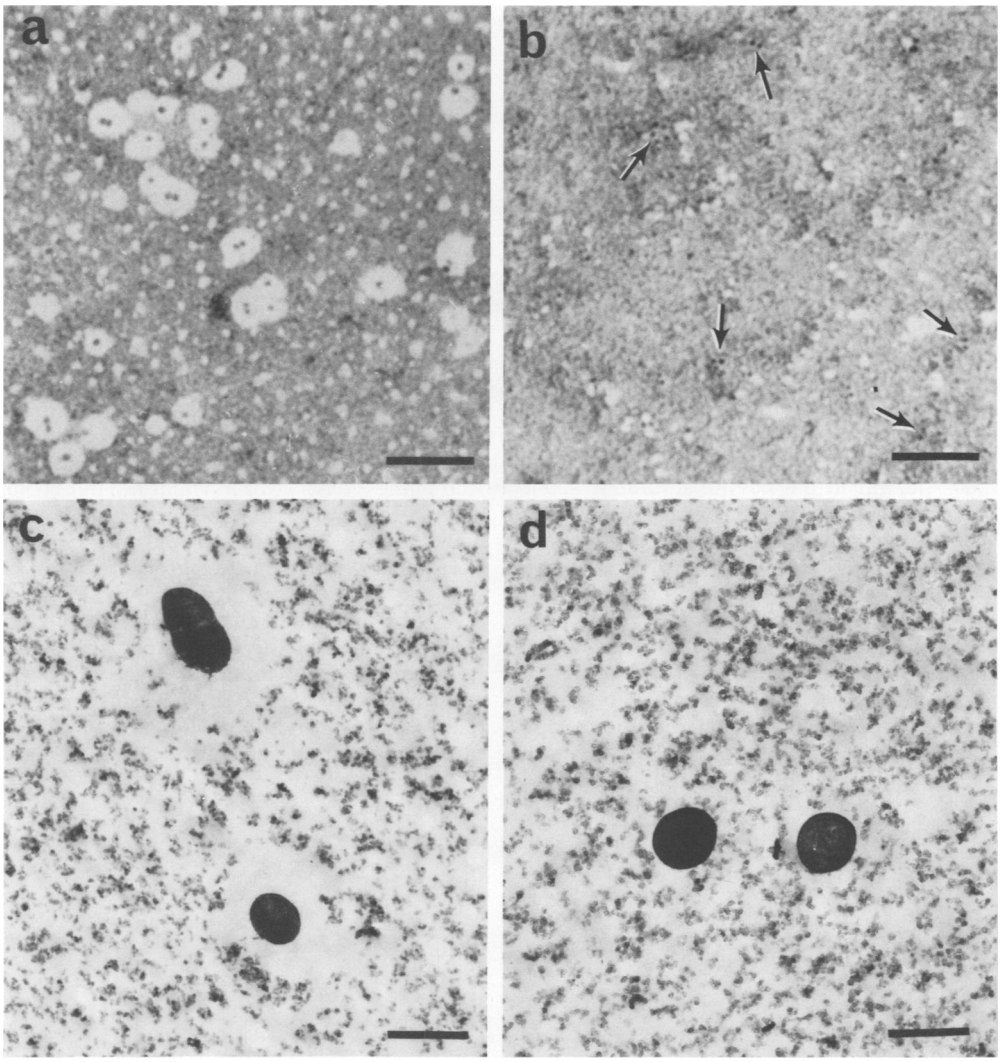

FIG. 1. *Encapsulation of S. simulans strain 76 demonstrated by a clear halo in India ink preparations. Examination by light microscopy (a) and by electron microscopy (c). Unencapsulated S. aureus cells are not surrounded by a clear halo in light (b, arrows) or electron (d) microscopy. Bar: a, b, 10 μm; c, d, 1 μm.*

ganisms were slightly rough coated, and the rest were normal. All of the mice were normal 48 h after inoculation and were allowed to survive for 6 days.

Encapsulated strain 76 of *S. simulans* was less virulent than the encapsulated strain of *S. epidermidis* described by Yoshida and Minegishi (15). They found that approximately 10^9 cells in saline produced 100% mortality in mice after intraperitoneal inoculation, whereas we found that 5×10^8 cells of strain 76 inoculated by the same route failed to kill any mice. Although differences in the breed and weight of the mice involved must be considered, the difference in lethality is significant. Likewise, strain 76 was less virulent than encapsulated strain M of *S. aureus*. Scott (11) gave the 50% lethal dose of strain M as $5 \times 10^6 \pm 6 \times 10^3$ colony-forming units and it was found that 10^5 colony-forming units of strain M killed 6 of 10 Compton white mice after intraperitoneal inoculation (J. C. Anderson, Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Suppl., in press). Thus, with death following intraperitoneal inoculation of live organisms as the criterion, encapsulated strain 76 of *S. simulans* is considerably less virulent than encapsulated strain M of *S. aureus*.

In a further experiment, *S. aureus* strain M60, *S. epidermidis* strain 25, and *S. simulans* strain 76 were each inoculated (10^8 colony-forming

units) into the peritoneal cavities of 30 mice. Five mice were killed 1, 2, 3, 6, 8, and 10 days after inoculation for determination of the number of organisms in the peritoneal cavity and examination of cytocentrifuge preparations of peritoneal washings. To determine the number of organisms in the peritoneal cavity, each mouse was killed, the skin over the abdomen was reflected, and 3 ml of sterile saline was rapidly inoculated into the peritoneal cavity. Peritoneal fluid was then removed via a Pasteur pipette inserted in the abdominal wall. The number of viable organisms was counted, and the result was expressed as the total number of organisms in the peritoneal cavity. The number of somatic cells in samples of peritoneal fluid was estimated by using a hemacytometer, and the number was adjusted to yield not more than 10^6 cells per ml in 5% bovine serum. A 200-μl sample was then placed in the reservoir of the cytocentrifuge (Shandon Scientific Company Ltd., Willesden, London) and spun for 10 min at 60 $\times$ *g*. This technique produced a monolayer of cells confined to a 6-mm-diameter spot on a microscope slide (12). Slides were dried in air, fixed in methanol, and stained with Giemsa stain (30%) in distilled water for 45 min. Dry preparations were examined in a light microscope. The bacteriological results are shown in Fig. 2.

S. epidermidis strain 25 was rapidly eliminated from the peritoneal cavity; at day 3, organisms were not recovered from three of five mice. *S. aureus* strain M60 was less well removed than strain 25, but at day 6 organisms were recovered from only one of five mice. The number of strain 76 cells remained elevated for 2 days and then declined; at day 6, organisms were recovered from four of five mice, and none was recovered from mice at day 8.

Cytocentrifuge preparations showed that neutrophils predominated in the peritoneal washings 24 h after inoculation of organisms, but macrophages were also present. Strain 25 and M60 cells were seldom found outside neutrophils or macrophages, whereas strain 76 cells were seldom found within these somatic cells (Fig. 3). There were more macrophages in the preparation made 2 days after inoculation, but strain 76 cells were again seldom found within phagocytic cells. At day 3, strains 25 and M60 were only occasionally found in the cytocentrifuge preparations. Macrophages predominated in the samples from mice inoculated with strain 76 and, although most of the organisms were extracellular, many were found on the cell walls of macrophages. At day 6 there was a considerable decrease in the number of strain 76 cells, and most were within macrophages.

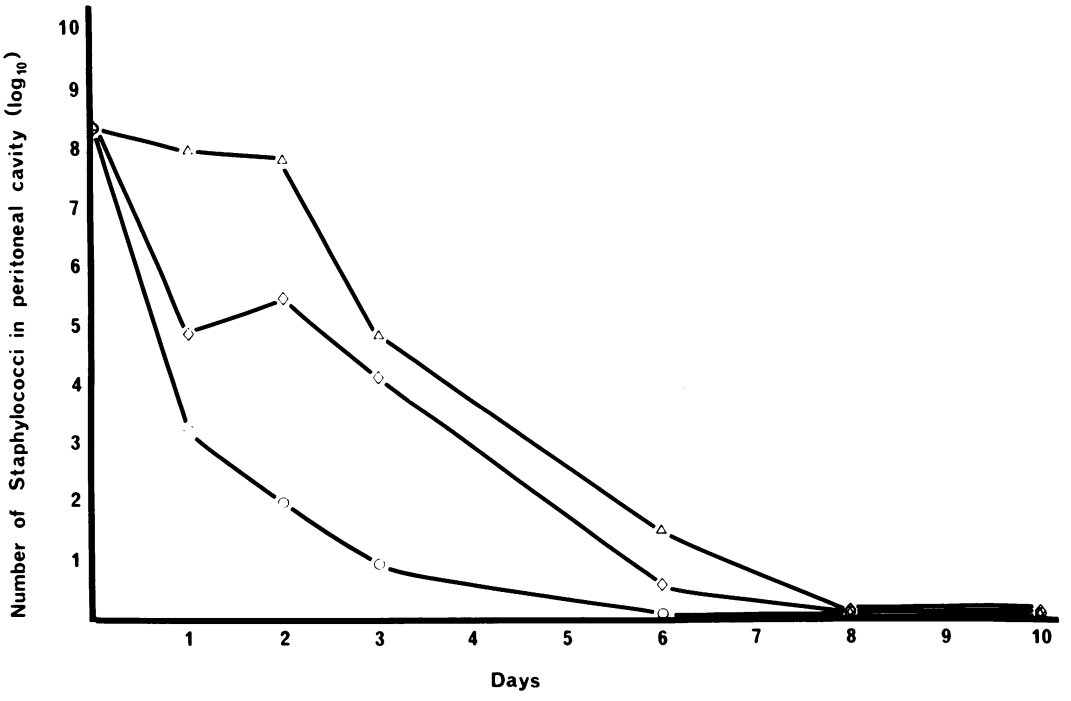

FIG. 2. *Number of staphylococci in the peritoneal cavity after inoculation of S. simulans strain 76 (△), S. aureus strain M60 (◇), and S. epidermidis strain 25 (○).*

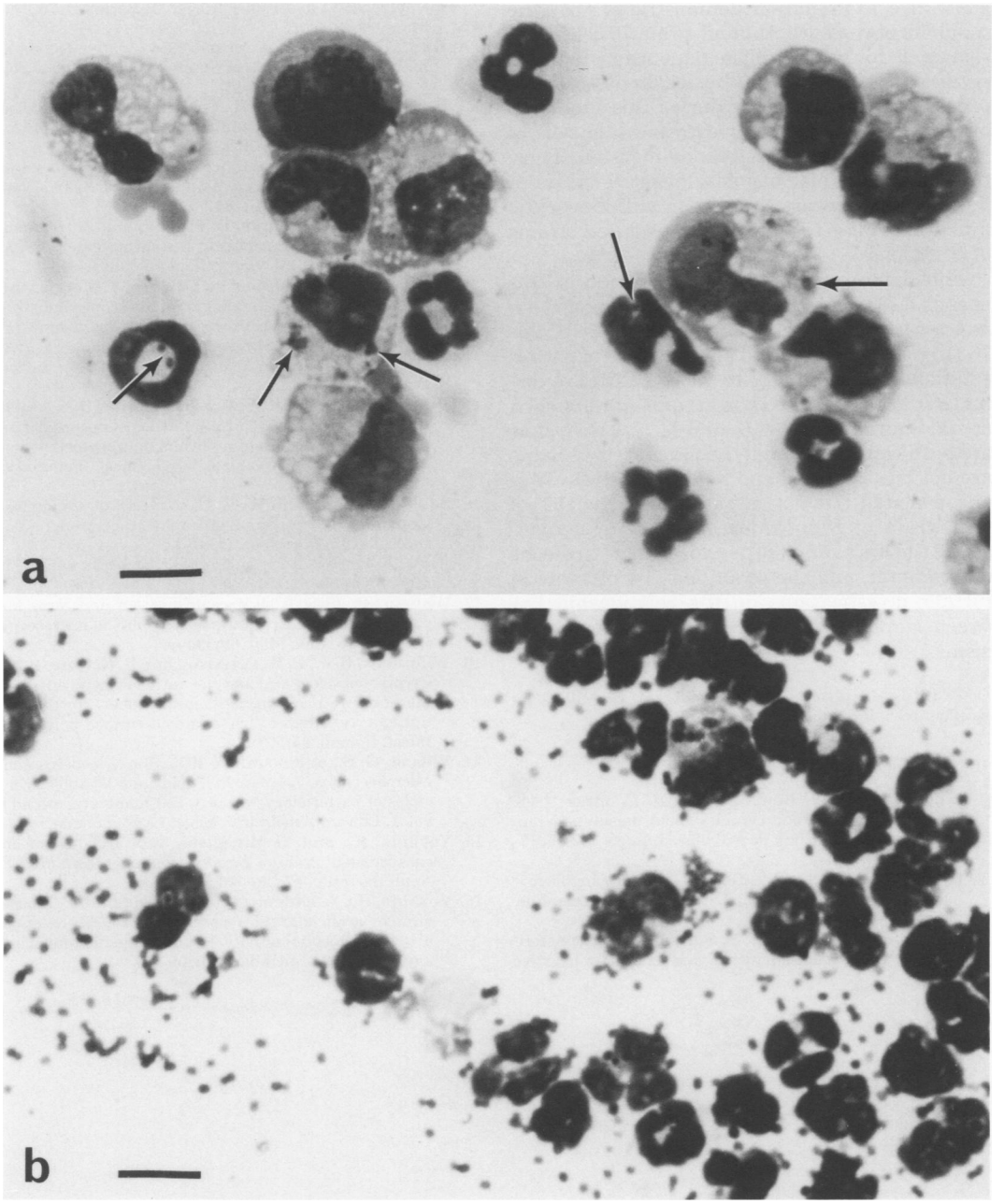

FIG. 3. *Cytocentrifuge preparations of peritoneal washings 24 h after inoculation of S. aureus strain M60 (a) and S. simulans strain 76 (b). Cells of strain M60 are within phagocytic cells (arrows), whereas cells of strain 76 are extracellular. Bar, 10 μm.*

The presence of a capsule on *S. aureus* strains makes the organism relatively resistant to phagocytosis (6, 7). The capsule interferes with opsonization by both classical and alternative pathways of complement as well as by heat-stable opsonic factors in nonimmune serum (9). The peptidoglycan of the staphylococcal cell wall, with which the C3b fragment of complement and the Fc portion of antibody molecules react, is masked by the capsule (13). The results of intraperitoneal inoculation of *Staphylococcus* strains followed by bacterial counting and cyto-

centrifuge examination demonstrated that the numbers of the encapsulated strain 76 cells were sustained for 48 h, unlike nonencapsulated *S. epidermidis* and *S. aureus* cells. Cytocentrifuge preparations showed that during this time cells of strain 76 resisted phagocytosis while cells of strains 25 and M60 were readily ingested. Thus, the capsule of this coagulase-negative strain of *S. simulans* appears to have an antiphagocytic function similar to that of encapsulated strains of *S. aureus*.

The significance of the identification of this encapsulated, coagulase-negative strain of *S. simulans* is that in this strain, unlike strain SMU-76 (15), the property and consequence of encapsulation and the ability to cause death are dissociated. Encapsulated strains of *S. aureus*, such as the Smith diffuse (4, 6) and M (7, 11) strains, are both capsulate and lethal, and their variants are not encapsulated and not lethal; strain 76 is encapsulated but not lethal. The properties of this strain of *Staphylococcus* help to demonstrate the limitation of the capsule as a virulence determinant, and the strain may be of value in further investigations on the relationship between staphylococci and host defense mechanisms.

We thank G. Hill, H. S. Anger, and I. M. H. Jebbett for excellent technical assistance.

LITERATURE CITED

1. **Butt, E. M., C. W. Bonynge, and R. L. Joyce.** 1936. The demonstration of capsules about hemolytic streptococci with India ink or A20 blue. J. Infect. Dis. **58**:5-9.
2. **Devriese, L. A.** 1979. Identification of clumping-factor-negative staphylococci isolated from cows' udders. Res. Vet. Sci. **27**:313-320.
3. **Finkelstein, R. A., and S. E. Sulkin.** 1958. Characterization of coagulase-positive and coagulase-negative staphylococci in serum-soft agar. J. Bacteriol. **75**:339-344.
4. **Hunt, G. A., and A. J. Moses.** 1958. Acute infection of mice with Smith strain of *Staphylococcus aureus*. Science **128**:1574-1575.
5. **Kloos, W. E., and K. H. Schleifer.** 1975. Simplified scheme for routine identification of human *Staphylococcus* species. J. Clin. Microbiol. **1**:82-88.
6. **Koenig, M. G.** 1962. Factors relating to the virulence of staphylococci. Comparative studies on two colonial variants. Yale J. Biol. Med. **34**:537-559.
7. **Melly, M. A., L. J. Duke, D. F. Liav, and J. H. Hash.** 1974. Biological properties of the encapsulated *Staphylococcus aureus* M. Infect. Immun. **10**:389-397.
8. **Melly, M. A., Z. A. McGee, R. G. Horn, F. Morris, and A. D. Glick.** 1979. An electron microscopic India ink technique for demonstrating capsules on microorganisms: studies with *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Neisseria gonorrhoeae*. J. Infect. Dis. **140**:605-609.
9. **Peterson, P. K., B. J. Wilkinson, Y. Kim, O. Schmeling, and P. G. Quie.** 1978. Influence of encapsulation of staphylococcal opsonization and phagocytosis by human polymorphonuclear leukocytes. Infect. Immun. **19**:943-949.
10. **Schleifer, K. H., and W. E. Kloos.** 1975. A simple test system for the separation of staphylococci from micrococci. J. Clin. Microbiol. **1**:337-338.
11. **Scott, A. C.** 1969. A capsulate *Staphylococcus aureus*. J. Med. Microbiol. **2**:253-260.
12. **Watson, P.** 1966. A slide centrifuge: an apparatus for concentrating cells in suspension onto a microscope slide. J. Lab. Clin. Med. **68**:494-501.
13. **Wilkinson, B. J., P. K. Peterson, and P. G. 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.
14. **Wilson, G. S., and A. Miles.** 1975. Staphylococcus and Micrococcus, p. 764-801. *In* Topley and Wilson's principles of bacteriology, virology and immunity, 6th ed., vol. 1. Edward Arnold (Publishers) Ltd., London.
15. **Yoshida, K., and Y. Minegishi.** 1979. Isolation of an encapsulated strain of *Staphylococcus epidermidis*. J. Appl. Bacteriol. **47**:299-301.
16. **Yoshida, K., Y. Ichiman, and T. Ohtomo.** 1976. Mouse virulent strain of *Staphylococcus epidermidis*. Relation of antiphagocytic activity to the protection-inducing antigen. Jpn. J. Microbiol. **20**:209-217.