

## CONCERNING THE SURFACES OF CELLS OF STAPHYLOCOCCUS PYOGENES\*

### I. A PSEUDOCAPSULATION PHENOMENON UNDER CERTAIN EXPERIMENTAL CONDITIONS

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The fact that neither antibiotics nor hygiene of themselves have provided adequate means of controlling the carrier state or infection with *Staphylococcus pyogenes*, suggests reexamination of the specific interactions of this parasite with defensive mechanisms of its hosts (1).

Even in exhaustive monographs (2, 3), however, definitive descriptions of the critical surfaces at which staphylococcal cells interact with their specific antibodies are not found. It has therefore seemed necessary to reinvestigate the antigenic surfaces of *S. pyogenes*. This communication represents one small step in this reexamination.

Gilbert (4) described an encapsulated strain of *Staphylococcus aureus*, which formed translucent mucoid colonies. Lyons (5) presented evidence of an encapsulated phase of staphylococcal growth. Capsule formation was demonstrated in young cultures of both toxigenic and non-toxigenic strains. More recently Price and Kneeland (6), Cohn and Morse (7, 8), and Wiley (9) have reported the occurrence of encapsulated staphylococcal organisms obtained from mucoid colonies.

Duguid (10) demonstrated the ability of *Bacterium aerogenes* to produce large capsules when the organisms were cultivated on a medium containing a high concentration of sugar and a very low concentration of nitrogen nutrients. The possibility of association of such a nutritional imbalance (*i.e.*, a high carbohydrate-low nitrogen medium) with a capsule-like structure of *S. aureus* appeared attractive.

#### *Methods and Materials*

The two strains of *S. aureus* employed were obtained from Dr. Frank Kapral (11).

Strain A, the parent strain, phage type 42B/52/80/81, possessing soluble and bound coagulase, fibrinolysin, hyaluronidase, alpha and delta hemolysins, and leucocidin.

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Strain C, a mutant derived from Strain A, which is identical in all respects with the exception that it does not produce soluble coagulase and fibrinolysin.

*Media.*—a. Bacto-tryptone 3 per cent, bacto-yeast extract 0.75 per cent, bacto-gelatin 9 per cent, Noble agar 9 per cent. b. Lactose 10 per cent, Noble agar 3 per cent. c. Mannitol 10 per cent, Noble agar 3 per cent.

The three solutions were autoclaved separately at 30 pounds for 30 minutes. Prior to use they were mixed together in equal parts. The broth medium consisted of the same three solutions in the same concentrations with the exception of the absence of Noble agar.

*Stains.*—India ink (Higgins) was mixed with equal volumes of 1.5 per cent aqueous gelatin solution and Ballotini beads. The mixture was shaken vigorously for 15 10-second periods in a Nossal shaker. After mild centrifugation the resulting homogeneously suspended particles of carbon in gelatin were stored at room temperature in sterile screw cap vials.

*Wet Mount.*—One loopful (3 mm.) of broth culture or a small portion of a colony picked from an agar plate, mixed with one loopful of sterile 2 per cent gelatin and a loopful of India ink, was immediately overlaid with a cover slip which was cemented in place with collodion.

Dry smears were prepared in the same manner as the wet mounts with the exception that the cover glass was drawn over the India ink mixture with a gentle but firm even movement of the thumb. The smear was allowed to air-dry, fixed by gentle heating, stained for approximately 5 seconds with 0.5 per cent aqueous crystal violet, and blot-dried.

*Mercurochrome Stain.*—A drop of 2 per cent aqueous mercurochrome was placed directly on colonies as they grew on the agar in Petri dishes; after 30 minutes at room temperature an impression smear of the stained colony was made on a slide. The smear was fixed in vapors of 2 per cent osmic acid for 30 seconds, counterstained with 0.5 per cent aqueous crystal violet for 5 seconds, and blot-dried.

*Electron Microscope Preparation.*—Microcolonies of the organism were grown on a collodion membrane overlaying the surface of the agar medium contained in a Petri dish. After a suitable incubation period, usually 6 hours at 37°C., the microcolonies on the collodion membrane and agar were cut out and the microcolonies (on the collodion membrane) were separated from the agar by flotation on either water, 0.33 per cent cadmium nitrate, or dilute solutions of rabbit anti-staphylococcal serum. When the specimens were floated off on the cadmium nitrate solution or dilute antisera, a drop of the respective solution was placed on the surface of the microcolony and allowed to react at room temperature for 30 minutes. The specimens were picked up on phosphor bronze grids prepared in the manner described by Ribi *et al.* (12), and fixed by freeze-drying in a manner similar to that of Williams (13). The grids were placed on a pre-cooled bronze block which in turn was placed by means of long mechanical fingers into the sublimation tube and subjected to a pressure of  $10^{-3}$  mm. of mercury for 15 to 30 minutes. The micrographs were taken with a Philips 75B electron microscope.

*Antigen Preparation.*—Two antigens were employed: a. Whole cells. 18-hour-old cultures at 37°C. were used to inoculate agar plates, which were incubated at 37°C. The organisms were washed from the plates with 1.5 per cent sterile aqueous bacto-gelatin and diluted with the gelatin to give a Klett reading of 190 ( $3 \times 10^8$  cells/ml.). Equal volumes of the cell suspensions and "Freund's incomplete adjuvant" were mixed vigorously until homogeneous. b. Extracellular material was removed from the cells, which were grown in the manner previously described, by suspending the cells in sterile saline and aspirated and discharged a number of times with a syringe and a small gauge needle. The suspension was centrifuged and the resulting supernatant was passed through a Swinny Millipore filter. The filtrates were mixed with equal volumes of "Freund's incomplete adjuvant" until homogeneous.

*Antibody Production.*—2 kg. white, male rabbits were injected subcutaneously with either the extracellular material or the whole cells of the two strains of *S. aureus*, at 2-week intervals for a 10 week period. The rabbits were initially bled 10 days following the last injection. Two

weeks later the animals received a booster and were exsanguinated by cardiac puncture 10 days later. The sera from the two bleedings were pooled.

#### RESULTS

Strain A, the parent strain, when grown on the yeast extract, tryptone, gelatin agar containing lactose and mannitol for a minimum of 6 hours at 37°C. produced a capsule-like substance which was discernible either in a wet India ink mount or a dried smear (Figs. 1 and 3). The soluble coagulase fibrinolysin negative mutant, strain C, did not produce this substance (Figs. 2 and 4). Electron microscopic examination of microcolonies of the strains which were floated on water and "washed" by several passages through different water baths and fixed in the conventional manner with OsO<sub>4</sub> vapors, revealed tightly packed organisms of the mutant strain (Fig. 6), whereas the organisms from the parent strain were not so packed (Fig. 5).

Various strains were employed in an attempt, by cytochemical techniques, to identify the extracellular material. Alcian blue (McKinney, 14) and saccharated iron oxide (Berenbaum, 15) which are used for staining acid mucopolysaccharides, yielded negative results. Similarly, Menschik's (16) phospholipid stain, as well as Sudan black B, did not stain the extracellular substance. Dubious results were obtained with Mazia (17) mercuric-bromphenol blue protein stain. Mercurochrome, with its affinity for disulfide linkages, stained the extracellular substance, which appears as a matrix, a distinct pink. The protoplasm of the individual organisms took the counter stain, crystal violet. Electron microscopic examination revealed a rather large amount of amorphous material deposited around the individual cells. The mutant, when stained with mercurochrome, revealed a rather shallow pink outline, which is assumed to be the cell wall, while the cytoplasm took the crystal violet stain (Figs. 7 and 8).

Individual cells of the microcolonies of the parent strain (Fig. 9) when fixed by freeze-drying, appeared to be embedded in an amorphous substance. This substance was absent in the microcolonies of the mutant (Fig. 10).

Duthie and Haughton (18) found that soluble coagulase was easily precipitated by cadmium salt. Since the mutant strain differs from the parent strain by absence of soluble coagulase in addition to fibrinolysin, microcolonies of the two strains were treated with 0.33 per cent cadmium nitrate for 30 minutes at room temperature and fixed, without washing, by freeze-drying and examined with the electron microscope. The cells from the parent strain, when so treated, were found to be enveloped by sharp electron scattering bands which immediately surrounded the individual cells, and in addition the cells had undergone plasmolysis. Neither enveloping bands nor plasmolysis were found when the mutant cells were so treated (Figs. 11 and 12).

The production of the adherent extracellular material by the parent strain

is dependent on lactose, mannitol, gelatin, tryptone, and yeast extract. Neither glucose nor galactose can be used in place of lactose. Nor is mannitol able to be replaced by sorbitol, glycerol, and/or mannose. Carboxymethyl cellulose (or small amounts of agar in the broth medium) were unable to replace gelatin. Casein hydrolysate, trypticase soya extract, or brain-heart infusion could not be used in lieu of yeast extract or tryptone. However, the yeast extract could be substituted with 1 per cent medium 199 (BBL) Morgan *et al.* (19).

The extracellular material can very easily be washed off the surface of the individual cells. It is obvious from the agglutination tests (Table I) that the antigenic make-up of the cell walls of the parent and mutant strains is such that they react to antisera against both strains. The unwashed parent cells reacted

TABLE I  
*Agglutination Tests with Rabbit Sera*

Antiserum vs.	Parent cells		Mutant cells	
	Unwashed	Washed	Unwashed	Washed
Parent cells.....	640	1280	1280	1280
Mutant cells.....	320-640	1280	1280	1280
Washings of parent cells.....	80-160	160-320	160	320-640
Washings of mutant cells.....	160	1280	1280	1280
Normal serum.....	20	40	20	640-1280

The numbers are reciprocals of highest dilutions giving definite tube agglutination.  
The above results are representative of replicate experiments.

to lower titer than the washed parent cells in all sera tested. Apparently, the extracellular material associated with the unwashed parent strain impedes its interaction with antigens of the cell wall, since washing the cells free from the extracellular material makes them more reactive.

These inferences from the agglutination tests are supported by the electron micrographs reproduced as Figs. 13 to 18. Both strains, after exposure to normal rabbit serum and washing, show clearly delimited cell walls (Figs. 13 and 14). The washed parent cells and the mutant cells treated with immune rabbit serum (Figs. 15, 16, and 18) even in very low concentration, show cell walls slightly blurred and not sharply delimited, we believe owing to interaction with antibodies (Mudd and Anderson, 20). The unwashed microcolonies of the parent strain treated with antisera show extracellular interaction product about the cells (Fig. 17).

#### DISCUSSION

The role of yeast extract, or the vitamin pool, is not clearly understood. However, Porter and Pelczar (21) reported that certain strains of *S. aureus*

which were unable to grow in the synthetic medium developed by Gladstone (22) required biotin in addition to nicotinic acid and vitamin B. Still other strains required yeast extract.

The action of cadmium salt is rather specific in that sodium nitrate and aluminum nitrate in similar osmotic and molar concentrations did not produce the same effect. The concentration of soluble coagulase is considerably greater when the parent strain is cultivated under the described condition than when it is cultivated on glucose (substituted for lactose and mannitol), yeast extract, tryptone, gelatin, agar medium. (Sall, data to be published). When compared with the Smith strain (Cohn and Morse, 7), which has a well defined capsule (Morse, 8), the structure demonstrated by the parent strain does not appear as a true capsule. It neither appears as firm, as well delimited, nor as large as that demonstrated with the Smith strain. The probability exists that the structure depicted in this study represents a "build-up" of extracellular material, which, under the conditions in question, cannot diffuse into the medium. We think of this as a phenomenon of pseudocapsulation.

The phenomenon of pseudocapsulation described in this communication is exhibited *in vitro* under very special conditions. Whether or not similar conditions can occur *in vivo* is uncertain. It does seem safe to conclude, however, that this phenomenon of pseudocapsulation, if it occurs at all *in vivo*, is of less significance in pathogenesis than the occurrence of true capsulation in a mucoid organism such as the Smith strain studied by Cohn and Morse (7, 8).

#### SUMMARY

A strain of pathogenic *Staphylococcus aureus*, grown under special conditions, accumulates extracellular metabolites about the bacterial cells. This phenomenon may simulate, but is differentiable from, the capsulation of, for instance, the mucoid "Smith" strain. The special nutrient requirements, namely lactose, mannitol, vitamin, and gelatin containing medium, promote the production and accumulation about the cells of, in particular, soluble coagulase. A mutant of this parent strain, deficient in capacity to elaborate soluble coagulase and fibrinolysin, does not accumulate metabolites about its cells, even under the special growth conditions. To avoid confusing this phenomenon, which at least *in vitro* is essentially artifactitious, with true capsulation, we suggest the term pseudocapsulation.

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## EXPLANATION OF PLATES

## PLATE 74

FIG. 1. Parent strain, grown on special lactose-mannitol agar for 8 hours at 37°C. India ink wet mount, anoptral photomicrograph.  $\times 2,350$ .

FIG. 2. Mutant strain, same conditions as in Fig. 1

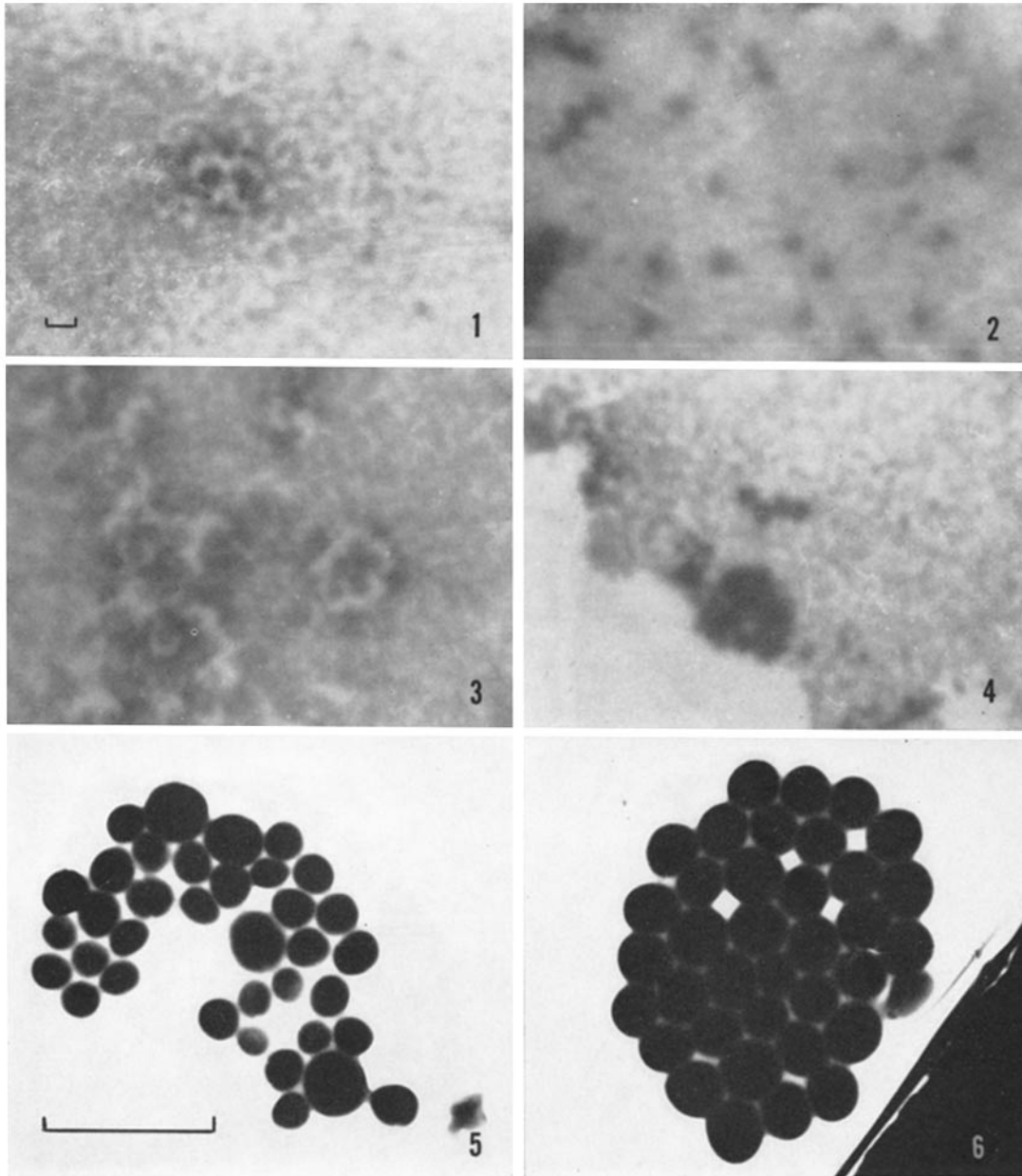
FIG. 3. Parent strain, same growth conditions as in Fig. 1. India ink dried smear counter-stained with crystal violet, parallel light photomicrograph.  $\times 2,350$ .

FIG. 4. Mutant strain, same conditions as in Fig. 3

FIG. 5. Parent strain, microcolony grown on a collodion membrane, overlaying the special lactose-mannitol agar, at 37°C. for 8 hours, washed by 3 passages through sterile distilled water over a 45 minute period, and fixed in vapors of 2 per cent osmic acid. Electron micrograph.  $\times 11,700$ .

FIG. 6. Mutant strain, same conditions as in Fig. 5



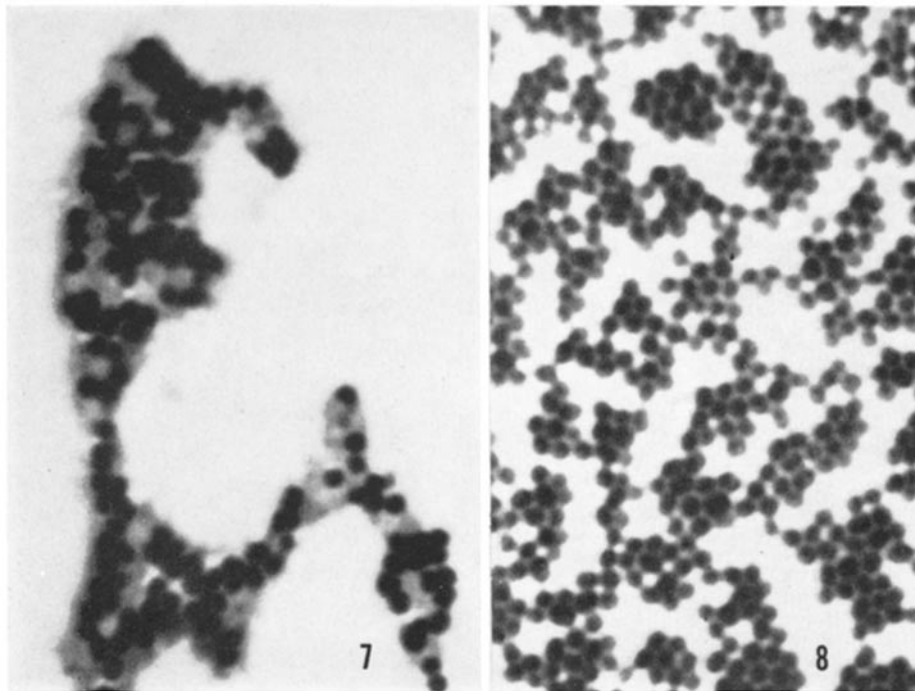


(Sall *et al.*: Surfaces of cells of *Staphylococcus pyogenes*. I)

PLATE 75

FIG. 7. Parent strain, grown on special lactose-mannitol agar for 8 hours at 37°C. The microcolonies were stained *in situ* with 2 per cent aqueous mercurochrome for 30 minutes at room temperature, and fixed in vapors of 2 per cent osmic acid. Impression smears made of the stained colonies were counter-stained with 0.5 per cent crystal violet. Parallel light photomicrograph.  $\times 3,600$ .

FIG. 8. Mutant strain, same conditions as in Fig. 7



(Sall *et al.*: Surfaces of cells of *Staphylococcus pyogenes*. I)

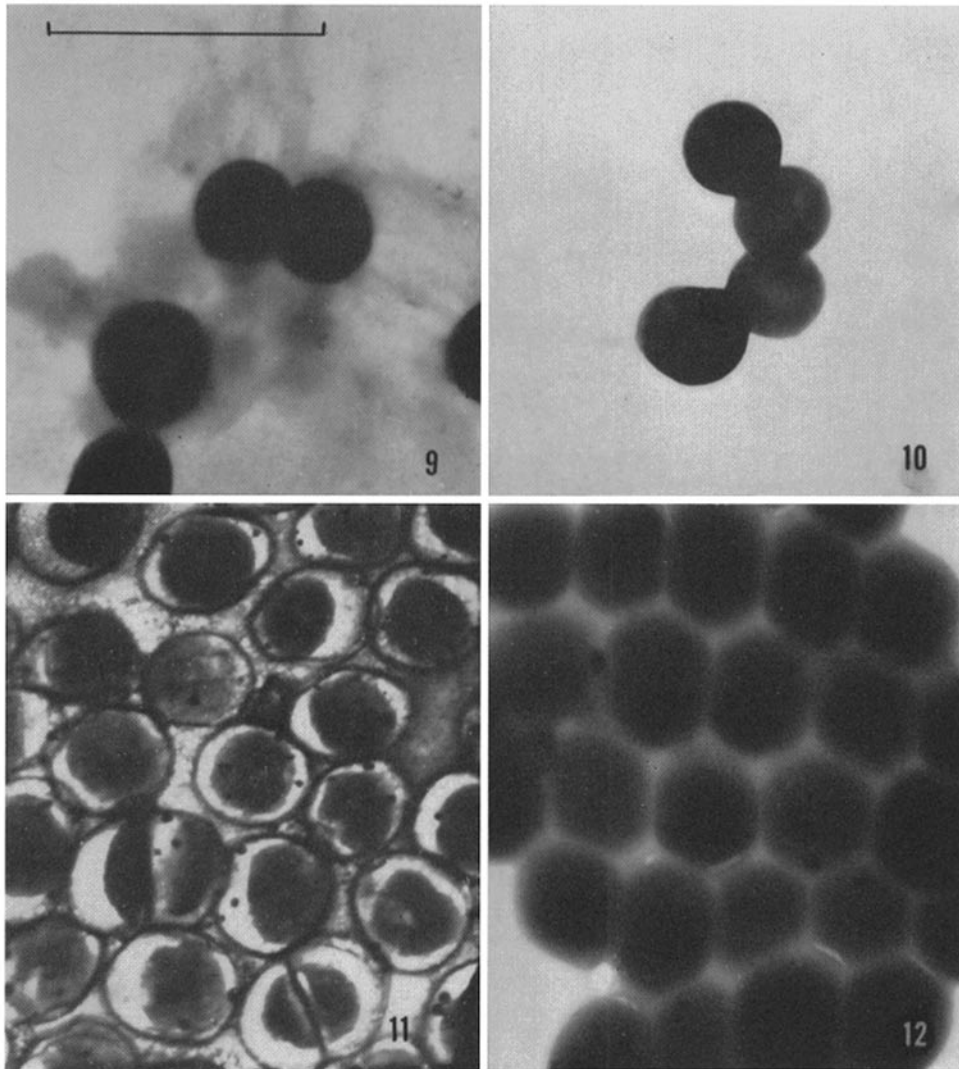
PLATE 76

FIG. 9. Parent strain, same growth conditions as in Fig. 5. The microcolonies on the collodion membrane were fixed by freeze-drying without "washing." Electron micrograph.  $\times 36,000$ .

FIG. 10. Mutant strain, same conditions as in Fig. 9

FIG. 11. Parent strain, same growth conditions as in Fig. 5. The microcolonies were stained with 0.33 per cent cadmium nitrate and fixed by freeze-drying. Electron micrograph.  $\times 36,000$ .

FIG. 12. Mutant strain, same conditions as in Fig. 11



(Sall *et al.*: Surfaces of cells of *Staphylococcus pyogenes*. I)

PLATE 77

FIG. 13. Parent strain, same growth conditions as in Fig. 5. The unwashed microcolonies were treated with normal rabbit serum (1:1,500) for 30 minutes at room temperature and fixed by freeze-drying. Electron micrograph.  $\times 19,000$ .

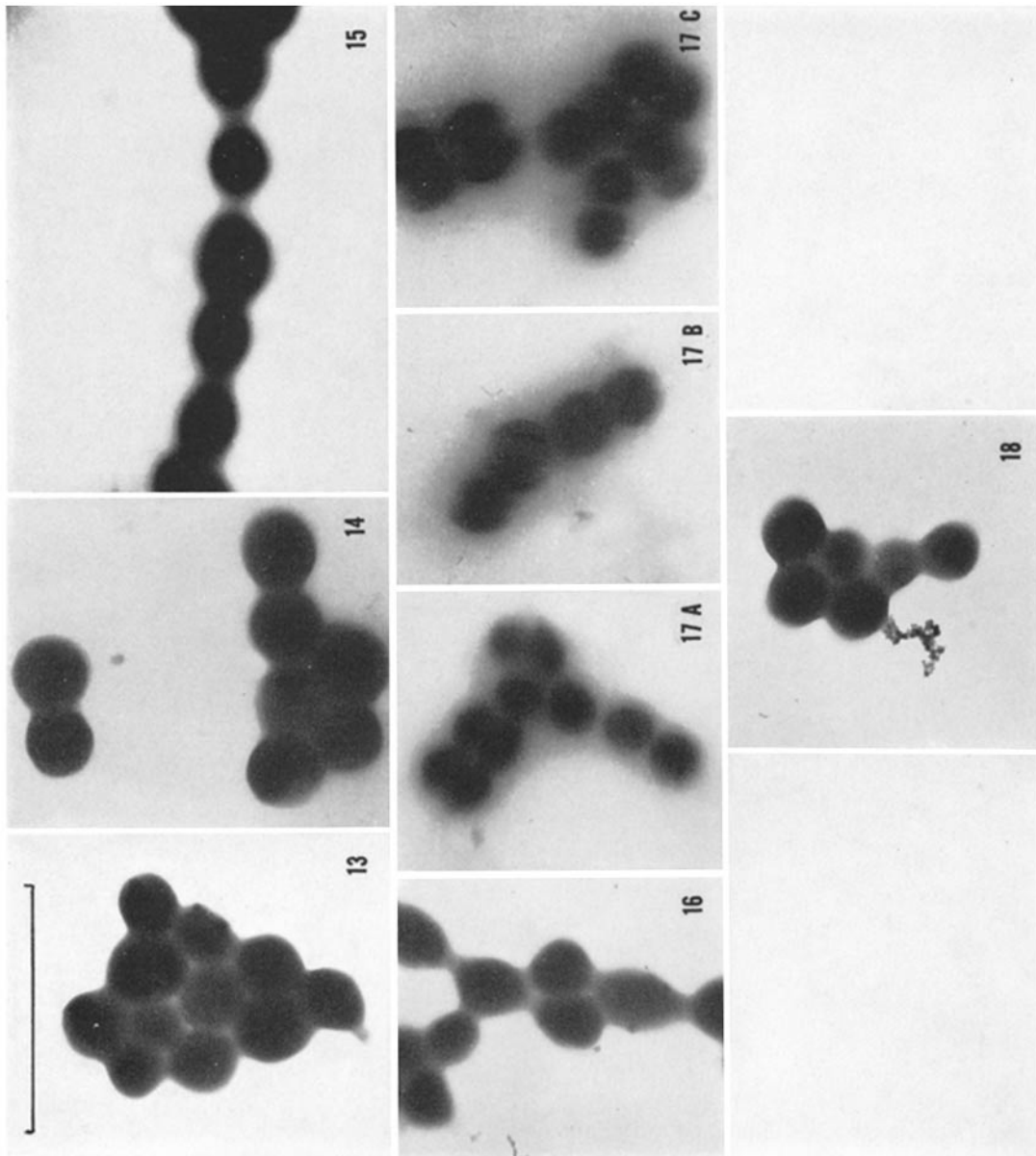
FIG. 14. Mutant strain, same conditions of growth and treatment as in Fig. 13

FIG. 15. Parent strain, same growth conditions as in Fig. 5. The microcolonies were washed by 3 passages through sterile distilled water over a 45-minute period and treated with rabbit serum (1:1,500) against the parent whole cells for 30 minutes at room temperature and fixed by freeze-drying. Electron micrograph.  $\times 19,000$ .

FIG. 16. Mutant strain, same growth conditions as in Fig. 5. The unwashed microcolonies were treated with rabbit serum (1:1,500) against the mutant cells for 30 minutes at room temperature and fixed by freeze-drying. Electron micrograph.  $\times 19,000$ .

FIG. 17. Parent strain, same growth conditions as in Fig. 5. The unwashed microcolonies were treated with rabbit serum against washing of the parent cells, at room temperature for 30 minutes, and fixed by freeze-drying. Electron micrograph.  $\times 19,000$ .

FIG. 18. Mutant strain. Same conditions of growth and treatment as in Fig. 17. The scale lines on the illustrations may be ignored.



(Sall *et al.*: Surfaces of cells of *Staphylococcus pyogenes*. I)