Properties of a Novel Pleiotropic Bacteriophage-Resistant Mutant of Staphylococcus aureus H

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Received for publication 15 September 1969

A phage-resistant mutant of Staphylococcus aureus H (SmR), S. aureus 52A5, was previously shown to lack polymeric teichoic acid. This paper characterizes other phenotypic differences between the strains. In broth cultures the mutant cells grew more slowly, were larger, and formed much larger clumps than the parent strain. The clumps of cells appeared to be covalently linked and could only be separated by mild sonic energy—a process which yielded viable cells. Mutant and parent cells autolyzed at equal rates, whereas isolated cell walls of the mutant strain autolyzed faster than the wild type. Nevertheless, the specific activity of the autolytic enzyme in the wild type soluble fraction was much higher than in the mutant. In contrast to the parent, strain 52A5 failed to accumulate nucleotide-bound murein precursors when treated with penicillin. Mutant strains with these characteristics were repeatedly isolated both spontaneously and by chemical mutagenesis. Strain 52A5 was shown to be fully reversible. Thus, it appears to be a pleiotropic mutation, and the possible nature of the defect which causes these varied effects is discussed.

Bacterial mutants, shown to be phage-resistant because phage would not adsorb to them, have an altered cell wall composition. Thus, phage-resistant mutants of Bacillus subtilis, isolated by Glaser et al. (5) and Young (20) lacked D-glucose in their cell wall teichoic acid. Similarly, Chatterjee isolated phage-resistant mutants of Staphylococcus aureus H which did not adsorb phages 52A 79, and 80; these mutants lacked N-acetyl-d-glucosamine (GlcNac) in their cell wall teichoic acid (2). Two of these resistant strains, 52A5 and 52A7, contained reduced amounts of phosphate in their cell walls. Strains 52A5 and 52A7 also differed from the other mutant strains in being resistant to the virulent phage k and a host-range mutant phage, 52Ah (2). On further investigation, the mutant strain S. aureus 52A5 was found to have additional specific properties distinguishing it from the parent strain. Apart from the differences in cell wall composition noted above, the mutant exhibited the following major phenotypic differences: larger cell size, a tendency to grow in large clumps of cells in liquid culture, failure to accumulate murein precursors when treated with penicillin, and an altered location of autolytic enzymes in the cells. The present paper describes these phenotypic differences and demonstrates that a single mutational event is involved, i.e., that it is a pleiotropic mutant.

MATERIALS AND METHODS

Bacterial strains. The isolation and maintenance of the streptomycin-resistant parent strain S. aureus H (SmR) and of the phage-resistant mutant strain S. aureus 52A5 was described previously (2). Conditions of growth were as follows. Both strains were grown in 0.5% phytone (BBL), 0.5% yeast extract (Difco), 0.3% K2HPO4, and 0.2% glucose (PYK medium, pH 7.2) in a gyratory shaker at 37 C. In all growth experiments, 3 to 5% of a 13- to 15-hr culture was used as an inoculum, and the cells were harvested after about 4 generations. Growth was monitored by optical density (OD) of the culture in a Zeiss PMQ II spectrophotometer (585 nm, 1.0-cm light path). Viable cell numbers were determined by standard plate counting techniques.

Accumulation of nucleotide-bound murein precursors. Cultures were grown in 50 ml of PYK medium to OD 1.8. At this point one of the following antibiotics...
was added: penicillin G, 6 μg/ml; vancomycin, 10 μg/ml (Eli Lilly and Co.); bacitracin, 50 μg/ml (California Corp. for Biochemical Research). After 90 min of further incubation, the cells were harvested by centrifugation, homogenized with 10 ml of 5% (or 25%) trichloroacetic acid, and kept in the cold with occasional mixing for 30 min. The cell suspension was then centrifuged at 12,000 × g for 20 min, and the trichloroacetic acid extract was extracted three times with 25 ml of diethyl ether to remove trichloroacetic acid. The remaining aqueous solution was lyophylized and concentrated in the cold. After extracting the trichloroacetic acid with ether, the material from each strain was contained in 10 ml, placed on a Sephadex G-25 column (medium bead, 2.3 by 110 cm), and eluted with 0.01 N acetic acid. Bound N-acetyl amino sugars, as a measure of nucleotide-bound murein precursors, were determined by the increase in Morgan-Elson color (13) after the material was hydrolyzed in 0.02 N HCl for 10 min at 100 C. GlcNAc was used as standard.

The nature of the materials which accumulated in the presence and in the absence of penicillin was studied. Cells from 2 liters of culture of the wild type and strain 52A5 were harvested at OD 3.0 and extracted with 100 ml of 5% trichloroacetic acid in the cold. After extracting the trichloroacetic acid with ether, the material from each strain was contained in 10 ml, placed on a Sephadex G-25 column (medium bead, 2.3 by 110 cm), and eluted with 0.01 N acetic acid. Bound Morgan-Elson positive materials appeared in the void volume (15). They were identified by high-voltage paper electrophoresis (Whatman 3MM, buffer pyridine 1.2 m acetic acid, pH 6.5, 40 v/cm, 120 min) and by paper chromatography (Whatman no. 1 paper; solvent, isobutyric acid-concentrated ammonia-water at a ratio of 5:3:1 (v/v)). As markers, bound N-acetylaminosugars, as a measure of nucleotide-bound murein precursors, were determined by the increase in Morgan-Elson color (13) after the material was hydrolyzed in 0.02 N HCl for 10 min at 100 C. GlcNAc was used as standard.

Radioisotopic cell walls and measurements of radioactivity. S. aureus H cell walls labeled with 14C-L-lysine were prepared by incubation of the cells for 5 hr in a defined medium containing the four cell wall amino acids (3) in the presence of 10 μg of penicillin G per ml and subsequent isolation of the partially uncross-linked cell walls (details on the preparation of this substrate and its use for assay of lytic enzyme activity, I. Takebe, H. J. Singer, E. M. Wise, Jr., and J. T. Park, submitted for publication). Strain 52A5 cell walls labeled with 14C-L-lysine were prepared from 52A5 cells grown in PYK medium containing 50 μCi of 14C-L-lysine per ml. Radioactivity was measured in a scintillation counter using a scintillation solution for aqueous samples (6).

Other methods. N-terminal amino acids were identified and quantitatively estimated by the methods of Ghuysen et al. (4, 7). The optical rotations of isolated dinitrophenyl-alanine and authentic dinitrophenyl-L-alanine (Calbiochem) in glacial acetic acid (12) were determined in a Perkin-Elmer 141 automatic polarimeter using 10 cm cells and sodium D line. Reducing sugars were determined by the Shickman modification of the Park and Johnson method (18). Cells were sonicated with the small probe (4 mm) of a Biosonic III apparatus (Brownwill Scientific, Roches-
ter, N.Y.) which was immersed about halfway in 1.0 ml of cell suspension in a test tube (13 by 100 mm) to be cooled in ice water. A power setting of 20 was used.

RESULTS

Phenotypic differences between S. aureus H (Sm1) and strain 52A5 are compared below.

Growth and morphology. In a variety of complex media, e.g., Trypticase Soy Broth (Difco), nutrient broth (Difco), or PYK-broth, the mutant strain 52A5 grew more slowly than S. aureus H (Sm1). For example, its generation time at 37 C in PYK-medium was 39 min as compared to 30 min for S. aureus H (Sm1). On nutrient agar (Difco) or PYK-agar colonies of S. aureus H (Sm1) were evenly rounded, smooth, and yellow, whereas strain 52A5 colonies were more irregular in shape and appeared rough and gray when examined in a stereoscopic dissecting microscope under low magnification.

A striking difference in the ability of the two strains to separate into individual cells was observed. In PYK-broth culture cells of S. aureus H (Sm1) occurred singly or in pairs with occasional longer chains, whereas the cells of strain 52A5 occurred mostly in large clumps or aggregates of 5 to 100 cells which were present at all stages of the growth cycle (Fig. 1). The basis for the aggregation has been studied to some extent. It is unlikely that the clumps are caused by ionic or other noncovalent bonds since homogenization of strain 52A5 cell suspensions in a tissue homogenizer with 1.0 M KCl, 1.0 M phosphate buffer (pH 7.0), 6 M urea, 0.1% sodium deoxycholate, 0.1 M KOH (30 min, 37 C), or 5% trichloroacetic acid (5 min, 90 C) had no effect on the size of the clumps. Moreover, isolated cell walls of 52A5 found to be similarly aggregated and failed to disperse when subjected to the various treatments mentioned above. An indication that the cells remained joined by their cell envelope was observed by electron microscopy. Thin sections of whole cells of strain 52A5 showed the presence of a cell envelope bridge joining two adjacent cells. No such phenomenon was observed in thin sections of S. aureus H (Sm1). Strain 52A5 cells also had a thicker cell wall with a rougher surface than S. aureus H (unpublished electron micrographs).

Sonication of 52A5 cell suspensions for only 1 min caused dispersion of the cell clumps as observed under the phase-contrast microscope. Cells of S. aureus H (Sm1) and strain 52A5 were sonicated for various periods and the number of viable cells remaining was assayed by plate count. The viable count of S. aureus H (Sm1) increased by about 50% after 3 min of sonication, whereas
the viable count of strain 52A5 increased by more than 10-fold over a similar period (Fig. 2). The number of colony-forming units obtained from the two sonicated cell suspensions having the same OD (OD 0.10 after 2 min of sonication) differed. Strain 52A5 produced $5.3 \times 10^7$ colonies per ml, whereas the suspension of S. aureus H (SmR) produced almost twice as many colonies ($1.1 \times 10^8$ per ml). This finding was confirmed by direct count of the cells in a Petroff-Hauser counter. About 1,000 cells of S. aureus H (SmR) and strain 52A5 were counted. For suspensions of equal turbidity, S. aureus H (SmR) preparations contained twice as many cells as those of strain 52A5. Phase-contrast microscopic observations of individual strain 52A5 cells indicated that they were larger than S. aureus H (SmR). The cell wall thickness, as measured from thin sections in the electron microscope, was about 40 nm for strain 52A5 and about 25 nm for S. aureus H (SmR). The dry weights for cell suspensions of S. aureus H (SmR) and strain 52A5 of the same OD were found to be identical. All these data indicate that S. aureus 52A5 cells are bigger in size and contain more mass per cell than S. aureus H (SmR).

**Autolytic activity.** S. aureus H (SmR) log-phase cells autolyze when suspended in 0.1 M phosphate buffer at pH 7.3. The autolytic enzyme of S. aureus H has been characterized as an amidase which splits the bond between MurNAc and L-alanine (Singer et al., unpublished data). No significant difference was observed in the rate of autolysis between whole cells of S. aureus H (SmR) and strain 52A5. The results with isolated cell walls differed markedly from this. Cell walls for autolytic studies were prepared by breaking exponential-phase cells in the Servall Omnimixer by the method of Sharon and Jeanloz (16). The cell walls were treated with ribonuclease and deoxyribonuclease; debris and whole cells were sedimented by centrifugation at 1,000 $\times$ g for 5 min, and the cell walls were recovered by centrifugation at 12,000 $\times$ g for 10 min. The walls were then washed in 0.03 M phosphate buffer pH 6.8, and resuspended in the same buffer for use the same day. All these operations were carried out in the cold. These walls differed from other wall...
preparations since they were not boiled to inactivate enzymes. As can be seen in Fig. 3, 35% of the $^{14}$C-lysine of strain 52A5 cell walls was released in 60 min at 37 C (in 0.1 M phosphate buffer at pH 7.3), whereas 3.5% of the label from S. aureus H (SmR) walls was released over the same period. Similar results were obtained where the rate of lysis of cell walls was followed by determination of OD (Fig. 3). It is conceivable that differences in rates of autolysis could be due to a redistribution of the lytic enzyme during preparation. The autolytic enzyme was firmly adsorbed or bound to the wall since extraction of S. aureus H (SmR) or strain 52A5 cell walls with 25% NaCl for 10 min in the cold, or sonication for 2 min, did not affect the rate of autolysis of the cell walls. Furthermore, no lytic activity was released by these methods, nor did repeated freezing and thawing of the cell walls cause any release of lytic enzyme.

In the following experiments the activity of the crude autolytic enzyme present in the soluble fraction was assayed by the release of $^3$H-L-lysine from cell walls prepared by the method of Park and Hancock (11) or by observing the decrease in OD of purified cell walls in which the autolytic enzyme was inactivated by boiling. Whether S. aureus H (SmR) or strain 52A5 cell walls were used as substrate, the specific lytic activity of the crude enzyme preparation of strain 52A5 was only 5 to 15% that of S. aureus H (SmR). This indicates that different rates of lysis are not due to
differences in substrate susceptibility, but to differences in the enzyme concentration. Thus, strain 52A5 contained much less active autolytic enzyme in soluble form than S. aureus H (SmR)

The main chemical bond cleaved by the autolytic enzyme in the mutant strain was the same as that cleaved by the S. aureus H (SmR) autolytic enzyme, namely, the amide bond between MurNAc and L-alanine. Dinitrophenylation studies of S. aureus 52A5 cell walls before and after autolysis showed a 3-fold increase in the level of dinitrophenyl (DNP)-alnine. No increase was observed in the small amount of DNP-glycine present in nonautolyzed cell walls. No other cell wall DNP-amino acids were present.

The configuration of the isolated DNP-alanine was established by measuring its optical rotation in glacial acetic acid solution. The value for the molar rotation was $[\alpha]_D^{25} = +36.6$ as compared to $[\alpha]_D^{25} = +41.2$ obtained for authentic DNP-L-alanine in the same solvent.

The reducing power released during autolysis for 18 hr was between 5 to 10% of the theoretical reducing power of the disaccharide repeating units (GlcNAc-MurNAc) present in the wall, and this value did not increase on further incubation. The hexosamine chains in strain 52A5 glycan have been shown to contain an average chain length of 6 to 7 disaccharide units (D. Mirelman, A. N. Chatterjee, H. J. Singer, D. R. D. Shaw, and J. T. Park, Bacteriol. Proc., p. 47, 1969). Therefore, the reducing power found after autolysis could be due to the solubilization of hexosamine chains containing terminal reducing groups initially present in the cell wall. The culture supernate of both of these strains was tested for lytic activity. No lytic activity was found in culture supernates or a 90% saturated ammonium sulfate precipitate of the culture supernate of either S. aureus H (SmR) or strain 52A5.

**Accumulation of murein precursors.** Since it appeared that the mutation affected cell wall biosynthesis, a number of experiments were performed to determine whether any difference could be found in membrane functions, composition, or permeability. S. aureus H is known to accumulate nucleotide-bound murein precursors when treated with penicillin G, vancomycin, and bacitracin. The major precursor accumulated has been identified as UDP-MurNAc-pentapeptide (10). Both strains, S. aureus H (SmR) and 52A5 were found to be equally sensitive to these antibiotics (minimum inhibitory concentration: penicillin G, 0.05 µg/ml; vancomycin, 0.7 µg/ml; bacitracin, 15 µg/ml). A striking difference in the level of nucleotide accumulation within the cells was observed in the presence of penicillin G (Table 1). Penicillin induced a 10-fold increase in the accumulation of nucleotide-bound N-acetylated amino sugar in cells of S. aureus H (SmR), whereas it caused no detectable change in strain 52A5 cells. Similar results were obtained on varying the penicillin G concentration (1 µg/ml to 120 µg/ml), the period of treatment with penicillin G (10 to 120 min), or the concentration of trichloroacetic acid (5 to 25%) for extracting the nucleotides. In contrast to this result, bacitracin and vancomycin caused accumulation of comparable amounts of UDP-MurNAc-pentapeptide in both S. aureus H (SmR) and strain 52A5. It may be noted that the basal level of nucleotide-bound murein precursor in strain 52A5 was more than twice than in S. aureus H (SmR), i.e., 5.7 nmoles/ml as compared with 2.2 nmoles/ml. Because inability to accumulate precursors could have been due to leakage of these compounds from the cells of strain 52A5 when treated with penicillin G, we analyzed the spent media for these precursors. Owing to the difficulties in assaying for bound N-acetylated amino sugar in the PYK-medium, cells were treated with 6 µg of penicillin per ml for 60 min in a defined medium containing the four cell wall amino acids and glucose (3). After removal of cells, the concentrated culture medium was desalted on Sephadex G-25 and the nucleotide precursors estimated. No nucleotide-bound N-acetylated amino sugars were found in the spent medium from S. aureus H (SmR) cultures with or without penicillin G, or from the medium from S. aureus 52A5 cultures incubated without penicillin G. About 4.0 to 7.0 nmoles/ml of nucleotide-bound N-acetylated amino sugar were found in the culture medium of strain 52A5 treated with penicillin G. This accounts for about 25% of the precursor which failed to accumulate inside the cells. The nature of the nucleotide-bound murein precursors was determined by paper chromatography. The compounds were detected on paper both by their UV absorption and by ninhydrin reaction. The principal nucleotide precursor identified in the cells and culture media of strain 52A5 as well as in S. aureus H (SmR) cells was UDP-MurNAc-pentapeptide.

**Membrane observations.** Since no accumulation of murein precursors occurred in the presence of penicillin, a defect in the membrane system of strain 52A5 seemed possible. However, growth inhibition tests with a number of chemical agents known to be deleterious to membranes failed to reveal differences between the strains in sensitivity to the following agents: methylene blue, polymyxin B, gramicidin S, versene, and sodium deoxycholate. Furthermore, no difference in
the membrane phospholipid composition between the two strains exists since mobilities of the phospholipids extracted by methanol-chloroform mixture (8) were identical when chromatographed in methanol-chloroform-water (35:75:4, v/v). Two major phospholipids had the same mobilities as phosphatidyl glycerol and cardiolipin. A minor spot had the mobility of phosphoethanolamine.

Nature of mutation. The nature of the mutation was studied to determine if the described pleiotropic effects were due to a single point mutation. Phage-resistant mutants with properties identical with those of \textit{S. aureus} 52A5 have been independently isolated from reisolated clones of \textit{S. aureus} H (Sm\textsuperscript{R}) after mutagenesis with \textit{N}-methyl-\textit{N}'-nitro-\textit{N}-nitrosoguanidine (NTG) as described before (2). This class of phage-resistant mutants deficient in teichoic acid were selected directly on a lawn of 5 $\times$ 10\textsuperscript{8} host range mutant phage 52Ah.

Mutants with strain 52A5 phenotype have also been isolated from \textit{S. aureus} H (Sm\textsuperscript{R}) without mutagenesis. About 2 $\times$ 10\textsuperscript{8} cells of the parent strain were spread on nutrient agar plates containing a lawn of host range mutant phage 52Ah. After 18 hr at 37\textdegree C about 15 colonies per plate were observed with the characteristic colonial morphology of 52A5. These proved to be similar to strain 52A5 in all the other properties tested.

The phage-resistant strain 52A5 was reverted to phage sensitivity after mutagenesis with NTG. Detection of phage-sensitive clones was by the nibbling technique described by Revel (14). About 1 $\times$ 10\textsuperscript{8} 52A phage particles and 200 bacterial cells were spread on plates of nutrient agar. After incubation at 37\textdegree C for 18 hr, about 1\% of the colonies were found to be nibbled. "Wild-type" cells were recovered from these colonies.

Wild-type phage-sensitive revertants were also obtained spontaneously. Advantage was taken of the clumping property of mutant cells. These aggregates tend to settle rapidly to the bottom of the culture fluid, whereas the \textit{S. aureus} H (Sm\textsuperscript{R}) cells remain in suspension. A 16 $\times$ 150 mm tube containing 10 ml of PYK broth and 0.1\% glucose was inoculated with strain 52A5. A freshly isolated colony rather than a slant culture was the source of inoculum. After 18 hr of incubation at 37\textdegree C in a stationary condition, the broth was still clear, though a heavy growth of cells was present at the bottom of the tube. A sample of the culture fluid (0.1 ml) from the top of the tube was transferred to another tube containing broth, and the incubation continued. This serial transfer was repeated; after the fifth transfer, the growth medium was uniformly turbid.

### Table 1. Accumulation of nucleotide-bound N-acetyl amino sugars in the presence of antibiotics

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Strain\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\textit{S. aureus} H</td>
</tr>
<tr>
<td>None</td>
<td>2.2</td>
</tr>
<tr>
<td>Penicillin (6 $\mu$g/ml)</td>
<td>24.2</td>
</tr>
<tr>
<td>Vancomycin (10 $\mu$g/ml)</td>
<td>25.0</td>
</tr>
<tr>
<td>Bacitracin (50 $\mu$g/ml)</td>
<td>19.3</td>
</tr>
</tbody>
</table>

* Results are expressed in nanomoles of N-acetyl glucosamine per ml of culture. Extraction of nucleotides was done with 5\% trichloroacetic acid in the cold and the N-acetyl hexosamines were estimated, after mild hydrolysis, by the modified Morgan-Elson assay.

A sample from the top of this tube was spread on nutrient agar plates. After overnight incubation at 37\textdegree C, colonies characteristic of the parent strain and the mutant were observed. This process has been repeated independently three times with identical results. The colonies that had the appearance of the parent strain proved to be phage-sensitive and possessed the other properties of \textit{S. aureus} H (Sm\textsuperscript{R}). Controls with noninoculated broth were carried through identical procedures in every experiment and no growth was detected in any of these control tubes.

All of the mutant and revertant strains retained their streptomycin resistance (1 mg/ml). In addition, all of them were coagulase-positive and fermented mannitol in a high salt medium.

### DISCUSSION

Mutants with the phenotype of \textit{S. aureus} 52A5 were readily obtained by spontaneous mutation and chemical mutagenesis. Furthermore, revertants could be obtained by selection for different properties of the parent strain (phage sensitivity or growth without clumping) in which all of the other mutant characteristics also reverted to wild type (Table 2). This strongly indicates strain 52A5 is mutant in a single gene which affects a variety of cell functions.

A summary for the properties of the strains \textit{S. aureus} H (Sm\textsuperscript{R}), 52A5, and of the various mutants and revertants isolated from these two strains, is shown in Table 2. The most striking characteristic of the mutant is the apparent absence of polymerized teichoic acid even though the polymerizing enzymes are present (Mireman et al., \textit{Bacteriol. Proc.}, p. 47, 1969). This lack of teichoic acid is the basis of its resistance to phage (2). Some of the other altered characteristics...
TABLE 2. Properties of *S. aureus* H (SmR), strain 52A5, and of various mutants and revertants derived from them

<table>
<thead>
<tr>
<th>Strain</th>
<th>Method of selection</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em> H (SmR)</td>
<td>Wild type</td>
<td>Sensitive to phages 52A, 52Ah, and K Resistant to 1 mg/ml of streptomycin Generation time 30 min Cells occur singly and in pairs Colonies smooth, shiny, and yellow Autolytic enzyme mostly in supernatant fraction Accumulates nucleotide-bound murein precursors in presence of penicillin G</td>
</tr>
<tr>
<td>52A5</td>
<td>Original mutant isolated after mutagenesis with NTG from <em>S. aureus</em> H (SmR)</td>
<td>Resistant to phages 52A, 52Ah, and K Resistant to 1 mg of streptomycin per ml Generation time 39 min Cells occur mostly in large clumps Colonies irregular, rough, and gray Autolytic enzyme mostly wall bound Little nucleotide-bound murein precursor in the presence of penicillin G Cells larger in size and mass than those of <em>S. aureus</em> H (SmR)</td>
</tr>
<tr>
<td>M1, M9</td>
<td>Isolated from <em>S. aureus</em> H (SmR) by NTG induction; selected on basis of resistance to phage 52Ah</td>
<td>All characteristic similar to 52A5</td>
</tr>
<tr>
<td>SH1</td>
<td>Isolated from 52A5 by NTG induction; selected on basis of sensitivity to phage 52Ah (nibbling technique)</td>
<td>All characteristics similar to <em>S. aureus</em> H (SmR)</td>
</tr>
<tr>
<td>RA RA2</td>
<td>Isolated from 52A5 spontaneously; selected on the property of wild-type cells to remain suspended in culture, in contrast to 52A5 cells which tend to settle to bottom</td>
<td>All characteristics similar to <em>S. aureus</em> H (SmR)</td>
</tr>
<tr>
<td>1, 3</td>
<td>Isolated from <em>S. aureus</em> H (SmR) spontaneously on the basis of resistance to phage 52Ah</td>
<td>All characteristics similar to 52A5*</td>
</tr>
</tbody>
</table>

* Nucleotide accumulation and autolytic activity have not been checked in these strains.

of the mutant appear to be related to a defect in the mechanism of cell separation. Thus, the cells remain attached to each other to form large clumps; the cell septa are not uniform; the cells are larger and possess a thicker cell wall with a rougher surface; and the growth rate is slowed. Yet another difference is that, although intact cells of both strains autolyze equally well, isolated mutant cell walls autolyze much more rapidly than walls from the parent strain. Nevertheless, the parent strain contains much more autolytic activity in soluble form than the mutant strain. These results may suggest that the lytic enzyme in strain 52A5 is present or acts at the wrong place or time so that the normal cell separation is impaired. This, of course, does not mean that the altered distribution of the enzyme is the primary defect rather than a consequence of the primary defect.

The activity of the autolytic system in *Streptococcus faecalis* is apparently regulated in such a way that the enzymes are mainly active at the zone of new cell wall synthesis (17). The liberation of hemispherical fragments of cell walls from *S. aureus* under suitable conditions due to the action of autolytic enzymes likewise suggests a selective, localized action by the autolytic enzymes of *S. aureus* (9). These observations...
suggest that location and timing of autolytic activity in the mutant may be responsible for various changes in morphology and growth characteristics. It is important to note that viable cells could be recovered from the clumped cell aggregate of strain 52A5 by sonication (Fig. 2). This indicates that the cells inside a clump have a functional cross wall and thus differ from the nonseptate division mutants in Escherichia coli (1).

Other properties of strain 52A5 cannot presently be explained (or rationalized) as consequences of the reduced teichoic acid content of its walls or its altered autolytic properties. Specifically, the mutant does not accumulate nucleotide-bound cell wall precursors in the presence of penicillin. This is a relatively unique property limited to the parent and other strains of S. aureus. The mutant also utilizes lactose at a much reduced rate, though it ferments normally other sugars used by the parent strain (Chatterjee, A.N., unpublished data). These last two observations might suggest some general membrane defect. A general membrane defect arising from an alteration in the structural membrane protein of Neurospora was observed by Woodward and Munkres (19).

We have not yet succeeded in pinpointing the single gene defect involved and it is difficult to understand how a single factor affects so many properties of the cell. It should be noted, however, that all of these phenotypic differences are in some way related to the structure or function of the cell envelope. The gene involved could affect one of the following: (i) the biosynthesis of teichoic acid—the resulting cell wall lacking teichoic acid would have a reduced net charge which might, of itself, indirectly produce the observed profound effects on the cell; (ii) regulation of the functions of the cell envelope during cell division; or (iii) a membrane protein that alters permeability and the activity of enzymes related to the cell wall. Further study of mutants of this type could lead to a better insight into the control of cell wall synthesis and cell division, the relation of the membrane to these processes, and the role of teichoic acid.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant AI-05090 from the National Institute of Allergy and Infectious Diseases. Howard J. Singer was a recipient of Public Health Service Predoctoral fellowship 5-F01-6M-35, 288 from the National Institute of Medical Sciences. David Mirelman was recipient of a Fulbright-Hayes Travel Grant.

We are indebted to John Robinson for the electron micrographs and helpful discussions.

We thank Evelyn Ekmejian and Olga Silins for excellent technical assistance.

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