Transfection of Lysostaphin-treated Cells of
Staphylococcus aureus

H. G. RIGGS, JR.1 AND E. D. ROSENBLUM
Department of Microbiology, The University of Texas Southwestern Medical School, Dallas, Texas 75235

Received 11 September 1968

After treatment with 1 unit of lysostaphin per ml for 3 min, two strains of Staphylococcus aureus, 233 and PS 44A HJD, were transfected with phenol-extracted deoxyribonucleic acid (DNA) from the staphylococcal bacteriophages, 53 and 44A HJD, respectively. The number of transfected cells was low in both systems, approximately two in 106 enzyme-treated cells. There was a saturation effect at high concentrations of DNA; optimal results were obtained at concentrations between 10 to 25 μg/ml. Growth curves and fluctuation tests indicated that cells of strain 44A HJD infected with phage, then converted to protoplasts by a 10-min treatment with lysostaphin, produce only one phage particle and lose their ability to lyse spontaneously in hypertonic media.

The infection of spheroplasts and protoplasts by isolated bacteriophage deoxyribonucleic acid (DNA) with the subsequent production of whole phage particles has been reported for several bacterial species: Escherichia coli (1, 4, 6, 11), Aerobacter aerogenes (11), Streptomyces kanamyceticus (7), and Bacillus subtilis (12). However, this phenomenon has not been previously reported for strains of Staphylococcus aureus.

The discovery of lysostaphin (9), which contains a peptidase that lyses the pentaglycine bridge in the staphylococcal cell wall, has facilitated the preparation of spheroplasts and protoplasts of this organism. Incubation with 1 unit (29.1 μg) of enzyme per ml of cell suspension for 10 to 15 min in a hypertonic tris(hydroxymethyl)amino methane (Tris) buffer completely solubilizes the cell wall of many staphylococcal strains; with shorter exposure times, a varying number of colony-forming units (CFU) can be recovered. This paper describes a procedure for transfection of lysostaphin-treated cells of two strains of S. aureus with isolated staphylococcal bacteriophage DNA.

MATERIALS AND METHODS

Bacterial strains. The two strains of S. aureus, 233 and PS 44A HJD, were obtained from the stock collection of the Department of Microbiology, The University of Texas Southwestern Medical School, Dallas.

Phages. The staphylococcal bacteriophages used in this study, 53 and 44A HJD, were also obtained from the above source.

1 Present address: Department of Microbiology, The University of Missouri Medical School, Columbia, Mo.

Preparation of phage DNA. Phage stock was added to a 4-hr Trypticase soy broth (TSB) culture of the propagating strain (233 for phage 53 and PS 44A HJD for phage 44A HJD) to give a multiplicity of infection of approximately 1. This mixture was incubated for 4 to 6 hr at 37 C and then was placed at 4 C overnight to allow lysis to continue but to prevent readsorption of the free phage. The mixture was centrifuged at 3,020 x g for 10 min at 4 C, and the supernatant fluid was filtered through an HA membrane filter (Millipore Corp., Bedford, Mass.). The sterile filtrates were centrifuged at 55,000 x g for 30 min to sediment the phage. The phage pellet was resuspended in 0.1 M NaCl-0.01 M Na2HPO4 solution, pH 7.0. An equal volume of phenol, neutralized to pH 7.0 with NaOH, was added, and this mixture was rotated at 60 rev/min in a relatively horizontal position for 30 min. The mixture was centrifuged at 1,930 x g at 4 C for 15 min. The aqueous layer was removed and fresh phenol was added to it. The tube was rotated for 15 min at 60 rev/min and centrifuged. The aqueous layer was removed and dialyzed at 4 C against sterile 0.01 Na2HPO4-0.001 M ethylenediaminetetraacetic acid (EDTA) solution overnight. The dialyzed solution was stored at 4 C as the stock phage DNA. The concentration of the DNA was determined by the diphenylamine method (3) with salmon sperm as the standard.

Preparation of lysostaphin-treated S. aureus cells. A 4-hr TSB culture was sedimented at 1,560 x g for 10 min, and the cells were resuspended in 0.05 M Tris buffer at pH 7.5. One unit of lysostaphin per ml of cell suspension was added, and the mixture was incubated for 3 min in shaking water bath at 37 C. The mixture was then placed in an ice bath for 10 min, and the treated cells were sedimented at 12,100 x g at 4 C. The treated cells were washed twice with the hypertonic Tris buffer and 2.5% trypsin and then were resuspended in TSB containing 5% NaCl.

Transfection procedure. The lysostaphin-treated
cells were incubated at 37°C with 10 μg of phage DNA per ml of cell suspension to which sodium citrate had been added to a final concentration of 0.09 M. Samples were removed at 15, 30, 45, 60, and 90 min and were assayed for infectious centers (IC) by the agar overlay method (5). Both the hard Trypticase soy agar (TSA) base layer and the soft TSA (TSB plus 0.7%, w/v, agar) overlay used in the assays contained 5% NaCl to protect the osmotically fragile treated cells. Indicator cells (a broth suspension of untreated cells of the same strain) were added to the soft agar overlay. Trypsin was added to the soft agar (final concentration, 2.5%) for a direct count of IC, but was omitted in the indirect assay method.

**Indirect assay method for transfection.** The soft agar overlay was mechanically removed from the assay plate. A 5-ml amount of TSB was used to wash the hard agar base surface and was then added to the harvested soft agar. The mixture was agitated on a Vortex Jr. mixer until a slurry formed. After centrifugation at 1,560 × g for 7 min at 4°C, the supernatant fluid was filtered through an HA membrane filter (Millipore Corp.). The filtrate was assayed for plaque-forming units (PFU) by the agar overlay method.

**RESULTS**

**Effect of lysostaphin treatment.** Approximately 10⁶ CFU/ml were treated with lysostaphin. After a 3-min exposure, this number decreased to between 10⁴ and 10⁵ CFU/ml as determined by the agar overlay method. The variation in yield was possibly the result of cells clumping together before treatment or the result of aggregation of cells after treatment. After addition of the enzyme, there was an immediate increase in the viscosity of the cell suspension; after centrifugation following washing, the cells formed long stringy masses which were difficult to resuspend.

In an attempt to obtain a more consistent yield of lysostaphin-treated cells, 10-fold dilutions of 1 unit of enzyme were added to cell suspensions. There was a more consistent recovery of CFU with the lower concentrations; however, when phage DNA was incubated with 0.1 unit of lysostaphin per ml or less, no transfection resulted. In a subsequent treatment, 0.25 unit/ml did give positive transfection.

**Transfection.** In the transfection of **S. aureus** strain 233 with phage 53 DNA, the maximal yield of IC was 150 by direct count, indicating that 1.5 cells in 10⁹ initially lysostaphin-treated CFU were infected with a complete phage genome. Similarly, in the transfection of strain PS 44A HJD with phage 44A HJD DNA, the number of infected cells was low; i.e., three IC in 10⁷ treated cells.

The results of these experiments are presented in Fig. 1 and 2. Two different methods were used to quantitate transfection: (i) an indirect method which involved harvesting the assay soft agar overlay after a 24-hr incubation period and enumerating the PFU present and (ii) a direct method which involved counting the IC produced by samples of the treated cells after incubation with phage DNA. The indirect method was used in initial experiments because there was sufficient residual lysostaphin in the incubation mixture to degrade the assay bacterial lawn, making it impossible to count the IC. In later experiments, the addition of trypsin to the assay soft agar destroyed the residual lysostaphin (10) and IC could be counted directly.

As shown in the figures, there is an apparent lack of correlation between the two assay methods; i.e., the increase in PFU at 120 min of incubation and the continuous decrease in IC. In the assay of overlays from plates containing residual lysostaphin (indirect assay method), we speculated that the phage particles produced and liberated from the infected cells were "inactivated" by cell wall material in the incubation mixture and that the residual lysostaphin continued to degrade this wall material, releasing infective phage which formed PFU in the assay. To test this hypothesis, cells were treated with lysostaphin and then were incubated for an additional 45 min. Whole phage were then added with 25 μg of chloramphenicol per ml to prevent phage production. Samples of the mixture were assayed for free phage at various time intervals (Fig. 3). There was an initial drop in the number

![Figure 1. Number of PFU produced by the indirect assay method (○) and number of IC produced by the direct assay method (O) from lysostaphin-treated cells of **S. aureus** strain 233 incubated with 10 μg of phage 53 DNA per ml for various time intervals. Controls containing (i) untreated **S. aureus** strain 233 cells plus 10 μg of phage 53 DNA per ml (solid line), and (ii) lysostaphin-treated **S. aureus** strain 233 cells plus deoxyribonuclease-treated phage 53 DNA (dashed line) were consistently negative. The latent period was determined for whole cells of **S. aureus** strain 233 infected with intact phage 53.]
of free phage, which suggests that the treated cells still retain the ability to adsorb phage, but there was no increase in phage up to the end of a 120-min incubation period, indicating that there was no release of adsorbed phage by continued exposure to residual lysostaphin.

**Effect of phage DNA concentration.** There is a DNA saturation effect in the transfection system of *S. aureus* strain PS 44A HJD and phage 44A HJD DNA. Results of experiments determining the effect of DNA concentration on plaque-forming ability are presented in Fig. 4. There is an apparent linear relationship between concentration and number of IC at concentrations ranging from 0.1 to 10.0 μg/ml; no increase in IC occurred between 10 and 25 μg/ml, and the number of IC decreased markedly at a concentration of 50 μg/ml. A concentration of 10 μg/ml was used in the transfection experiments.

A study was made to determine the effect that a mixture of the two types of phage DNA would have when added to lysostaphin-treated cells of strain PS 44A HJD. This strain is normally resistant to phage 53. We found that the mixture produced fewer IC than one would expect from an infection with phage 44A HJD alone and that none of the cells would produce phage 53.

**Growth curves for untreated and treated cells.** A one-step growth curve for *S. aureus* strain 233 infected with phage 53 was determined. The latent period was 60 min, the rise period was 15 min, and the burst size was 16. A growth curve for untreated *S. aureus* strain PS 44A HJD infected with phage 44A HJD is shown in Fig. 5. The latent period was 45 min, the rise period was 20 min, and the burst size was 16. In comparison, strain PS 44A HJD cells which were first infected with whole phage 44A HJD and then were treated for 10 min with lysostaphin did not show any increase in phage titer up to 120 min of incubation. To determine whether these infected protoplasts were inhibited from lysing before plating,
0.1 ml of the infected organisms was osmotically ruptured in distilled water and assayed for PFU. The results were similar to those obtained with unruptured cells (see Fig. 5). The comparable titer between whole and osmotically ruptured protoplasts suggested that an infected protoplast produced a single phage particle which was not spontaneously released, but required artificial rupture. The intact protoplasts were probably lysed during or after the pipetting of the sample in the assay procedure. There was a difference in the morphology of the plaques formed by cells plated directly and by osmotically disrupted cells; all of the plaques formed by unruptured protoplasts were large and had halos, whereas the plaques formed by the osmotically ruptured cells were large, clear plaques. There are several possible explanations for this difference, but it is most likely that the phage particle from the cells ruptured during plating is released slowly and the delay could be responsible for the plaque with a halo.

**Fluctuation test for single burst.** Cells of *Staphylococcus aureus* PS 44A HJD which were first infected with phage 44A HJD and then treated with lysostaphin for 10 min were dispensed in 50 tubes so that there was less than one infected bacterium per tube. The tubes were incubated for 90 min, and the entire contents of the tubes were plated for phage assay with a separate plate for each tube. The results of a typical experiment are shown in Table 1. The experimental results are in good agreement with the expected results calculated from the Poisson formula and show that there is approximately one phage particle produced per infected bacterium.

**DISCUSSION**

Lysostaphin-treated cells of two different strains of *S. aureus* can absorb isolated bacteriophage DNA and produce complete phage particles. The rate of transfection is low in both cases; however, it compares favorably with reported rates in other bacterial species (2, 8).

One aspect of this study indicated that the step or steps subsequent to the absorption of phage DNA determine the sensitivity of a bacterial strain to a phage. In a series of experiments, phage 53 DNA was unable to produce IC in lysostaphin-treated cells of strain 44A HJD, a strain normally resistant to phage 53. An excess (25 μg/ml) of phage 53 DNA also interfered with the uptake of phage 44A HJD DNA in these treated cells.

The degree of cell wall damage for DNA adsorption is still undefined as the incubation mixture contained intact cells as well as partially and completely denuded cells. However, from the low rates of transfection and the data indicating that

**Table 1. Distribution of yields from individual phage-infected strain 44A HJD cells treated with lysostaphin (single-burst distribution)**

<table>
<thead>
<tr>
<th>Determination</th>
<th>Distribution of yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Total no. of plates</td>
<td>50</td>
</tr>
<tr>
<td>(b) No. of plates found without plaques</td>
<td>30</td>
</tr>
<tr>
<td>(c) Avg no. of infected bacteria per plate (from b)</td>
<td>0.51</td>
</tr>
<tr>
<td>(d) Calculated no. of plates with one infected bacterium</td>
<td>15</td>
</tr>
<tr>
<td>(e) Actual no. of plates with one plaque</td>
<td>18</td>
</tr>
<tr>
<td>(f) Calculated no. of plates with two infected bacteria</td>
<td>4</td>
</tr>
<tr>
<td>(g) Actual no. of plates with two plaques</td>
<td>2</td>
</tr>
<tr>
<td>(h) Calculated no. of plates with three infected bacteria</td>
<td>0</td>
</tr>
<tr>
<td>(i) Actual no. of plates with three plaques</td>
<td>0</td>
</tr>
<tr>
<td>(j) Total no. of plaques</td>
<td>22</td>
</tr>
<tr>
<td>(k) Avg yield per infected bacterium</td>
<td>0.87</td>
</tr>
</tbody>
</table>
protoplasts produce only one phage particle, it can be speculated that only those cells with considerable wall damage are capable of absorbing DNA.

The inability of a cell to produce more than one phage particle after conversion to a protoplast may account for the discrepancy between the two assay methods. Even though the treated cells were washed twice with trypsin, there apparently was sufficient residual lysostaphin in the incubation mixture to continue degradation of the cell wall during the 120-min incubation period. A concentration of as little as 0.001 µg/ml has the capacity to reduce the CFU in a cell population to less than half. Therefore, since it appears that phage DNA absorption takes place during the initial 30-min incubation period, many of the more heavily damaged cells may be converted to protoplasts during that time by the residual lysostaphin and may lose their capacity to produce more than one phage particle as well as their ability to lyse spontaneously. The less heavily damaged cells may or may not retain their ability to produce several mature phage as well as their ability to lyse. After long periods of incubation, the entire infected cell population may be converted to protoplasts and may require some means of mechanical disruption for lysis. These assumptions would account for the discrepancy found in the two assay procedures: the increasing number of PFU and IC during the early part of the experiment could be the result of cells having the ability to lyse spontaneously. But as the cells are converted to protoplasts (this would have to occur before the end of the normal phage reproduction cycle), the only detectable IC would be those resulting from the small number of protoplasts lysed during the plating procedure. This would explain the continuous decrease in IC by direct count. However, when the agar overlays from the plates containing no trypsin were harvested (a harsh procedure for the fragile protoplasts), the infected protoplasts would be lysed. Some of these protoplasts may have produced many phage particles before losing their spontaneous lysis ability. An increase in phage particles due to mechanical disruption during harvest would account for the increase rather than the decrease in PFU. An analogous situation, i.e., a greater number of PFU produced from osmotically ruptured cells versus the number of IC by direct plating for the same cell suspension, has been reported for spheroplasts of *E. coli* W-3350/λ,1 infected with coliphage T1 DNA (2).

The inability of a protoplast to produce more than one phage particle as well as to lyse spontaneously is of great interest. These phenomena may be related to evagination of the mesosome when the cells are converted to protoplasts. This organelle is an invagination of the cell membrane and may require the rigid cell wall to maintain its integrity. Since this structure has been reported to be involved in the cell's DNA replication, its loss may prevent phage DNA replication. Nevertheless, the phage DNA molecule which has been absorbed could still be transcribed to messenger ribonucleic acid which would code for the production of phage protein. This protein and a single DNA molecule could then assemble into the one phage particle. Since production of the lytic enzyme is one of the late functions in intracellular phage development, a sufficient quantity of the enzyme may not be produced to overcome the osmotic protection given the protoplast by the hypertonic medium.

ACKNOWLEDGMENTS

We thank P. A. Tavormina, Mead Johnson Laboratories, for supplying the lysostaphin used in this study.

This investigation was supported by Public Health Service research grant AI-03093 from the National Institute of Allergy and Infectious Diseases and by Public Health Service training grant 2E-142 from the National Institute of General Medical Sciences.

LITERATURE CITED