Transfection of *Staphylococcus aureus* with Bacteriophage Deoxyribonucleic Acid

**JAN-ERIC SJÖSTRÖM, MARTIN LINDBERG, AND LENNART PHILIPSON**  
Department of Microbiology, The Wallenberg Laboratory, University of Uppsala, Uppsala, Sweden

Received for publication 29 June 1971

*Staphylococcus aureus* cells of strain 8325 (N) are competent for phage deoxyribonucleic acid (DNA) when harvested in the early exponential growth phase. Phenotypic expression of the competence requires divalent cations, and calcium ions are most effective. Treatment of phage DNA with deoxyribonuclease completely destroys infectivity and heat-denatured DNA is not infectious. The highest frequency of transfection is around 10^4 plaque-forming units per μg of DNA.

Infection of spheroplasts and protoplasts with isolated phage deoxyribonucleic acid (DNA) has been reported for several bacterial species: *Escherichia coli* (1, 4, 7, 17), *Aerobacter aerogenes* (17), *Streptomyces kanamyceticus* (13), and *Proteus* (20). Rigg and Rosenblum (16) reported transfection of *Staphylococcus aureus* protoplasts where the cell walls had been partially destructed by treatment with the enzyme lysostaphin. Transfection of cells with intact cell walls has been described for *E. coli* in the presence of helper phage (8, 10), for *B. subtilis* with cells competent for uptake of transforming DNA (18), and for *B. stearothermophilus* (19). Recently Mandel and Higa (11) described infection of competent *E. coli* cells by phage DNA in a reaction medium containing calcium ions.

The present paper reports a system for transfection of intact *S. aureus* cells with DNA from phage 80α. Transfection was not obtained with DNA from phage 53 under the same conditions.

**MATERIALS AND METHODS**

**Bacterial strains.** The strain 8325 (N) of *S. aureus* was obtained from M. H. Richmond, Department of Bacteriology, The Medical School, Bristol.

**Phages.** Two staphylococcal bacteriophages were used in this study: 80α provided by R. P. Novick, Department of Biochemistry, New York University, School of Medicine, New York, and the standard typing phage 53 obtained from The National Bacteriological Laboratory, Stockholm. Novick et al. (12) consider it uncertain whether phage 80α is a host-range mutant or a host-induced phenotypic modification of the typing phage 80. Both phage 80α and phage 53 belong to the serological group B. Phage stocks were propagated in Trypticase Soy Broth (TSB) and diluted and stored in BPD (3) which has the following composition: nutrient broth (Difco) containing 0.25% (w/v) K₂HPO₄ and 0.20% (w/v) glucose. The stock suspensions were sterilized by filtration (0.45 μm pore size, Millipore Corp.) and stored at 4°C.

**Propagation and purification of phages.** Phages were propagated on strain 8325 (N) in TSB. For phage 80α, a multiplicity of infection (MOI) of approximately 0.01 was used. For phage 53, MOI was 0.1. Calcium was added to a final concentration of 0.004 M. The mixtures of phages and bacteria were allowed to stand at 37°C for 10 min to allow adsorption of phages and then were further incubated with shaking at 37°C until lysis occurred. The lysates were purified from bacteria and cell debris by centrifugation at 16,300 x g for 15 min. The phages were pelleted at 107,000 x g for 2 hr. The pellets were suspended in 0.01 M (tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.1. The suspensions were again centrifuged at 12,100 x g for 15 min. Phage 80α was finally concentrated and purified on a cushion of CsCl (1.60 g/cm³) by centrifugation at 81,000 x g for 150 min. Phage 53 was concentrated by centrifugation at 81,000 x g for 150 min in a preformed CsCl gradient (1.40 to 1.60 g/cm³). The phage bands were recovered and dialyzed overnight at 4°C against sterile 0.01 M Tris-hydrochloride at pH 8.1.

**Preparation of DNA.** Phage DNA was extracted by phenol by a modified procedure of Green and Piña (6). Four volumes of concentrated phage suspension was mixed with one volume of Pronase (10 mg/ml in 0.01 M Tris-hydrochloride, pH 8.1) and incubated at 37°C for 60 min. Sodium dodecyl sulfate was then added to a final concentration of 0.3% and the incubation continued at room temperature for 30 min. The treated phage suspension was then mixed with an equal volume of phenol saturated with 0.01 M Tris-hydrochloride, pH 8.1, and the mixture was shaken for 15 min at 4°C. The resulting emulsion was separated into two layers by slow-
speed centrifugation and the upper aqueous phase was removed. The phenol treatment was repeated twice. The aqueous phase was then extracted with ethyl ether several times to remove traces of phenol. Finally, the ether was removed by nitrogen. The DNA was precipitated by slow addition of two volumes of cold ethanol. The DNA was collected by centrifugation at 12,100 × g for 15 min and dissolved in 0.01 dilution of SSC (0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.2) and stored at 4°C. The concentration of DNA was determined by the diphenylamine method (2) with calf thymus DNA as standard. The ultraviolet spectrum of the DNA preparation was read in a Zeiss spectrophotometer. The absorption ratio (260/280 nm) was 1.9 to 2.0.

Melting and annealing of DNA. Tubes with 2 ml of DNA solution containing 20 μg/ml in 0.01 dilution of SSC were treated for different times in boiling water. The heated solutions were divided into halves. The Ionic strength was raised to 1 × SSC in both samples. One of these was rapidly chilled in ice water. The other one was kept at 60°C for 2 hr. The melting point of phage 80c DNA in 0.01 × SSC was about 62°C as determined in a preliminary experiment. Native, melted and annealed DNA was assayed for infectivity as described below.

Enzymatic treatment of DNA. Deoxyribonuclease was dissolved in 0.01 M Tris buffer, pH 7.5, containing 0.05 M MgCl₂. Ribonuclease and Pronase were dissolved in the same buffer containing 10⁻⁴ M ethylenediaminetetraacetic acid (EDTA). Enzymatic treatment was carried out at 37°C for 4 hr.

Assay for infectious DNA. Competent bacteria were prepared as follows. Trypticase Soy Agar (TSA) plates were inoculated from a stock culture and the cells were allowed to grow at 37°C for 12 hr. Cells were collected in TSA to an OD₅₄₀ = 0.200 and diluted 10⁻⁴ fold in TSB which corresponds to 10⁶ colony-forming units (CFU)/ml. This culture was incubated at 37°C with shaking. When OD₅₄₀ reached a value of 0.100, the cells were centrifuged and washed twice with 0.1 M CaCl₂ in distilled water and finally suspended in the same solution to a cell concentration of 10⁷ to 10⁸ CFU/ml. The cell concentration used varied in different experiments.

The DNA was immediately added to the cells, and the mixture was incubated for 10 min at 37°C with slow shaking. Samples were then plated using the standard plaque assay method with 8325 (N) as the indicator bacteria. The bottom agar layer was TSA. The soft agar medium contained TSB plus 0.5% (w/v) agar. The plates were incubated at 37°C for 16 hr.

Chemicals and enzymes. Pronase B grade was obtained from Calbiochem, Calif. Deoxyribonuclease and ribonuclease A were purchased from Worthington Biochemical Corp. Phenol was pro analysis (Merck) which was redistilled before use. TSA and TSB were obtained from B-B-L.

RESULTS

Competence curve and the effect of divalent ions. The competence for transfection through the growth curve of S. aureus in TSB was first analyzed. The inoculum was collected from TSA plates incubated for 12 hr at 37°C. Samples were withdrawn at intervals during growth, and the cell density was adjusted to OD₅₄₀ = 0.200 in TSB. The cells were washed twice and suspended in 0.1 M CaCl₂ in distilled water to one-tenth volume (= 10⁵ CFU/ml). DNA was added to a final concentration of 10 μg/ml. Figure 1 illustrates the infectivity of 80c DNA at different points on the growth curve for S. aureus 8325 (N). The competence curve has a distinct peak in the early exponential growth phase. Figure 1 also shows the relationship between viable counts and the optical density at 524 nm.

Different cations in distilled water were tested for efficiency of competence promotion. As shown in Fig. 2, 0.1 M CaCl₂ is the optimal concentration for promotion of the ability of the cells to take up DNA. The frequency of transfection obtained with Mg²⁺ as competence promoting ion was 50 to 60% of that with Ca²⁺. Ba²⁺ could also promote competence but to a level much reduced as compared to the other two ions.

Effect of enzymes on the infectivity of 80c DNA. Table 1 reports the results from enzymatic treatment of DNA prior to assay of infectivity (see above). It is evident that the infectivity is unaffected by digestion with ribonuclease or Pronase, but deoxyribonuclease at a concentration of 5 × 10⁻³ μg/ml completely destroys the infectivity.

Attachment and penetration of DNA. To determine the optimal time of incubation required for maximal transfection, recipient bacteria and phage DNA were mixed, and, after different times, samples were assayed for plaque-forming units (PFU). After 8 to 10 min of contact between DNA and competent cells, the number of PFU reaches a maximum (Fig. 3). Incubation beyond this time reduces the number of transfected cells. This is caused probably by the lethal effect from the high concentration of calcium required for maximal competence, since both viable counts and reduction in PFU per milliliter follow the same slope when cells are incubated with calcium without addition of DNA (not shown).

The attachment of DNA to the cells is followed by transport of the adsorbed DNA to a state establishing deoxyribonuclease resistance (5). Studies were undertaken to examine the kinetics of the appearance of deoxyribonuclease resistance. At zero time, DNA and competent cells in 0.1 M CaCl₂ were mixed and incubated for 5 min at 37°C. The incubation period was terminated by washing the cells twice in TSB to eliminate unattached...
DNA. The cells with attached DNA were then incubated in TSB at 37°C with shaking, and the infective centers were examined at different times by plating parallel samples, one directly and the other after deoxyribonuclease treatment for 5 min. Figure 4 (curve A) shows that the adsorbed DNA molecules are irreversibly adsorbed to the cells, since the number of PFU is constant until lysis of the infected cells. The lower curve (curve B) shows that all the adsorbed infective DNA molecules which were sensitive to deoxyribonuclease treatment at 20 min gain resistance to deoxyribonuclease treatment after about 45 min of incubation in TSB.

**Effect of phage DNA concentration.** The effect of DNA concentration on infectivity was studied next. The number of recipient cells was 10^10 CFU/ml. Figure 5 shows that there exists at this cell concentration a linear relationship between the number of PFU and concentration of DNA in the range 0.1 to 10 µg/ml. Increasing the DNA concentration above this level does not markedly affect the number of PFU.

In experiments with a constant amount of DNA (10 µg/ml), there was a linear correlation between PFU and cell number in the range 10^4 to 5 x 10^6 CFU/ml.

**Production of phages from infected cells.** The release of free phages from cells infected with phage DNA was followed after allowing contact for 5 min between bacteria and DNA (Fig. 6). The infective process was terminated by deoxyribonuclease treatment. After washing in TSB, the cells were suspended in the same broth and incubated at 37°C with shaking.
Fig. 3. Kinetics of the appearance of transfectants. Time course of the reaction between phage 80α DNA at a concentration of 10 μg/ml and 8325 (N) cells at a density of 10⁶ colony forming units/ml. Symbols: ○, plaque-forming units; ●, colony-forming units.

Samples were plated at intervals for estimation of PFU. Lysis started at 65 min and free phages were released for a period of about 30 min. It can be calculated that about 30 free phages were liberated per infected bacterium. The rise period and burst size for the first infectious cycle with free phages were about the same as with DNA-infected cells, as shown in the later part of the curve. The 80α phage showed in one-step growth curves a latency period of about 40 min and a yield of 40 phages per bacterium over a rise period of about 20 min (not shown).

Infectivity of different DNA forms. A comparison of the infectivity of native, heat-denatured and renatured DNA is reported in Fig. 7. Heat-treatment at 100 C for a shorter time than 2 min yielded a low number of PFU indicating incomplete separation of the strands. After 2 min of denaturation, the infectivity is completely lost. Annealing of DNA resulted in recovery of infectivity. DNA treated at 100 C for a period of 4 min or longer was not infectious after annealing which may be caused by nicks on the single strands. In a separate experiment, it was verified that 100 C for 4 to 8 min gave a hyperchromicity corresponding to complete strand separation (not shown). These data indicate that native double-stranded DNA is infectious.

Infectivity test of phage 53 DNA. The DNA prepared from phage 53 was not infectious. Whether this depended on the quality of the DNA preparations or was caused by a different configuration of the DNA is not known.

DISCUSSION

The DNA of phage 80α has not yet been chemically and physically characterized. Elimination of the biological activity by treatment with deoxyribonuclease appears, however, to confirm that DNA was the infective agent. This is also supported by the infectivity of renatured DNA presented in Fig. 7.

Competent bacteria were prepared by a standard growth method. The expression of

Fig. 4. Kinetics of appearance of deoxyribonuclease-resistant transfectants. After a 5-min period of exposure to 10 μg of 80α DNA per ml, the cells were washed and incubated in Trypticase Soy Broth. Samples were assayed for infective centers (curve A) and for deoxyribonuclease-resistant infective centers (curve B). Time period of 0 to 20 min includes exposure time for DNA (5 min) and washing of the cells. The bars at the bottom of the graph indicate number of plaque-forming units (PFU) when the 80α DNA was digested with deoxyribonuclease prior to incubation with cells. These samples gave zero PFU.
TRANSFECTION OF S. AUREUS

Vol. 109, 1972

The competence required that the cells were suspended in a reaction medium containing divalent ions at high concentrations. Calcium ions gave the highest competence. The competence curve (Fig. 1) has a narrow peak in the early exponential growth phase. Although the competent cells have been prepared by a standard growth method, the number of transfectants varied with a factor 2 to 5 when the same DNA preparation was used in different experiments. Yet the peak of the competence curve was always located at the early log phase of the growth curve. The absolute requirement of Ca²⁺ to promote competence (Fig. 2) was recently reported for transfection of E. coli (11). Transformation frequencies in B. subtilis (21) and Micrococcus lysodeikticus (9) are also enhanced by divalent ions.

No data are yet available to explain the potential ability for competence of S. aureus in the presence of calcium in the early exponential growth phase. The action of the ions could be either on the cell surface or on the DNA molecules.

The competence is maximally expressed when the cells are exposed to DNA immediately after they have been suspended in the calcium solution. The number of transfectants decreases rapidly after the peak at contact for 8 to 10 min between cells and DNA (Fig. 3). This decrease could be explained by the simultaneous decrease in viable cell counts. After

**Fig. 5.** Relationship between infectivity and concentration of DNA. Cells at 10⁸ per ml were incubated for 10 min with different concentrations of DNA, and the number of plaque-forming units was measured.

**Fig. 6.** Time dependence for release of free phages after infection with phage 80a DNA (10⁷ cells/ml and 10 µg of DNA/ml). Time period of 0 to 20 min includes exposure time for DNA (5 min), treatment with deoxyribonuclease (5 min), and washing of the cells (2 × 5 min). (A) Rise period for DNA-infected cells; (B) rise period for cells infected by free phages released from DNA-infected cells.

**Fig. 7.** Infectivity of different DNA forms. Tubes containing 2 ml of DNA (20 µg/ml) in 0.01× SSC were treated for different times at 100 C. The ionic strength was raised to 1× SSC. One-half of the solution was then rapidly chilled. The other half was treated at 60 C for 2 hr for annealing of DNA. Equal volumes of bacteria (10⁸ colony-forming units/ml) and DNA (20 µg/ml) were mixed in the assay procedure. Denatured DNA (curve A) and renatured DNA (curve B). The unheated DNA control yielded 3 × 10⁸ PFU/ml.
the peak, the curve for plaque formation decreases nearly parallel with the cell count. Potentially plaque-forming cells and the remaining population may be equally susceptible to the high concentration of calcium.

The attachment of DNA to the cells is a rapid process as shown in Fig. 3. The next step is penetration of DNA resulting in resistance to deoxyribonuclease treatment. Figure 4 reports the results when cells have been exposed to DNA for 5 min in CaCl\(_2\) followed by washing and suspension of the cells in TSB. About 70% of the adsorbed DNA has gained deoxyribonuclease resistance at 20 min. After 45 min of further incubation, treatment with deoxyribonuclease no longer reduced the number of PFU. This suggests that all the DNA remaining attached to the cells after washing was irreversibly bound to the cells and gradually transported into the cells.

At low DNA-cell ratios, the number of transfectants was found to be proportional to the amount of DNA. At 10 \(\mu\)g of DNA/ml and 10\(^{10}\) CFU/ml (Fig. 5), a plateau was reached where the addition of more DNA did not markedly affect the number of transfectants. This saturation effect suggests that a limited number of sites are available for binding of the DNA.

The phage yield per DNA-infected bacterium was about 30 as calculated from data in Fig. 6. The burst size from bacteria infected by free phages or phages released from transfected bacteria was of the same order of magnitude. Riggs and Rosenblum (16) obtained only one phage particle per infected protoplast of \(S.\) \(aureus\). They proposed that this could be related to evaporation of the mesosome when the cells were converted to protoplasts. The present study indicates that the competent bacteria are intact cells which upon infection with a complete phage genome produce a normal burst. The first free phages were released after about 65 min postinfection with DNA. This time included 20 min for adsorption, deoxyribonuclease treatment, and washing of the cells at 4 C. The time required for lysis of the cells infected by intact phages from transfected bacteria was around 40 min, which corresponds to the latency period in one-step growth cycles with this phage. The retardation of the phage development cycle after DNA infection may be caused by osmotic or temperature shock during transfection.

The highest transfection value obtained in our experiments with 80\(\alpha\) DNA was 10\(^{4}\) PFU per \(\mu\)g of DNA. In these experiments, 10 \(\mu\)g of DNA and 10\(^{10}\) cells per ml were used. With the same amount of DNA and 10\(^{5}\) cells per ml, the number of transfectants was generally 2 \(\times\) 10\(^{2}\) to 10\(^{3}\) per \(\mu\)g of DNA. It is difficult to make comparisons with other transfection systems because of the different conditions used. Mandel and Higa (11) reported 10\(^{4}\) PFU per \(\mu\)g of DNA in their system for \(E.\) \(coli\). Reilly and Spizizen (15) obtained more than 10\(^{6}\) infectious centers per \(\mu\)g of DNA with \(B.\) \(subtilis\) and DNA from some different phages. Thus, the values obtained in our system seem to be somewhat lower. There are several possible explanations for this. The number of competent bacteria in the recipient population may be low. \(S.\) \(aureus\) is known to excrete a nuclease which hydrolyzes DNA. This enzyme has been considered to be responsible for the failure to transform in this species (14). The question also arises whether the DNA from phage 80\(\alpha\) is modified or mutated (12) to make it more resistant to nuclease than DNA from phage 53, which was not infectious under the present conditions. The configuration of the DNA may also influence the transfection frequency. At present, we only knew that denatured 80\(\alpha\) DNA are not infectious nucleic acid.

ACKNOWLEDGMENT

The excellent technical assistance of Solveig Karlsson is gratefully acknowledged.

LITERATURE CITED


