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Genetic Transformation in *Staphylococcus aureus*: Isolation and Characterization of a Competence-Conferring Factor from Bacteriophage 80α Lysates

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The competence-conferring activity in crude lysates of the staphylococcal bacteriophage 80α was concentrated and purified by $(NH_4)_2SO_4$ precipitation, differential ultracentrifugation and rate-zonal centrifugation through Ficoll. This concentrated preparation exhibited lytic activity toward assay cells of *Staphylococcus aureus* 8325-4 that could not be attributed to the residual 80α infectious particles present. Electron microscopic examination of the concentrated competence-conferring activity revealed an occasional intact but empty virion and large numbers of free phage tails. Sodium dodecyl sulfate-polyacrylamide gel analysis of this material confirmed that the competence-conferring activity contained only some, but not all, of the major virion proteins. The competence-conferring activity exhibited single-hit kinetics when assay cells and 80α transfecting deoxyribonucleic acid were present in excess. The competence-conferring activity thus seems to be a unique morphogenic precursor of the 80α virion that mediates transfection and transformation in the presence of 0.1 M $CaCl_2$.

The accompanying paper (19) summarizes the observations that led to the recognition of a factor unique to crude lysates of serological group B bacteriophages of *Staphylococcus aureus* that, in the presence of 0.1 M $CaCl_2$, enables *S. aureus* to become competent for transformation and transfection. This competence-conferring activity could be separated from infectious particles by a combination of isopycnic CsCl centrifugation and rate-zonal centrifugation through Ficoll and was shown to exhibit many of the characteristics of a proteinaceous subvirion structural component (19), probably a precursor of the adsorption organelle. Furthermore, these observations were inconsistent with the proposal (17) that an early gene product of the phage, made intracellularly, is responsible for competence. These experiments also suggested that the competence-conferring activity could be purified sufficiently to allow its further characterization. This study is concerned with this further characterization. The results strongly implicate the adsorption organelle of the serological group B bacteriophage as being responsible for the competence-conferring activity of crude lysates of these phages.

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MATERIALS AND METHODS

Bacteria and bacteriophage. The derivatives of *S. aureus* strain 8325 and the bacteriophage used in this study and the manner in which they were cultivated and maintained are described in the accompanying paper (19). In addition, strain RN2573 (=8325(80α)Ω34[Chr::Tn*551*] *pig-131*) was used in one experiment.

Transformations and transfections. Transforming DNA was prepared by the method of Pattee and Neveln (13), and DNA from phage 80α (used for transfections) was prepared by the method of Thompson and Pattee (18), but with a phenol extraction before precipitation of the DNA (10, 16). DNA was assayed by the diphenylamine reaction (4). Transformations were performed as described elsewhere (19). Transfection assays were performed in the same manner as transformations, except that after exposure of the competent cells to excess phage 80α DNA, the cells were resuspended in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and incubated for 10 min at 35°C before assaying the incidence of plaque-forming units (reflecting the transfected cells) with RN450 as indicator.

Preparation of competence-conferring activity from 80α lysates grown in broth. Large-volume lysates of phage 80α were propagated on RN450 in Trypticase soy broth supplemented with 5 mM $CaCl_2$ as follows. A 1,200-ml volume of Trypticase soy broth contained in a 1-liter Erlenmeyer flask was inoculated with cells of RN450 from overnight brain heart infusion agar (Difco Laboratories, Detroit, Mich.) slants to attain an initial optical density at 540 nm of 0.1.

The flask then was shaken at 180 rpm at 35°C on a New Brunswick model V platform shaker until the optical density attained a value of 0.5. Sterile 0.5 M $CaCl_2$ was then added, and after 5 min of additional shaking, 5×10^{12} plaque-forming units of 80α were added. The flask then was shaken at 35°C until the optical density (after increasing) dropped to 0.2; unlysed cells and cell debris were removed by centrifugation at 10,300 × *g* for 30 min at 4°C. This procedure consistently produced a crude lysate of phage 80α with a titer of approximately 10^{11} plaque-forming units per ml. These preparations of crude lysate usually contained about 10^3 units of competence-conferring activity per ml. One unit of competence-conferring activity is that amount required to bring excess assay cells (usually RN450) to a level of competence resulting in the recovery of one novobiocin-resistant (Nov^r) transformant under standard assay conditions.

Further concentration and purification of the competence-conferring activity in the crude lysate was accomplished as follows: to 1,200 ml of the crude lysate, 600 g of crystalline $(NH_4)_2SO_4$ was added and continuously stirred for 2 h at 4°C after all of the $(NH_4)_2SO_4$ had dissolved. The mixture then was centrifuged (10,300 × *g*, 30 min, 4°C), and the supernatant fluid was discarded. The precipitate was redissolved in 120 ml of phage suspension medium (20) and dialyzed against suspension medium at 4°C to remove residual $(NH_4)_2SO_4$. The dialyzed material then was centrifuged at 50,000 × *g* for 2 h at 4°C to pellet most of the plaque-forming units while leaving the majority of the competence-conferring activity in suspension. The phage contained in the pellet from this centrifugation, after gentle resuspension in suspension medium and banding in CsCl, was used as a source of purified phage 80α for electrophoretic analysis and DNA extraction. The supernatant fluid from this centrifugation then was centrifuged (135,000 × *g*, 5 h, 4°C), resulting in a pellet containing the bulk of the competence-conferring activity. This pelleted material was gently resuspended in 5 ml of suspension medium and is referred to hereafter as the competence-conferring activity-rich fraction. This fraction contained 10^{11} plaque-forming units and 5×10^5 units of competence-conferring activity per ml.

Electron microscopy. Purified samples of phage 80α and Ficoll-purified competence-conferring activity were concentrated by centrifugation and then suspended in several drops of deionized water. One drop of each sample was negatively stained in a solution containing 3 drops of 4% phosphotungstic acid, 1 drop of 0.5% bovine serum albumen (Cohn fraction V), and 8 drops of deionized water. The solutions were sprayed onto collodion- and carbon-coated grids with a nebulizer (Ted Pella Inc., Tustin, Calif.) and viewed with a Hitachi model HU-12A transmission electron microscope set at 75 kV.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Electrophoresis was performed with the buffers described by Laemmli (9) and the apparatus described by Kerchaert (8). Vertical slab gels (14 by 12.6 by 0.08 cm) were cast so that an acrylamide gradient range of 4% (top) to 12% (bottom) was achieved. The samples were mixed with equal volumes of sample buffer (6.25 ml of 1 M Tris, 5 ml of 2-mercaptoethanol, 10 ml of glycerol, 1 ml of 0.1% bromphenol blue, 2.3 g of sodium dodecyl sulfate, and deionized water in a final volume of 100 ml [pH 6.8]) and incubated for 10 min at 100°C, and 10-μl volumes were applied to the gel wells. Electrophoresis was performed for 2 h with a current of 60 mA. Proteins were detected with Coomassie brilliant blue.

Centrifugations. All centrifugation forces are expressed as average *g* forces.

RESULTS

Purification of competence-conferring activity on Ficoll. The competence-conferring activity-rich fraction obtained by prolonged high-speed centrifugation was layered on Ficoll gradients (prepared as described in reference 19, but in phage suspension medium). After centrifugation at 120,000 × *g* for 4 h at 4°C, 1.0-ml samples were removed from the top of the gradient and analyzed for plaque-forming units, competence-conferring activity, and 280 nm-absorbing material. When 0.1-ml samples of the Ficoll fractions were assayed for competence-conferring activity (Fig. 1A), it appeared that the recovery of competence-conferring activity was poor and that the bulk of this activity was spread through fractions 3 through 6. In contrast (Fig. 1B), when 0.01-ml fractions were assayed for competence-conferring activity, not only was there a marked increase in the recovery of competence-conferring activity, but also the peak of this activity clearly was in fractions 3 and 4. The difference in these results was understood when it became evident that the assay cells exposed to 0.1-ml (but not 0.01-ml) samples were undergoing lysis, as shown for fraction 3 in Table 1. This loss in viable cells at the lower dilutions could not be attributed to the activity of free plaque-forming units in the undiluted material (only about 4.5×10^6 plaque-forming units per ml) since RN2573 (lysogenic for phage 80α and immune to lysis by phage 80α at low multiplicities) (12) also exhibited a loss in cell viability when assayed in the same manner.

The competence-conferring activity recovered from the Ficoll gradients (and hereafter referred to as Ficoll-purified competence-conferring activity) was freed of residual Ficoll before being used further. Ficoll was removed by taking 1-ml portions of the Ficoll-purified competence-conferring activity (usually fraction 3, Fig. 1), adding 60 ml of deionized water, and pelleting the competence-conferring activity at 135,000 × *g* for 5 h at 4°C. The pellets were then resuspended in a small amount of suspension medium (or distilled water for subsequent electron microscopic examination).

Ficoll-purified competence-conferring activity was inactivated by incubation with 10% serolog-

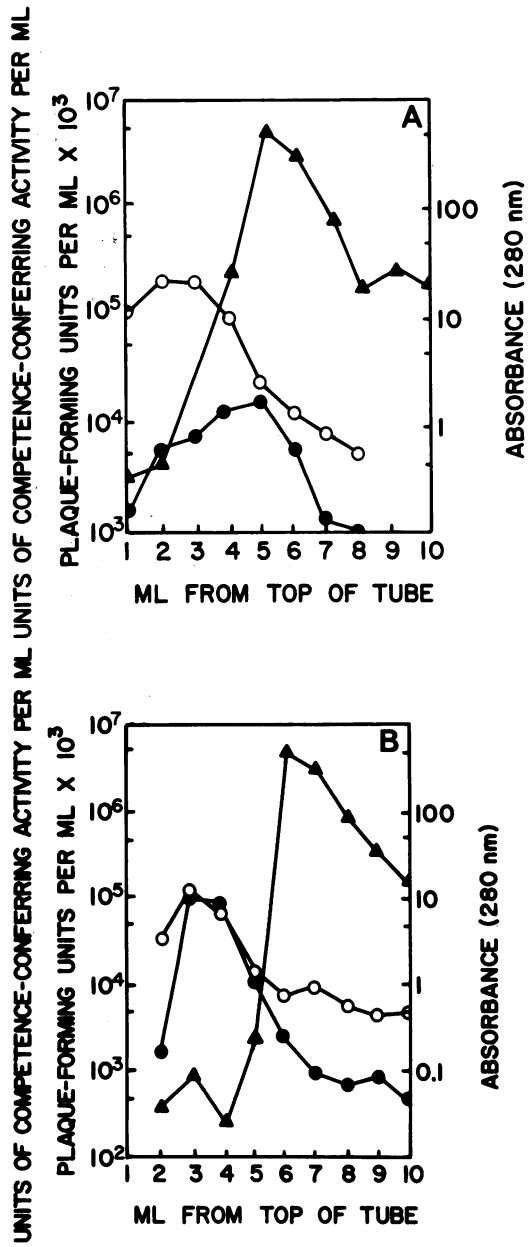

FIG. 1. *Distribution of plaque-forming units (▲) and competence-conferring activity (●) on Ficoll gradients when 1.0 ml of the competence-conferring, activity-rich fraction was centrifuged on a 5 to 25% Ficoll gradient at 120,000 × g for 4 h at 4°C. Fractions were removed from the top of the gradients, the optical density at 540 nm (○) was determined for each fraction, and each fraction was also assayed for plaque-forming units. In A, 0.1-ml samples of each fraction were assayed for competence-conferring activity using excess ISP2 DNA and RN450 as the assay strain. In B, 0.01-ml samples were assayed. A and B represent separate gradients assayed in separate experiments.*

ical group B anstiserum (k = 70) for 30 min at 35°C.

Incidence of competent cells. Transfection has been demonstrated to be a more effective means of estimating the incidence of competent cells than transformation (14) and therefore was used in this study to determine the incidence of cells made competent under conditions in which saturating DNA was used. The use of this procedure was limited to assaying only the Ficoll-purified competence-conferring activity since the low numbers of plaque-forming units in this preparation could be effectively inactivated with UV light (1 J/m² per s) for 5 min (18). After exposure to UV light, the Ficoll-purified competence-conferring activity was serially diluted and assayed for competence-conferring activity. The results (Fig. 2) reveal maximum competence-conferring activity at a dilution of 1:100 (with evidence of lysis of the assay cells seen at a 1:10 dilution). Extrapolation of the activity curve to zero dilution reveals that 1 ml of the Ficoll-purified competence-conferring activity has the ability to make about 2×10^7 colony-forming units (or about 1 cell in every 100) competent for transfection with phage 80α DNA. Moreover, the linear nature of these results reveals that a single competence-conferring factor is sufficient to convert a cell to the competent state when excess transfecting DNA is present.

Electron microscopy of purified competence-conferring activity. Cesium chloride-purified 80α virions and Ficoll-purified competence-conferring activity were negatively stained and examined by transmission electron micros-

TABLE 1. *Effect of dilution of a fraction enriched for competence-conferring activity by rate-zonal centrifugation in Ficoll on the lytic and competence-conferring activities of the fraction*

Assay	Dilution of fraction assayed	Colony-forming units/ml[a]	Competence-conferring activity[b]
1	1:10	6.0×10^8	8.0×10^3
2	1:50	1.9×10^9	2.0×10^4
3	1:100	4.5×10^9	5.0×10^4
4	1:1,000	4.7×10^9	6.1×10^4
5[c]	1:100	4.4×10^9	<10
6[d]		4.5×10^9	<10

[a] The colony-forming units were assayed at the completion of the transformation procedure.

[b] The competence-conferring activity of fraction 3 (Fig. 1) at each dilution was assayed by using 30 μg of ISP2 DNA and is expressed as Nov[r] transformants recovered per milliliter × the dilution factor. The undiluted competence-conferring activity (fraction 3) contained 4.5×10^6 plaque-forming units per ml.

[c] Received 0.01 ml of fraction 3 but no DNA.

[d] Received 30 μg of ISP2 DNA but no material from fraction 3.

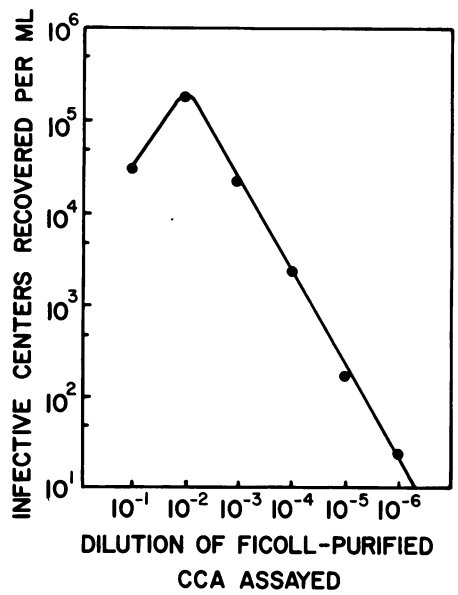

FIG. 2. *Effect of dilution of Ficoll-purified competence-conferring activity on the incidence of competent cells as detected by transfection with excess phage 80α DNA. Cells of RN450 were exposed to 10-μl samples of various dilutions of Ficoll-purified competence-conferring activity and then assayed for competence by transfection with phage 80α DNA. The results are expressed as transfectants per milliliter of the Ficoll-purified competence-conferring activity used in the assay. The transfection assay mixtures contained 20 μg of phage 80α DNA and 2.0 × 10^9 colony-forming units of RN450 per ml.*

copy. The Ficoll-purified competence-conferring activity (Fig. 3) consisted of large numbers of free phage tails and an occassional intact but empty virion. In addition, at the limit of resolution there were numerous structures that may have been aggregates of the distal end of the phage tail (i.e., adsorption organelles).

Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of competence-conferring activity. Although electron microscopy revealed that a large number of phage-derived structures were present in the Ficoll-purified competence-conferring activity, it was important to also analyze the protein composition of this material. If the Ficoll-purified competence-conferring activity contains proteins other than those of phage origin, it would be necessary to ascertain whether they had any function in the acquisition of competence by RN450. Also, if the Ficoll-purified competence-conferring activity was enriched for intact phage tails (as indicated by electron microscopy), then a change in the ratio of some of the virion-derived proteins should be apparent when compared with the intact 80α proteins.

Ficoll was removed from the Ficoll-purified competence-conferring activity by washing the fraction in suspension medium and sedimenting the competence-conferring activity by centrifugation. The proteins contained in the Ficoll-purified competence-conferring activity were then compared with those of purified phage 80α by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A large number of proteins were present in the Ficoll-purified competence-conferring activity that were absent from the purified phage preparation (data not shown), indicating that some cellular component or organelle was cosedimenting with the competence-conferring activity. When Ficoll-purified competence-conferring activity was prepared from a crude phage lysate using suspension medium that was devoid of Mg^{2+}, most of the contaminating proteins were eliminated by centrifugation at 135,000 × *g*. Competence-conferring factor thus purified contained approximately the same level of competence-conferring activity, as well as a similar number of plaque-forming units, as competence-conferring factor prepared with Mg^{2+}-containing suspension medium. We attribute the difference in protein concentration to the removal of ribosomes whose structural integrity requires the presence of Mg^{2+} (1).

Additional contaminating proteins were removed from the Ficoll-purified competence-conferring activity by passing 0.5 ml through a 5-ml Matrex Gel Orange A column (Amicon Corp., Lexington, Mass.) following the protocol supplied by the manufacturer. Neither the level of competence-conferring activity nor the level of infectious phage particles was altered, suggesting that passage of the material through the column did not enrich or select for particular phage components. Examination of the Matrex Gel Orange A-purified material by electron microscopy revealed the same distribution of phage components that had been observed in the untreated Ficoll-purified competence-conferring activity. This fraction was shown by electrophoresis to contain only proteins that were of the same mobilities as those of the intact phage (Fig. 4). The proteins in the Ficoll-purified competence-conferring activity, after removal of Ficoll and passage through Matrex Gel Orange A (all in the absence of Mg^{2+}), were thus considered to be of phage origin. Three of the proteins evident in the sodium dodecyl sulfate-polyacrylamide gel of the intact phage were significantly reduced in content in the purified competence-conferring factor (bands C, E and F, Fig. 4); this observation is consistent with these being proteins of the phage head, the presence of which is strikingly

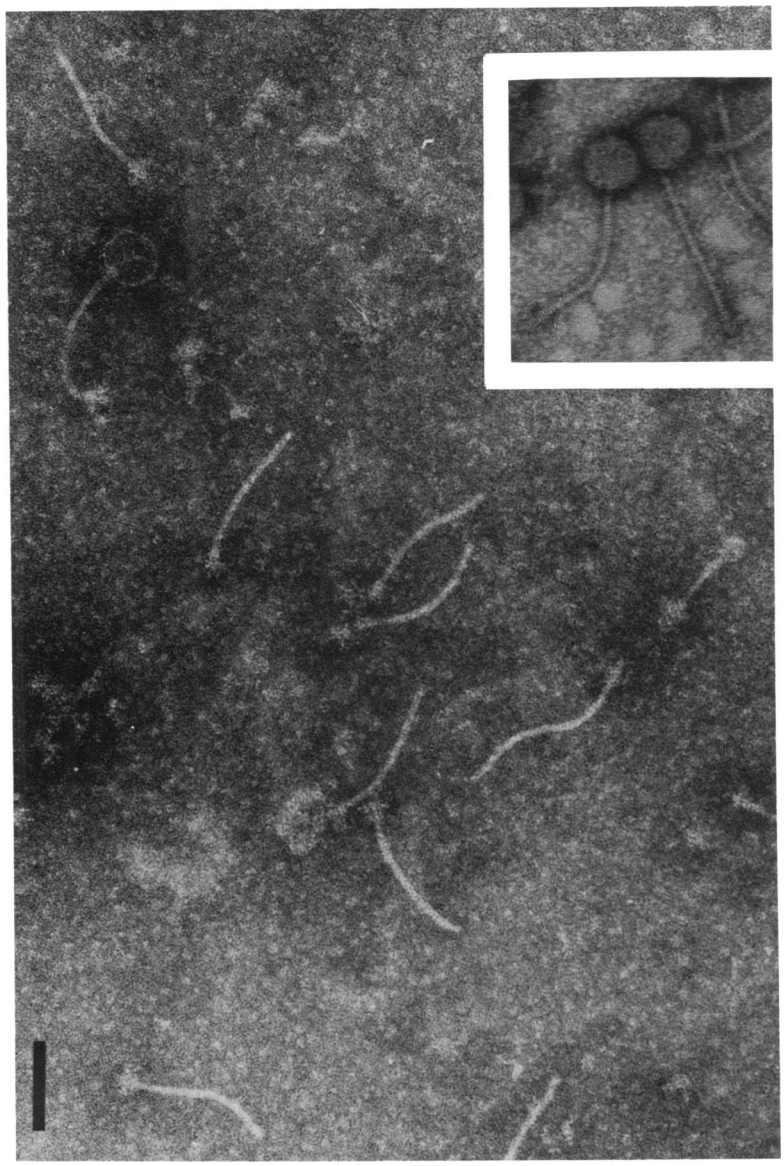

FIG. 3. *Negatively stained Ficoll-purified competence-conferring activity and (inset) purified phage 80α particles. Bar, 100 nm.*

reduced in electron micrographs of the competence-conferring activity after purification.

DISCUSSION

The preparation of large volumes of crude lysates of bacteriophage 80α by broth propagation and the efficient, nondestructive precipitation of the plaque-forming units and competence-conferring activity from these lysates with $(NH_4)_2SO_4$ enabled us to avoid many of the problems associated with soft-agar propagation and resulted in preparations containing competence-conferring activity that remained stable during subsequent manipulations. In particular, neither the plaque-forming units nor the competence-conferring activity exhibited the instability after migration through Ficoll gradients that had complicated the previous study (19). This instability apparently was a consequence of using CsCl in the purification of competence-conferring activity, a step that has been avoided in the present study. In addition, the recognition that the competence-conferring activity could be pelleted by prolonged high-speed centrifuga-

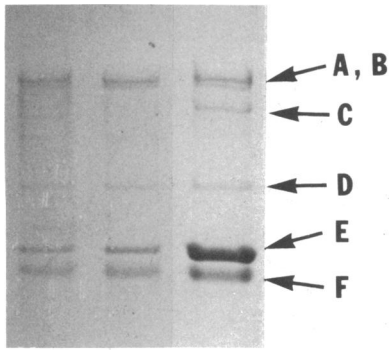

FIG. 4. *Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of purified phage 80α plaque-forming units and Ficoll-purified competence-conferring activity prepared from crude phage lysate using suspension medium devoid of Mg^{2+}. Channel 1 contains Ficoll-purified competence-conferring activity before, and channel 2 contains the same material after passage through Matrex Gel Orange A, and channel 3 contains the equivalent of 10^{11} plaque-forming units of phage 80α. The arrows identify the major protein bands referred to in the text.*

tion contributed significantly to the preparation of competence-conferring activity that was sufficiently concentrated for further purification by rate-zonal centrifugation in Ficoll and aided in freeing the competence-conferring activity from ribosomes before analysis on polyacrylamide gels.

The fractions from the Ficoll gradients that were enriched for competence-conferring activity (fraction 3, Fig. 1B) were shown to possess lytic activity toward the assay cells (Table 1; Fig. 2), whereas fractions containing higher numbers of plaque-forming units but less competence-conferring activity were devoid of this lytic activity. This observation and the susceptibility of the competence-conferring activity to inhibition by antiserum prepared against purified serological group B phage (19) encouraged us to examine the fractions enriched for competence-conferring activity by electron microscopy. The results (Fig. 3) do not define a single morphological structure as being the competence-conferring factor. Although rare intact but empty virions were seen, the predominant structures were free phage tails. However, at the limit of resolution were numerous structures that may have been the phage adsorption organelle; frequently these appeared to be aggregated. Because of the ability of specific antiserum to inhibit the competence-conferring activity (19), and considering the role of the adsorption organelle in the reproductive cycle of the phage, we strongly suspect that the competence-conferring factor is the adsorption organelle; however, whether the competence-conferring activity is evident when this organelle is assembled with or free from the major tail structure remains unknown.

Additional evidence that the competence-conferring factor was a component of the 80α virion derives from the results of polyacrylamide gel analysis (Fig. 4). Particularly when Mg^{2+} was deleted from the suspension medium during concentration of the competence-conferring activity after migration through Ficoll, the identity of the protein bands from 80α with those of the competence-conferring factor is striking. Whereas the competence-conferring factor and 80α preparations appeared to contain about equivalent amounts of material in bands A, B, and D (Fig. 4), the material in band C was virtually absent in the competence-conferring factor preparations, particularly after passage through Matrex Gel Orange A. Also obvious was the vastly greater amount of material in bands E and F from the 80α preparation. Presumably, these differences reflect the absence of the majority of the head proteins from the competence-conferring factor preparation.

The lytic activity associated with highly concentrated competence-conferring factor preparations cannot be explained on the basis of contaminating plaque-forming units. Not only were the numbers of plaque-forming units in these preparations insufficient to account for this lytic activity, but also lysis of the assay cells was observed when the assays were conducted with an 80α lysogen rather than with RN450. This lytic destruction of the assay cells, associated with highly concentrated preparations of the 80α tail (and, perhaps, the adsorption organelle), is suggestive of lysis from without as observed with high multiplicities of phage such as T4. Some bacteriocins also exhibit lytic activity toward susceptible cells, and they have been shown by electron microscopy to be apparently defective phage virions possessing contractile tails (2, 7, 11). It is likely that phage of Bradley group A, endowed with contractile sheathed tails, are all able to elicit lysis from without, this being a secondary manifestation of the manner in which these phages inject their DNAs into the host. In contrast, phages in Bradley group B (3), to which phage 80α (and the majority of other staphylococcal phages) (15) belongs, lack a contractile tail. The manner in which the DNA of these phages is transmitted into the cell remains unknown, at least among phages that are active on gram-positive bacteria. As suggested by Thompson and Pattee (19), the manner in which the competence-conferring factor facilitates the entry of exogenous DNA into a cell may be closely

related to the manner in which the phage virion facilitates the entry of phage DNA into the cell during the course of normal phage reproduction.

The demonstration that the competence-conferring activity exhibited single-hit kinetics when excess assay cells and transfecting DNA were present (Fig. 2) means than a single active 80α competence-conferring factor, in the presence of 0.1 M $CaCl_2$, can convert a single cell to the competent state. The effective collision of the competence-conferring factor with a susceptible cell is readily understood if, as seems to be the case, the factor uses the same receptors as the plaque-forming units. Chatterjee (5) and Coyette and Ghuysen (6) estimate that there are about 600 phage receptors per cell of *S. aureus*.

The failure of every cell in the assay to become competent in the experiments described in Table 1 and Fig. 2 is attributed to an insufficiency of competence-conferring factor to saturate all of the assay cells and to the lytic activity associated with the purified competence-conferring factor. This lytic activity, which is only associated with undiluted preparations of the purified competence-conferring activity (Table 1; Fig. 2), strongly indicates that the competence-conferring factor is not the primary component in these preparations. Rather, it is clear that one or more components in these preparations must contribute to the lytic activity toward the assay cells. The competence-conferring factor exhibits single-hit kinetics (Fig. 2), and 1 ml of the Ficoll-purified competence-conferring activity contains only sufficient competence-containing factor for about 1 cell in every 100 in an assay mixture. We favor the view that the competence-conferring activity is a manifestation of but one of several configurations of the adsorption organelle of phage 80α (and, by inference, of serological group B phages in general) (18, 19). At limiting concentrations, this component—the competence-conferring factor—is not lytic toward the cell. The most highly purified preparations of the competence-conferring factor (Fig. 3 and 4) also contain a variety of other 80α-related virion components (presumably also morphogenic precursors), at least some of which exhibit lytic activity toward the cells—probably as a consequence of each cell adsorbing several of these components. The relationship of the lytic component(s) and the competence-conferring factor to one another, and to the intact 80α virion, can only be resolved by further study of more homogeneous preparations of the competence-conferring factor.

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