Transduction of *Streptococcus pyogenes* K 56 by Temperature-Sensitive Mutants of the Transducing Phage A 25

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Experiments aimed at increasing the efficiency of transduction by the virulent group A streptococcal phage A 25 were carried out using temperature-sensitive phage mutants. Lysates of phages carrying ts mutations proved to transduce more efficiently than wild type lysates, the order of increasing effectiveness being $ts^{-} < ts_{1} \sim ts_{2} < ts_{1} - 2$. Differential survival of the recipient, K 56, appeared to be the principal reason for the improvement of transduction when cells decapsulated by hyaluronidase were transduced at the restrictive temperature. In transduction of encapsulated recipient cells, in which the superiority of ts lysates was less pronounced, the possibility was considered that mutant lysates might contain higher fractions of transducing particles than wild type lysates produced under parallel conditions.

The transducing activity of wild type lysates depended strongly on the temperature at which the phage had multiplied on the donor strain, the frequency of transduction increasing with decreasing temperature. Since the minimal latent period of the phage varied roughly in the same way with temperature, a connection is suggested between the time required for phage multiplication and the proportion of transducing particles formed.

Although the medical importance of group A streptococci makes a genetic analysis of these organisms highly desirable it was not until recently that ganisms highly desirable it was not until recently that a system of genetic exchange discovered. LEONARD, COLON and COLE provided evidence for transduction to streptomycin resistance of the type 12 strain, K 56, by several phages, the most efficient of which was the virulent phage A 25. Though high titer phage stocks may easily be produced with virulent phages the efficiency of transduction suffers from the fact that, due to the lack of immunity, superinfection of potential transductants by stray phage in the system tends to diminish the yield of transductants. To lessen superinfection, the multiplicity of infection could be kept small but this would unsuitably decrease the number of transductants per plate because the frequency of transduction is smaller than $10^{-5}$.

It is the aim of this paper to demonstrate that transduction by phage A 25 can be made more efficient if temperature-sensitive mutants of the transducing phage are employed. Concomitantly, it was found that the transducing activity of wild type lysates strongly depends on the temperature at which the phage was propagated on the donor strain.

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Materials and Methods

**Strains.** The streptomycin-sensitive *Str. pyogenes* strain K 56 (group A, type 12) was isolated by and obtained from KJEMS. Strain 56 X 188 (group A, type 6; streptomycin-resistant) and the virulent phage A 25 came from the laboratory of R. M. COLE and were made available by A. E. COLON. In the paper by LEONARD et al., these three organisms are called 61 X 101, 56 X 188 S6, and 12204, respectively. The number 12204 is the designation given by the American Type Culture Collection to phage A 25 (pens. communication by A. E. COLON) which was originally isolated by BOULGAKOV from Paris sewage and maintained by MAXTED.

**Media.** Serum broth (SB) was composed of 10 g proteose peptone No. 2 (Difco), 3 g NaCl, 2 g Na2HPO4 · 12 H2O, 0.2 g CaCl2, 1 g glucose, 1000 ml beef heart broth, and 10 per cent horse serum; pH 7.2. With the addition of 2 per cent agar, serum agar (SA) contained the same constituents as SB but the amounts of beef heart broth and serum were reduced to 20 and 2 per cent, respectively. To the soft agar (0.7 per cent agar) (SSA) beef heart broth and serum were added to concentrations of about 40 and 20 per cent, respectively.

**Phage techniques.** General methods were those described by ADAMS. To obtain transducing lysates, phage A 25 was generally propagated on log phase cells (10⁶ cells/ml) of the donor strain 56 X 188 grown in

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SB, using an input ratio of 2 phages per cell. After 4 hours incubation with agitation at the respective temperatures the lysates were centrifuged and filtered. For phage titration the agar layer method was used employing log phase cells of K 56 as indicator. Because this strain is encapsulated 15 units of hyaluronidase (Hylase, Impfstoffe Dessau) were added per ml SSA 1.[5]

Production of antiphage A 25 rabbit serum. 1 ml of a phage stock in 0.1 m ammonium acetate containing $10^{11}$ plaque forming units (PFU) per ml was intravenously injected twice weekly for 4 weeks. After a rest period of one week, the animals were bled by cardiac puncture and yielded a serum with an inactivating coefficient, $K$, of 100 units.

One-step growth experiments. Because the host strain 56 X 188 is non-encapsulated and the organisms tend to stick together in clumps, special pains were taken to make a uniform suspension consisting of single cells and short chains. Two ml of phosphate buffer were added to an overnight SA plate, the bacterial growth of which was rubbed off with a spatula and dispersed by repeated forcible blowing of the suspension through the fine orifice of a pipet tip. After appropriate dilution with SB to give a total count of $5 \cdot 10^{12}$ colony forming units (CFU) per ml, the suspension was allowed to incubate for 30 min at 37 °C. Simultaneously with handling for viable count, 0.9-ml samples were removed, equilibrated for 5 min at the respective temperatures, and infected with 0.1 ml of an A 25 suspension such that the multiplicity of infection (MOI) was 0.04. After adsorption (about 90–95 per cent within 10 min, regardless of the temperature used) and neutralization of free phage (5 min), the mixtures were diluted $10^{-3}$ for the first growth tube (FGT) and $2 \cdot 10^{-4}$ for the second growth tube (SGT). 0.1-ml samples were removed from FGT and SGT at suitable intervals and plated with K 56 for plaques.

Isolation of temperature-sensitive mutants of phage A 25. ts mutants were isolated from a phage stock propagated on the streptomycin-sensitive counterpart K, 56 +ts2. The plate containing K 56 alone was incubated at 29.5° and the other two were incubated at 37.5°. Spots that failed to appear at 37.5° indicated the presence of ts mutants in the lysates in question. The mutants were plated at 29.5°, one plaque was picked, and a stock was prepared on 56 X 188. Two mutants, ts1 and ts2, comprising 0.3 per cent of the plaques on the master plates, were isolated in this way.

Isolation of the double ts mutant, ts1–2. An overnight culture of K 56 was diluted 30-fold in SB+15 units/ml of hyaluronidase, grown to 2 – 3 $\cdot 10^8$ cells/ml, and received KCN at a final concentration of 1 mM. The bacteria were mixed with an equal volume of phage mixture containing $2.5 \cdot 10^9$ PFU/ml of each of ts1 and ts2, shaken at room temperature for 15 min, then received antiphage serum (final K~1), and 10 min later were diluted $10^2$, $10^3$, and $10^4$-fold in SB. After 3 hours incubation at 29.5° lysis was completed by the addition of chloroform. The lysates were assayed at 29.5° for total progeny and at 37.5° for wild type recombinants. The recombination frequency was about 3 per cent. Individual plaques from the 29.5° plates were randomly picked and tested for complementation with each of the parental mutants on 3 plates (see Fig. 1): One was seeded with K 56 alone, the second with K 56 +ts1 (10° PFU/plate), and the third with K 56 +ts2. The plate containing K 56 alone was incubated at 29.5° and the other two were incubated at 37.5°. Spots that failed to show lysis on both of the 37.5° plates were suspected to be derived from double ts mutants. Failure of the suspected double ts mutants to produce wild type recombinants in crosses with either of the parents was considered to confirm the identification.

Transduction. The standard procedure was to mix 1-ml portions of stationary recipient cells with equal volumes of phage. After 20 min of adsorption at the respective temperatures, the mixtures received 3 ml of SSA and were poured over SA plates. These were incubated for 2 hours at the corresponding temperatures and then overlaid with 8 ml of SSA containing 750 µg/ml of streptomycin. After further incubation for 24 hours, streptomycin-resistant transductants could be counted. As some residual growth of streptomycin-sensitive recipient cells was observed even after the addition of the top layer containing streptomycin, the 2 hours incubation period for phenotypic expression proved to be sufficient. Transduction of decapsulated bacteria was carried out by treating the recipient cells with 60 units/ml of hyaluronidase for 30 min at 37° before the infection with phage, the enzyme being permitted to continue working in the central agar layer. In cases where the transduction mixtures were plated with phage antiserum, this was added after the 20-min adsorption period to give a final K of about 2 in the central layer. Appropriate controls of cells with phage propagated on the streptomycin-sensitive counterpart of strain 56 X 188 were always included.

6 E. Freese, E. Bautz-Freese, and E. Bautz, J. molecular Biol. 3, 133 [1961].
Results

Characterization of the ts mutants. Since ts mutants of streptococcal phages have not yet been isolated and it seems relevant for the experiments to be presented, some properties of the ts mutants used will be described. Preliminary experiments revealed that the burst size of wild type A 25 is reduced at temperatures little higher than 37°, and the efficiency of plating decreased above about 38°. Thus, mutants which gave rise to plaques at 29.5° but not at 37.5° were isolated. Fig. 1 shows the lysis pattern of wild type and ts mutants on incubation at 29.5° and 37.5° when spotted on plates containing K 56 or K 56 together with either of the single ts mutants. Ignoring the possibility of intragenic complementation, the mutational defects of ts1 and ts2 were considered to be in different cistrons since they proved to be positive for complementation. Revertant frequencies defined as those phage capable of plaque formation at 37.5° were approximately 10⁻⁷ for the single mutants and less than 10⁻¹⁰ for the double mutant. To quantitate the degree of leakage, 56 X 188 was infected as in one-step growth experiments and phage production was measured after one hour of incubation at 37.5°. Appropriate controls running at 29.5° were included. Phage production at the non-permissive temperature was 0.06 (ts1), 0.10 (ts2) and less than 0.007 (ts1–2) phage per infected cell. The test for leakage was also carried out by a transmission test at 37.5°. The transmission values were less than the minimum detectable level of about 7·10⁻³ for all three mutants, meaning that fewer than 0.7 per cent of the infected cells produced infective centers on incubation at 37.5°.

Dependence of the transducing potential of wild type lysates on the temperature at which the phage has multiplied on the donor strain. Preliminary experiments designed to determine the yield of transductants were carried out by using wild type lysates obtained at 37° and mutant lysates produced at 26°. It was soon realised, however, that phages to be compared should have multiplied at the same temperature. When such lysates were employed the difference in the efficiency of transduction between ts⁺ and ts lysates appeared to become smaller. This observation prompted a more detailed study of the effect of the propagation temperature on the transducing potential of ts⁺ lysates.

10-ml portions of an exponential bulk culture of 56 X 188 grown at 37° were equilibrated for 10 min at temperatures ranging from 26° to 37°, and infected with A 25 at a multiplicity of 1–2 phages per cell. After 3 hours of incubation at the respective temperatures lysis was completed by the addition of chloroform. The resulting lysates had phage titers of approximately 10⁹ PFU/ml, regardless of the temperature used for propagation. The transducing activity was assayed simultaneously on stationary cells of K 56 at a multiplicity of about 0.3 phage per cell, adsorption and overnight incubation taking place at 37°.

As is evident from Fig. 2, the transduction frequency (operationally defined as the ratio of the number of transductants to that of PFU) depends strongly on the temperature at which the transducing lysates were produced, the transduction titers increasing with decreasing propagation temperature of the phage. Lysates obtained at 37° gave a frequency of transduction of approximately 5·10⁻⁷ while those produced at 26° showed a transducing activity greater by a factor of about 5; in between, there was a gradient of transducing activity of the lysates. Based on the results of one-step growth experiments carried out at different temperatures (see below), in another series of experiments the period between infection and completion of lysis was varied in such

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7 R. S. Edgar, G. H. Denhardt, and R. H. Epstein, Genetics 49, 635 [1964].
a way that phage growth was restricted to one cycle at each temperature. Assays for transduction of the resulting one-cycle lysates gave results corresponding with those illustrated in Fig. 2.

![Fig. 2. Transduction frequency and minimal latent period as functions of the temperature at which the transducing phage, A 25 ts+, was propagated on the donor strain (see text for details).](image)

To approach the question of the reason underlying the temperature dependence of the transducing potential of A 25 lysates, one-step growth experiments were performed at temperatures identical with those used in lysate production. Fig. 3 shows that the average burst size was practically insensitive to a change in temperature, equalling approximately 30 PFU per infected cell. However, temperature affected strongly the extent of the latent period which was about 39 min at 37°C and about 107 min at 26°C, with a gradient of length in between. The minimal latent period varied roughly in the same way with temperature, as the transducing activity of the lysates (Fig. 2). If this is not a mere coincidence it may suggest a connection between the time required for phage multiplication and the proportion of transducing particles formed.

The latent period at 29°C of the 3 mutants was identical with that of the wild type at this temperature.

**The effect of phage ts mutations on yields of transductants.** Lysates, the transducing potential of which was to be compared, were generally produced at 26°C and standardized to equal phage titers by appropriate dilution. Table 1 gives the results of transduction experiments in which the transduction mixtures were plated with and without hyaluronidase and antiphage serum at 37.5°C. The data represent average frequencies ± standard deviations as derived from 6 lysates of each phage, made on 6 separate cultures of 56 X 188.

<table>
<thead>
<tr>
<th>Phage</th>
<th>Transduction frequency ( \times 10^{-6} ) when plated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>with hyaluronidase</td>
</tr>
<tr>
<td>ts+</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>ts1</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>ts2</td>
<td>4.4 ± 0.6</td>
</tr>
<tr>
<td>ts1−2</td>
<td>14.5 ± 0.5</td>
</tr>
</tbody>
</table>

Table 1. Transduction of K 56 to streptomycin resistance by wild type and ts mutants of phage A 25. Transduction mixtures contained 3-10⁹ cells of K 56 and 6-10⁸ PFU. Incubation was at 37.5°C. The data represent average frequencies ± standard deviations as derived from 6 lysates of each phage, made on 6 separate cultures of 56 X 188.

mixtures were plated with and without hyaluronidase and antiphage serum at 37.5°C. The essential features are the following: At the same multiplicity of infection, transduction by ts mutants resulted in greater yields of transductants than that by wild type phage, the factors of increase depending on the particular conditions employed. When plated without hyaluronidase the transduction frequencies for the single and double ts mutants were, respectively, 1.7 and 4 times higher than for wild type. The presence of antiphage serum did not significantly increase the yield of transductants. Plating with hyaluronidase and without antiserum drastically reduced the efficiency of transduction by wild type, the yield of transductants for ts+ lysates being 0.3 per cent of

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that in the absence of the enzyme. This decrease was much less pronounced in cases of the single and double ts mutants where the transduction frequencies dropped to about 3 – 10 and 40 per cent, respectively. The presence of antiphage serum in the transduction mixtures of decapsulated bacteria compensated for the effect of hyaluronidase, though, with the exception of transduction by ts1 – 2, the transduction frequencies did not completely attain the values found in the experiments without the enzyme.

When these experiments were performed at 29.5°, in the absence of hyaluronidase similar results as at 37.5° were obtained. In experiments with hyaluronidase and without antiserum, however, the differences in the transduction frequency between wild type lysates and those of the single ts mutants disappeared, and the lysates of the double ts mutant were only 4 times more effective than those of the wild type (as opposed to 590 times more effective at 37.5°).

The results presented above apply to a constant multiplicity of infection of about 0.2 phage per cell. Table 2 shows the results of experiments in which the same amount of phage was added to a series of tubes containing decreasing numbers of cells to give multiplicities of infection ranging from 0.2 to 2.0. It can be seen that as the multiplicity of infection increased, the frequency of transduction decreased, presumably by a shift from single to multiple infection of cells. However, this decrease was 2 – 3 times less pronounced in cases of transduction by the ts mutants.

### Discussion

Lysates of phages carrying ts mutations prove to transduce more efficiently than wild type lysates, the order of increasing effectiveness being $ts^- < ts1 < ts2 < ts1 – 2$. There are several possibilities to account for this fact. The situation seems to be clear when decapsulated cells are to be transduced. As already suggested by LEONARD et al., the hyaluronic acid capsule protects the transductants from secondary infection and lysis by any new phage derived from lysed complexes in the transducing mixture. Hence, decapsulation by hyaluronidase results in severe losses of transductants (Table 1). Conditions that prevent or lessen the emergence of stray phage should allow the system to give rise to transductant colonies even in the presence of the enzyme. This is what can be found when transduction is performed by ts mutants at the non-permissive temperature (Table 1). It appears that a single ts mutation is not sufficient to produce an optimum effect, but two ts mutations (in different cistrons) must be present in order to do so. In trying to explain this, the occurrence of revertants, some degree of leakiness and, above all, the fact that temperature control is not as good on plates as in liquid culture may be considered. Thus, short pulses of permissive temperature at the proper time during the lytic cycle may allow some complexes to give rise to progeny phage which, in turn, could kill potential transductants. It thus appears that higher survival of the recipient cells in the transduction mixture is the principal factor that leads to improvement of the efficiency of transduction when decapsulated bacteria are transduced by ts mutants at the restrictive temperature. The differential effect of the various phages on the survival of the recipient cells correlates well with the degree of the bacterial background growth in the central agar layer, e.g. there is no background growth if decapsulated cells are transduced by $ts^-$ but there is strong background growth if transduction of these cells is performed by ts1 – 2 at the restrictive temperature.

Differential survival of the recipient does not seem to be mainly involved in transduction of encapsulated bacteria. This assumption is based on two facts: The first is that, under the conditions of these experiments, the yield of transductants cannot be

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Table 2. Effect of multiplicity of infection on yield of transductants. Transduction mixtures containing neither hyaluronidase nor antiphage serum were composed of 6-10$^8$ PFU and appropriate amounts of K56 cells. Incubation was at 37.5°. The transduction frequencies represent the average of duplicate assays for one lysate of each phage. Assays for transduction were made simultaneously.

<table>
<thead>
<tr>
<th>Phage</th>
<th>Actual (upper values) and relative (lower values)</th>
<th>$\times 10^{-6}$ at a MOI of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>$ts^+$</td>
<td>2.9</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.35</td>
</tr>
<tr>
<td>$ts1$</td>
<td>5.0</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.64</td>
</tr>
<tr>
<td>$ts2$</td>
<td>5.1</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.76</td>
</tr>
<tr>
<td>$ts1 – 2$</td>
<td>14.7</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.81</td>
</tr>
</tbody>
</table>

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increased significantly when transducing mixtures containing the wild type phage are plated with antiphage serum. The second is that transduction by ts mutants at the permissive temperature continues to be more efficient than that by wild type. Since different adsorption rates of the phages may be ruled out by experiment, one is left with the possibility that wild type and mutant lysates produced under parallel conditions contain different fractions of transducing particles.

Considering the protective function of the capsule, the question remains to be answered how infection of cells which were not artificially decapsulated is possible. Experiments of Warren and Gray indicate that streptococci which produce hyaluronic acid also elaborate hyaluronidase. Coexistence of the enzyme and its substrate leads to disappearance of hyaluronic acid on continued incubation. Since cells to be transduced came from the stationary phase of growth, they are likely to possess no pro-


nounced capsule. The relatively high serum content of the transducing mixture could provide conditions that are favourable for abundant capsule formation, a point also made by Maxted and Leonard et al.1.

As for the temperature dependence of the process that results in the formation of transducing particles, further work is necessary to find out the underlying mechanism. Among others, the following hypothesis is attractive. As the temperature decreases from 37°C to 26°C, the reproduction and/or maturation of progeny phage is increasingly delayed (see Figs. 2 and 3) in favour of either a genetic interaction between vegetative phage and host genome or some kind of phenotypic mixing, as a result of fragmentation of the bacterial chromosome to pieces of proper size for incorporation into phage heads.

I thank Drs R. M. Cole and A. E. Colón who generously provided bacterial strains and phages and critically read the manuscript. The dependable technical help of Mrs. I. Hoffmann is gratefully acknowledged.

Separation of poliovirus specific RNA structures by analytical gelelectrophoresis

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Die analytische Gelelektrophorese an Agarose-Polyacrylamidgelen gestattet eine Auftrennung der poliovirus-spezifischen RNS-Strukturen: Einzelstrang-RNS, doppelsträngige replikative Form (RF) und replikative Intermediatform (RI). Unter den verwendeten Bedingungen werden die RNS-Arten nach ihrer Molekülgröße getrennt. Auch in dieser Trennmethode zeigt sich die Inhomo­genität der mehrsträngigen RI-Struktur.

Three types of virus specific RNA have been found in poliovirus infected cells: a single stranded viral RNA with a molecular weight of 2 × 10^6, a double stranded replicative form (RF) with a molecular weight of 4 × 10^6 and a replicative intermediate form (RI) with a molecular weight of 4–8 × 10^6.

This communication reports about the application of the gel electrophoresis by W. Davies for that purpose as it has been described already for


the separation of RNA structures from the Qβ-bacteriophage, Reovirus RNA and Influenza virus. The method proves useful for a qualitative and quantitative analysis of the three types of poliovirus specific RNA. Similar results by Noble et al.9 appeared recently. In their case RI did not enter the 2.25% acrylamide gel used and then would be separated from the two other and smaller viral structures. We also observed this behaviour of RI in order to analyze the expected inhomogeneity of RI