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# Transfection of *Streptococcus pneumoniae* with Bacteriophage DNA

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It was possible to transfect *Streptococcus pneumoniae* with DNA obtained from a newly isolated bacteriophage, diplophage-4 (Dp-4). Optimal frequency of transfection (0.9%) required the use of a nuclease-defective mutant; with wild-type bacteria, the transfection frequency was about 100-fold lower. Transfection requires physiological conditions that appear to be similar to the competent state needed for genetic transformation (A. Tomasz, J. Bacteriol. 91:1050-1061, 1966).

Recent reports on the isolation and characterization of pneumococcal bacteriophages (3, 8, 10, 11, 13, 16) opened up the possibility of developing a transfection system in this bacterium. The usefulness of transfection systems for a variety of genetic and physiological studies have been amply demonstrated in the work with *Bacillus subtilis* transfection (for a review, see reference 20).

Porter and Guild (Fed. Proc. 35:1595, 1976) have recently reported successful transfection of pneumococci. In this system superior transfecting ability was associated with a replicating intermediate of the phage DNA (Porter and Guild, Fed. Proc. 35:1595, 1976). Another unique feature was the apparent lack of saturation in the yield of transfection, even at very high concentrations of the phage DNA (up to 400 μg/ml) (W. R. Guild, personal communication).

In this communication we briefly describe a relatively simple isolated phage, diplophage-4 (Dp-4). The critical factor for obtaining relatively high transfection frequencies appears to be the use of a nuclease-defective pneumococcus mutant. However, the nature of bacteriophage also seems important, since DNA isolated from two other members of the diplophage series (Dp-1 and Dp-2) had only a poor transfecting capacity even when the nuclease-defective bacteria were used.

## MATERIALS AND METHODS

**Bacterial strains.** The wild-type pneumococci used in some experiments were derived from *Streptococcus pneumoniae* strain R36A (Rockefeller University stocks). The nuclease-deficient mutant (end 1-exo 2) (7) was kindly supplied by S. Lacks (Brookhaven National Laboratory). This mutant is lacking about 90% of the wild-type nuclease activity and is capable of normal levels of genetic transformation.

**Phages.** Bacteriophage Dp-1 has been previously described and characterized (8, 10, 13). Dp-2 and Dp-4 were isolated from throat samples of patients with upper respiratory symptoms in the Ciudad Sanitaria La Paz, Madrid, Spain (by C. Fernandez). Details of the isolation technique and the preparation and purification of phage have been published elsewhere (8, 10).

**Preparation of DNA.** A volume (1 to 5 ml) of purified bacteriophage suspension (containing about $10^{11}$ PFU/ml) in TBT solution (0.1 M Tris-hydrochloride [pH 7.8]-0.5 M NaCl-10 mM $MgCl_2$) was mixed with an equal volume of phenol (reagent grade, bidistilled before use) saturated with TBT, and the mixture was shaken for 15 min at room temperature. After low-speed centrifugation, the upper aqueous phase was removed. This treatment was repeated twice. The pooled extracts were dialyzed for 2 days against several liters of 0.15 M NaCl and 0.015 M sodium citrate with stirring at 4°C. DNA carrying the streptomycin resistance marker, obtained and purified as previously described (19), was used as donor DNA for transformation experiments. DNA concentration was determined from UV absorbance at 260 nm.

**Assay for infectious DNA and transformation procedures.** Competent cells were prepared by standard methods used for *S. pneumoniae* (18); several of the experimental procedures used have been described previously (i.e., assay of transformants and viable cells [19]). Transfection was tested as follows. Cultures of exponentially growing, competent wild-type or competent nuclease-deficient cells were diluted 1:10 with C medium containing albumin and yeast extract (17), and then DNA was added at the appropriate concentration. This mixture was incubated at 30°C, without shaking, for various time periods, and the reaction was stopped by adding DNase (10 μg/ml; Worthington, 2× recrystallized). Samples were taken and plated (each in a total of 7 ml of 0.8% agar), using the phage assay described previously (10). Catalase ($10^3$ to $2 \times 10^3$ U/plate) was added to each assay mixture, and, after solidification of the soft-agar layer, each plate received an additional (5 ml) layer of 0.8% agar in C medium. This procedure was necessary to obtain a confluent lawn of bacteria and distinct plaques. Transfection frequency was calculated as the number of transfectants per milliliter times 100 divided by the total number of viable cells per milliliter.

**Chemicals and enzymes.** DNase I and RNase A were purchased from Worthington Biochemicals Corp., Freehold, N.J. RNase was heated at 80°C for 15 min to destroy traces of contaminating DNase. Pronase, B grade (Calbiochem, La Jolla, Calif.), was prepared as recommended by Hotta and Bassel (6).

## RESULTS AND DISCUSSION

Early attempts to obtain transfection in *S. pneumoniae* using DNA from Dp-1 (the first bacteriophage of the diplophage series) were unsuccessful. We have recently isolated and purified three additional pneumococcal phages (Dp-2, Dp-3, and Dp-4), which differ from one another in size, host range, specificity for neutralizing antibodies, and capacity to transfect wild-type or nuclease-defective pneumococci. A more detailed characterization of these phages will be described elsewhere. In this communication, we only report on the relative transfecting abilities of the phage nucleic acids.

Only DNA extracted from Dp-4 could produce transfection (with a low frequency) in wild-type *S. pneumoniae*. Transfected cells started to appear after 50 min of incubation with competent wild-type cells. However, when the nuclease-defective mutant was used as a recipient of the phage DNA, a high level of transfection (about 0.9%) was achieved (Fig. 1). This finding is reminiscent of the greatly improved transfection frequencies observed with *Escherichia coli* spheroplasts prepared from mutants defective in the *recBC* nuclease (2). Pneumococci and phage DNA (prepared from phage Dp-1, Dp-2, or Dp-4) were mixed, and after different times, samples were withdrawn, treated with DNase for 5 min at 37°C, and assayed for PFU (Fig. 2). The increase in the number of viable cells during the 60 min (maximal incubation time) ranged from $6 \times 10^6$ to $1.2 \times 10^7$ colony-forming units/ml. Using Dp-4 DNA, a maximum transfection frequency was reached after 55 min of incubation. Figure 3 illustrates that the latent periods of phage infection for Dp-1, Dp-2, and Dp-4 range from 60 to 85 min. Thus, the relatively long incubation (55 min) with transfecting DNA does not run the risk of a superimposed phage infection cycle.

Figure 4 shows transfection as a function of the phage DNA concentration; saturation of the yield of transfection appears to occur at DNA concentrations ranging from 10 to 16 μg/ml.

The shape of these curves suggests that more than one DNA molecule (or fragment) must interact in a competent cell to produce one infective center, as previously suggested for other systems (for review, see 20). A plateau was reached at 16 μg of DNA per ml, and the addition of more DNA did not significantly affect the

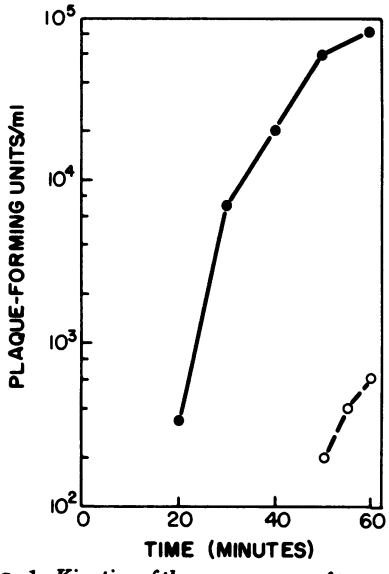


FIG. 1. *Kinetics of the appearance of transfectants. Phage DNA (15 μg in 0.1 ml) was added to 1 ml of growth medium containing competent R6 (○) or nuclease-deficient (●) cells at a concentration of $7 \times 10^7$ colony-forming units/ml. At intervals, 0.1-ml samples of these cultures were added to 0.9 ml of medium with DNase (10 μg), incubation was continued for 5 min at 37°C, and transfection was assayed.*

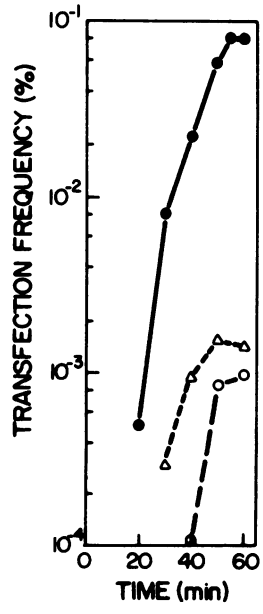


FIG. 2. *Kinetics of the appearance of transfectants using DNA obtained from different phages. Competent nuclease-deficient cells ($7 \times 10^7$ colony-forming units/ml) were incubated with phage DNA (15 μg/ml) obtained from Dp-1 (○); Dp-2 (△); or Dp-4 (●). At different times, samples were taken and treated as described in Fig. 1.*

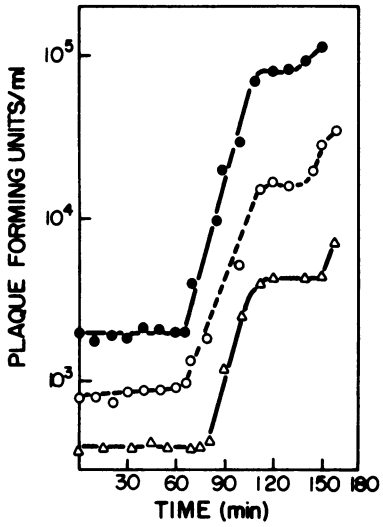


FIG. 3. *One-step growth curves of bacteriophages Dp-1, Dp-2, and Dp-4. The nuclease-deficient strain, at $10^8$ to 1.5 × $10^8$ colony-forming units/ml, was infected at a multiplicity of about 0.003 at 30°C; after 5 min the suspensions were diluted $10^3$ times in fresh C medium containing albumin and yeast extract at the same temperature. At intervals, samples were withdrawn and assayed for infectious centers: (●) Dp-4; (○) Dp-1; (△) Dp-2.*

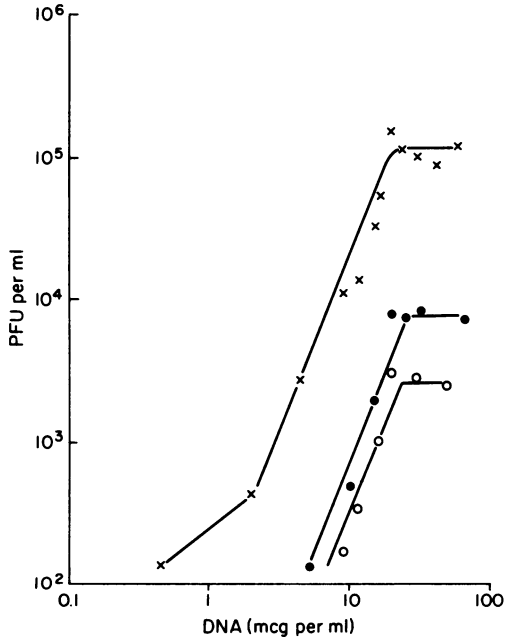


FIG. 4. *Effect of DNA concentration on the yield of transfection. Nuclease-defective pneumococci (7 × $10^7$ colony-forming units/ml) were incubated with various concentrations of DNA from phage Dp-1, Dp-2, or Dp-4 at 30°C for 55 min. DNase (10 μg/ml) was added, and the number of infectious centers was determined. Symbols: Dp-1 (○); Dp-2 (●); Dp-4 (×).*

number of transfectants. The highest transfection found for Dp-4 was about $10^4$ PFU/μg of DNA. This value is in the range of those obtained for *Staphylococcus aureus* (14) and streptococci (9).

The level of transfection with Dp-4 DNA was about 100 times higher than that with DNA extracted from Dp-2 and Dp-1. These results suggest that successful transfection in this system depends on both the use of the nuclease-defective mutant and some special, as yet undetermined characteristics of the phage DNA. The sensitive dependence of infectivity on the structure of transfecting DNA has already been described in the case of coliphages (2).

Although a detailed analysis of the nucleic acid of Dp-4 has not yet been carried out, the results reported in Table 1 demonstrate that DNA is the active component of this phage. In contrast to the infectivity of diplophages, transfection with the phage DNAs was insensitive to Pronase treatment.

**Competence for transfection.** The competence for transfection through the growth curve of *S. pneumoniae* was studied and compared with that obtained for transformation. At different cell concentrations samples were taken and incubated with either 15 μg of Dp-4 DNA per ml (for 55 min at 30°C) or 1.0 μg of DNA per ml carrying the streptomycin resistance marker (for 15 min at 30°C). The reactions were stopped with DNase (10 μg), and the samples were treated as described in Materials and Methods. Figure 5 demonstrates that the peaks of competence for both transfection and transformation are reached at a cell concentration of about 7 × $10^7$ colony-forming units/ml.

Table 2 summarizes a number of additional experiments which show that transfection re-

TABLE 1. *Effect of enzymatic treatment on the infectivity of Dp-4 DNA*[a]

| Enzyme | Concn (μg/ml) | PFU/ml | % of remaining infectivity |
|---|---|---|---|
| Control | | $7.7 \times 10^4$ | 100 |
| DNase | 5 | 0 | 0 |
| DNase | $5 \times 10^{-1}$ | 0 | 0 |
| DNase | $5 \times 10^{-2}$ | 0 | 0 |
| DNase | $5 \times 10^{-3}$ | 0 | 0 |
| DNase | $5 \times 10^{-4}$ | $2.2 \times 10^1$ | 0.03 |
| RNase | 50 | $7.4 \times 10^4$ | 100 |
| Pronase | 100 | $7.6 \times 10^4$ | 100 |

[a] Samples (1 ml) containing 10 μg of Dp-4 DNA were treated with the different enzymes at 37°C over 15 min. After that, 0.1 ml of the competent nuclease-deficient strain and 0.8 ml of C medium containing albumin and yeast extract were added to the samples and reincubated at 30°C for 55 min. DNase (10 μg/ml) was added to stop the reaction, and the samples were treated as described in Fig. 1.

quires the same competent state that is known to be essential for the genetic transformation of pneumococci (17, 18). Thus, successful transformation and transfection both require activation of the recipient bacteria by the pneumococcal activator in a process that is pH dependent, sensitive to trypsin, and inhibited by chloramphenicol.

Results summarized in Tables 3 and 4 demonstrate the competition between double-stranded bacterial (homologous) DNA and the transfecting phage DNA. Single-stranded bacterial DNA was less effective, and tRNA showed relatively little competition even at very high concentrations. The data also show that bacterial DNA appears to have a substantially higher specific activity than phage DNA in the competition experiments. The reason for this observation is not known.

No plaques were observed when competent cells of *Streptococcus sanguis* (strain Wicky) were treated with Dp-4 DNA. (In this experiment, $10^7$ viable cells of *S. sanguis* strain Wicky were activated to competence with the streptococcal competence factor [5]. Incubation of

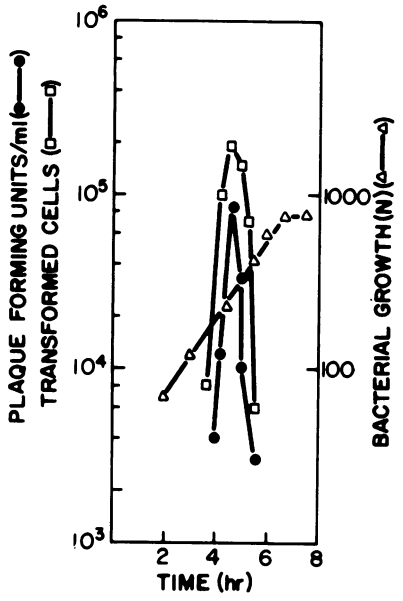


FIG. 5. *Competence for transformation and transfection. The nuclease-defective mutant of S. pneumoniae and the DNA of Dp-4 (15 μg/ml) or DNA carrying the streptomycin resistance marker (1.0 μg/ml) were used. At the times indicated, samples (0.1 ml) of the culture were removed and assayed for transfection and transformation, according to the procedures described in the text. Symbols: (△) growth in nephelometric units; (○) total number of infectious centers; (□) total number of transformed cells.*

TABLE 3. *Competition between bacterial and phage DNA in transfection*[a]

| Nucleic acid | Competing polynucleotide (μg/ml) | Phage DNA (μg/ml) | Residual transfection (%) |
|---|---|---|---|
| None | None | 16 | 100 |
| Pneumococcal DNA | | | |
| Double stranded | 0.5 | 16 | 21.5 |
| | 1.5 | 16 | 10.5 |
| | 3.0 | 16 | 2.3 |
| | 5.0 | 16 | 1.5 |
| | 10.0 | 16 | 0.5 |
| Single stranded | 0.5 | 16 | 92 |
| | 1.5 | 16 | 80 |
| | 3.0 | 16 | 25 |
| | 5.0 | 16 | 15 |
| | 10.0 | 16 | 10 |
| tRNA | 10.0 | 16 | 75 |

[a] Competent cells of the nuclease-defective mutant were incubated with mixtures of bacterial plus phage DNA at different relative concentrations. Transfection was assayed after 55 min of incubation, whereas transformation was tested after 15 min of incubation (to limit cellular lysis caused by phage production). Data in Fig. 2 clearly show that addition of DNase within 15 min of incubation of the bacteria with the phage DNA suppresses transfection. Thus, the low transformation cannot be due to the killing of potential transformants by phage, but, rather, it must represent a genuine competition of the bacterial and phage DNAs for a common step in cellular uptake.

TABLE 2. *Transfection and transformation require a similar competent state*

| Recipient cells[a] | Transfection[b] (PFU/ml) | Transformation[c] (transformants/ml) |
|---|---|---|
| Incompetent[d] | $<0.001 \times 10^4$ | $<0.001 \times 10^6$ |
| + Activator, pH 8 | $1.2 \times 10^4$ (100%) | $3 \times 10^6$ (100%) |
| + Activator, pH 6.6 | $0.03 \times 10^4$ (2.5%) | $0.01 \times 10^6$ (0.3%) |
| + Activator + Trypsin (50 μg/ml) | $0.025 \times 10^4$ (2.1%) | $<0.001 \times 10^6$ |
| + Activator + Chloramphenicol (100 μg/ml) | $<0.001 \times 10^4$ | $0.03 \times 10^6$ (1%) |

[a] Nuclease-defective mutant pneumococci were grown in C medium to a cell concentration of $5 \times 10^7$ viable units/ml.

[b] Transfection was assayed with 16 μg of Dp-4 DNA per ml, according to the procedure described in the text.

[c] Transformation was assayed using 0.1 μg of DNA per ml carrying the streptomycin resistance marker.

[d] Bacteria in the incompetent state were grown in C medium at pH 6.6.

TABLE 4. *Competition between bacterial and phage DNA in transformation*[a]

| Nucleic acid | Competing polynucleotide (μg/ml) | Transforming DNA (μg/ml) | Residual transformation (%) |
|---|---|---|---|
| None | None | 0.05 | 100 |
| Dp-4 phage DNA | 0.2 | 0.05 | 81 |
| | 1.0 | 0.05 | 21 |
| | 10.0 | 0.05 | 4 |
| Pneumococcal DNA | | | |
| Double stranded | 0.5 | 0.05 | 13 |
| Single stranded | 0.5 | 0.05 | 60 |
| tRNA | 10.0 | 0.05 | 95 |

[a] See footnote to Table 3.

these [competent] cells with DNA carrying the streptomycin resistance markers yielded $3 \times 10^5$ transformants per ml. No PFU could be detected when the same bacteria were incubated with 15 μg of Dp-4 DNA per ml for 55 min and were assayed for transfection, as described in Materials and Methods.)

The availability of this transfection system should be of considerable help in our ongoing studies concerned with the mechanism of DNA binding and uptake in competent pneumococci.

## ACKNOWLEDGMENT

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