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TRANSFECTION OF VIBRIO CHOLERAE BY BACTERIOPHAGE ϕ149 DNA

Meenakshi Balganesh and Jyotirmoy Das[1]

Department of Biophysics
Indian Institute of Experimental Medicine
Calcutta - 700 032. INDIA

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SUMMARY

DNA isolated from Choleraphage ϕ149 of Group IV was infectious when mixed with competent V. cholerae cells. The cells were competent during mid-log phase of growth. The infectivity of phage DNA was destroyed by deoxyribonuclease but not by ribonuclease or pronase. About 5 min is required for the establishment of the DNase resistant state. The dose response curve for transfection suggested that 2 to 3 molecules of DNA are required to produce one infectious center. An infectivity of 5×10^4 infectious center per μg of DNA was obtained.

INTRODUCTION

Lack of suitable defined growth medium and of demonstrable genetic exchange systems have frustrated studies on biology of Vibrio cholerae, a highly pathogenic gram-negative bacterium (1). The use of non-genetic phenotypic markers have greatly hampered work on genetics of this organism (2). Genetic analysis of V. cholerae has, so far, been restricted to conjugation (3) and attempts to demonstrate transformation have not yet been successful.

Transfection (4), defined as infection of cells by isolated nucleic acid from viruses resulting in the production of mature viruses, has been reported for several bacterial and animal virus systems (5-9). This phenomenon can be considered as a simplified model of bacterial transformation. Each transfection system examined has its own characteristic features and for proper evaluation of this model, the recognition of these features is essential (10).

We report in this paper a transfection system in V. cholerae by DNA isolated from a Group IV Cholera phage, ϕ149. Phages infecting V. cholerae have

[1] To whom correspondence should be addressed.

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been isolated and serologically (11) and morphologically (12) classified into four groups. Group IV phages are of special interest as these phages are routinely used in differentiating V. cholerae from V. el tor (13). The data presented here show that isolated phage DNA is infectious when mixed with competent cells.

MATERIALS AND METHODS

Bacteria and phage : Vibrio cholerae Og 154 obtained from Cholera Research Center, Calcutta, India, was used for phage propagation and as an indicator strain in these studies. V. cholerae strains R1 and R3, the two phage resistant mutants of V. cholerae In 569B were kindly provided by Dr. A.N. Chatterjee, Bose Institute, Calcutta, India. V. el tor 620, was obtained from Cholera Research Center, Calcutta, India.

Choleraphage Ø149 of Group IV (11) obtained from Cholera Research Center, Calcutta, India, was used in all of these studies.

Media and buffer : The bacteria and phages were cultivated using nutrient broth containing 1 per cent Oxoid Bacto-peptone, 1 per cent Oxoid "Lab Lemco" and 0.5 per cent NaCl. The pH of the medium was adjusted to 7.5. Cells and viruses were assayed as colony forming units (CFU) and plaque forming units (PFU) respectively on nutrient agar plates containing 2 per cent Difco bacto-agar in nutrient broth.

The Tris-HCl-Mg buffer (pH 7.4) was 50 mM Tris containing 5 mM $MgCl_2$. Tris-EDTA-Mg buffer (pH 7.4) was Tris-HCl-Mg buffer containing 1 mM EDTA. Sodium-saline-citrate (SSC) used in DNA extraction was 0.15 M NaCl and 0.015 M sodium citrate.

Preparation of phages : The phage Ø149 was propagated on V. cholerae Og 154 lawns. Phages were washed off the agar plates with Tris-HCl-Mg buffer. The suspension was centrifuged at 12,000 g for 10 min in a Sorvall RC-2B centrifuge to remove cell debris. The supernatant was centrifuged for 1 hr at 100,000 g in a Beckman Model L5-65 centrifuge. The pellet was resuspended in Tris-HCl-Mg buffer.

Extraction of phage DNA : Phage DNA was isolated by phenol extraction following the method of Thomas and Abelson (14). The phage suspension was mixed with an equal volume of phenol saturated with Tris-HCl-Mg buffer. Mixing was done by gently rolling the suspension and vigorous pipetting was avoided in all steps. The emulsion was centrifuged at 5,000 g for 5 min and aqueous phase was removed. The phenol phase was re-extracted twice and aqueous layers were pooled. The aqueous phase was extensively dialyzed against Tris-EDTA-Mg buffer and subsequently against SSC. DNA was precipitated by adding 2 volumes of ethanol and the precipitate was resuspended in SSC. The DNA solution was extensively dialyzed against SSC and stored at 4°C. DNA concentration was determined from the absorbance at 260 nm using an absorbancy per mole of nucleotide of 6,000 cm^{-1}. The ratio of the absorbancy at 260 nm to 280 nm for the DNA used in these studies was 1.7.

Transfection assay : Phage DNA was mixed with competent cells (3 hr old culture) and incubated for 30 min at 37°C. The infectious centers were assayed using soft agar overlay method (15).

Enzymatic treatment of phage DNA : Bovine pancreatic deoxyribonuclease 1 (DNase), bovine pancreatic ribonuclease (RNase) and pronase were obtained from Sigma Chemical Co., St. Louis, Mo. DNase was dissolved in Tris-HCl-Mg buffer and RNase and pronase solutions were made in Tris-EDTA-Mg buffer. All reactions were carried out for 15 min at 37°C.

RESULTS

Identification of the infective agent :

DNA isolated from ϕ149 was tested for infectivity by mixing with log-phase culture of *V. cholerae* Og 154 and was found to produce plaque-forming units when assayed by soft agar overlay method as described in Materials and Methods. To rule out the possibility of infection by surviving phage contaminants or by partially degraded phage, the infective agent was treated with DNase, RNase and pronase. While the transfecting agent was totally insensitive to RNase and pronase treatment, infectivity was completely lost by treatment with 50 μg DNase per ml for 15 min (Table 1). Treatment with 10 μg DNase reduced the transfecting activity by about 85 per cent. Three cycles of freezing and thawing of the infective agent reduced the transfecting ability by more than 90 per cent. Intact phages under similar condition lose only 15 per cent of infectivity (Das, J., unpublished data). Shearing of the infective agent also reduced the transfecting activity.

Table 1 : Effect of various treatments on the infectivity of ϕ149 DNA.

Treatment	Percentage activity remaining
None	100
DNase (10 μg/ml, 15 min, 37°)	15
DNase (50 μg/ml, 15 min, 37°)	0
RNase (1 mg/ml, 15 min, 37°)	106
Pronase (1 mg/ml, 15 min, 37°)	126
Freezing and thawing (1 Cycle)	56
Freezing and thawing (2 Cycle)	11
Freezing and thawing (3 Cycle)	4.2
Vortex (1 min)	63

All assays were carried out at a DNA concentration of 6 μg/ml. Three hour old culture grown as described in Materials and Methods was used for infection and the number of plaque forming units (PFU) was taken as the measure of activity. With no treatment the infectious center titer was 2.4×10^4 PFU/ml .

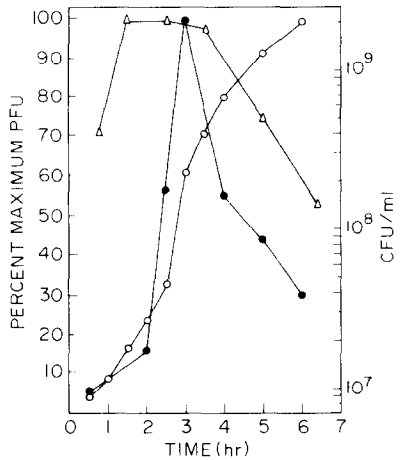


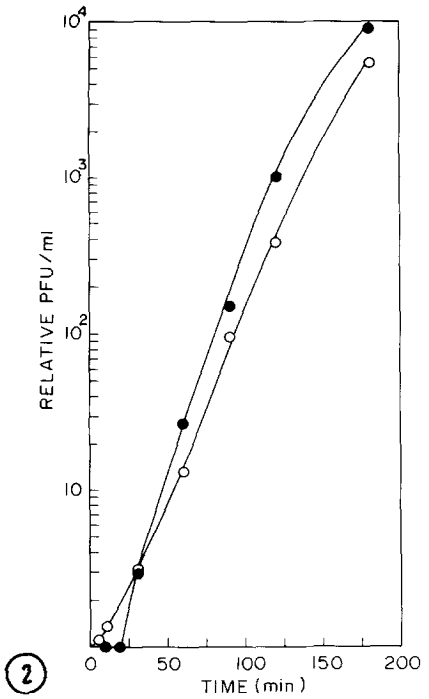
Fig. 1 : Efficiency of formation of infectious centers by V. cholerae Og 154 cells of different ages infected with ø149 (Δ) or ø149 DNA (●). The cell growth was assayed as CFU/ml (○). At intervals during cell growth samples were removed and infected with either ø149 at a multiplicity of infection (m.o.i) of 0.1 or with ø149 DNA at a final concentration of 6 μg/ml. Five minutes for phage and 30 minutes for phage DNA were allowed for adsorption. Phage infected samples were centrifuged at 5000 g for 5 min to remove unadsorbed viruses and the pellet assayed for PFU/ml. Samples infected with phage DNA were treated with 20 μg/ml DNase for 5 min at 37°C and assayed for PFU/ml. PFU at each time is expressed as the per cent of the maximum PFU/ml.

Dependence of transfection on age of the culture :

To examine whether cells of different ages can be transfected by phage DNA with equal efficiency, infective centers were assayed by mixing ø149 DNA with cells at various times during growth. Cells in mid-log phase of growth were most competent for infection by phage DNA (Fig. 1). Under the present experimental conditions cells during 2.5 to 3.5 hr of growth were considered competent and in all subsequent experiments 3 hr old cultures were used for transfection. For comparison, dependence of infection by intact phages on the age of culture was examined and no change in the number of infective centers was observed for cells 1 to 4 hr old (Fig. 1).

Production of phage by ø149 DNA infected cells :

One step growth curves obtained by phage and phage DNA infection are shown in Fig. 2. Infection by DNA has a latent period of about 20 min compared to 5 min latent period for infection by intact phage. This may be due to the fact that



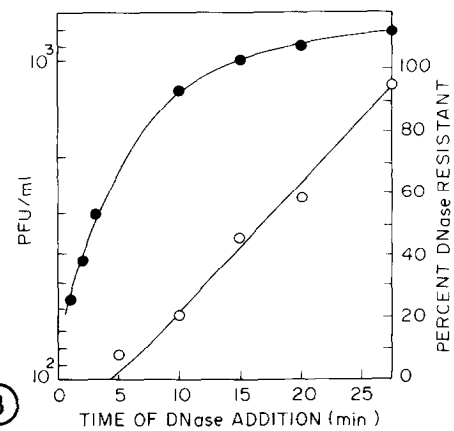


Fig. 2 : One step growth curve obtained by ø149 (○) and ø149 DNA (●) infection. Competent cells of V. cholerae Og 154 were infected either with phage or phage DNA. Ten minutes at 37°C was allowed for adsorption. Infection by DNA was terminated by 25 µg/ml DNase. Phage or DNA infected cells were centrifuged at 5000 g for 5 min. The pellets were resuspended in prewarmed nutrient broth (1 : 10 dilution). At different times during incubation at 37°C samples were removed and assayed for PFU.

Fig. 3 : Kinetics of infection by ø149 DNA. Phage DNA (6 µg/ml) was added to competent V. cholerae Og 154 cells and at different times during infection samples were removed. One part of the sample was immediately assayed for PFU (data not shown) and the other part was treated with 50 µg DNase per ml for 15 min at 37°C and thus assayed for PFU (●). The fraction of infectious centers resistant to DNase was calculated from the DNase treated and untreated PFU/ml at each time (○).

phage adsorption is more rapid than the DNA uptake by competent cells. The nature of the growth curves obtained by phage or phage DNA are similar. Phages produced by DNA infection were examined and found to have the same properties as ø149. A phage resistant mutant of V. cholerae, Strain R1, unable to adsorb ø149 was found to be sensitive to phage DNA. Strain R1 was resistant to phages produced by transfection in this strain. To examine whether the inability of V. el tor, to serve as host for Group IV choleraphage is an adsorption problem, the cells at log-phase growth were

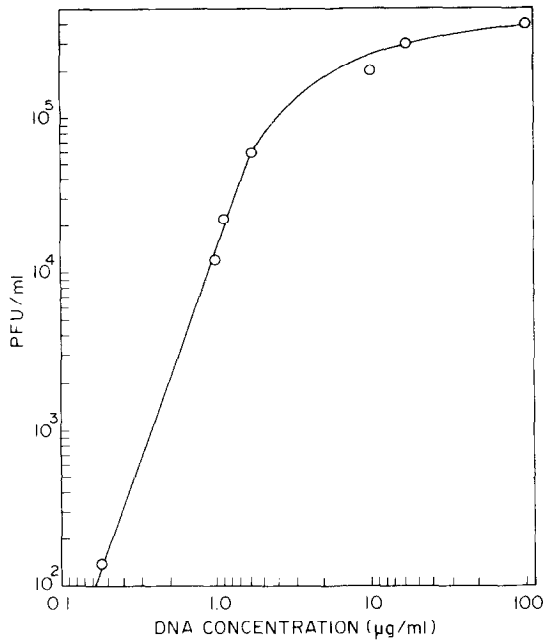


Fig. 4 : Effect of DNA concentration on infectious center formation. DNA was added to 2.5×10^8 competent V. cholerae Og 154 cells per ml and incubated at 37°C for 20 mins. At the end of this period 50 µg DNase per ml was added to terminate the reaction and after 10 min assayed for PFU.

mixed with 50 µg ø149 DNA per ml and assayed for PFU. No infectious centers were observed.

Kinetics of infection by ø149 DNA :

6 µg ø149 DNA per ml was added to the competent cells and at intervals assayed for DNase resistant infectious centers (Fig. 3). At each time, the percentage of DNase resistant transfectants was calculated. About 5 min is required for the establishment of the DNase resistant state in V. cholerae transfection system (Fig. 3). 50 per cent of the infectious centers achieve DNase resistance within 15 min of the appearance of the first DNase resistant cell.

Dependence of infective centers on DNA concentration :

The dose-response curve is an important characteristic of transformation and transfection systems(16). Various concentrations of ø149 DNA were added to 2×10^8 competent cells and after 20 min at 37°C assayed for PFU. The dose-response curve (Fig. 4) was linear between DNA concentration 0.5 to 6 µg. At higher concen-

trations the number of infective centers stopped increasing linearly and saturation is reached at about 50 μg DNA per ml. The slope of the linear part of the log-log plot was 2.4 . Hence the number of transfectants is proportional to second or third power of the DNA concentration.

DISCUSSION

The data presented here show transfection of V. cholerae by choleraphage ø149 DNA. From the saturation value of PFU/ml (4×10^5) and the corresponding dose (6 μg/ml) (Fig. 4), the amount of DNA required to make one infectious center was found to be 1.7×10^{-5} μg. Assuming the molecular weight of ø149 DNA as 36×10^6 (17), the number of viral DNA equivalent per PFU comes to about 10^6. This value is comparable to those reported for other systems (18 - 20). The low efficiency of transfection is mostly due to the low percentage of competent cells (less than 5 per cent, in the present study, as estimated from the ratio of the number of infected centers at saturation of the dose-response curve to the input number of cells) and partly due to damage to the viral DNA during isolation or during storage. Studies to improve the efficiency of transfection are in progress.

Although transformation and transfection may share only a few of the common steps, demonstration of transfection in V. cholerae by phage DNA might help studies involving DNA mediated transformation in this system.

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