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Factors Affecting Infection of Protoplasts with Deoxyribonucleic Acid of Actinophage PK-66

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To establish a method for transmission of genetic materials in the genus *Streptomyces*, the conditions of infection of protoplasts of *S. kanamyceticus* by actinophage PK-66 deoxyribonucleic acid (DNA) were studied. The protoplasts of *Streptomyces* were prepared by treatments with lysozyme and trypsin. The infectivity of the phage DNA was enhanced by the presence of NaCl in the medium. The optimal concentration of the protoplasts for infection with DNA was 7×10^7 to 4×10^8/ml. A proportional relationship was found between the infectivity and the DNA concentration within a certain range. The maximal production of mature phage was achieved after 19 hr of incubation. The number of phage propagated in the infection mixture reached 10^4 plaque-forming units per ml under the appropriate conditions. The phage DNA infected not only protoplasts prepared from *S. kanamyceticus* but also those prepared from *S. violaceoniger* and *S. acidomyceticus*, which were resistant to intact phage PK-66.

Single-stranded deoxyribonucleic acid (DNA) extracted from ϕX174 phage, when transferred to spheroplasts of *Escherichia coli*, was found to produce infective phage particles (4, 5, 13, 14). Subsequently, similar studies on infection of *E. coli* spheroplasts with DNA of λ were reported (9). Penetration of homologous DNA and its recombination with host DNA were accomplished in protoplasts of *Bacillus subtilis* (3). These results indicate that the highly polymerized DNA penetrates and replicates itself in the recipient protoplasts to demonstrate its inherent information. In the *Streptomyces* system, the efficiency of infection was found to depend on the conditions of the infection; a marked effect of *p*H on the efficiency was reported in our previous paper (11).

The present investigation deals with the conditions suitable for infection of *Streptomyces* protoplasts with phage DNA, phage production in relation to the concentration of phage DNA, time course of multiplication of phage, and infectivity of the DNA to protoplasts prepared from streptomycetes resistant to intact phage particles.

MATERIALS AND METHODS

Organisms. A subculture of *S. kanamyceticus* K-2J, designated NIHJ At-463, was used for preparation of protoplasts and for phage count. Protoplasts were also prepared from *S. acidomyceticus* At-85, *S. violaceoniger* At-161, *S. erythrochromogenes* At-475, and *S. tanashiensis* At-97. Isolation, characteristic properties, and procedure for assay of actinophage PK-66 were described in a previous paper (10).

Purification of phage. Phage PK-66 was propagated in medium AC (glucose, 10 g; yeast extract, 10 g; $CaCl_2 \cdot 2H_2O$, 0.73 g; and distilled water, 1 liter; *p*H 6.8) with *S. kanamyceticus* as the host. After 20 hr of incubation, the lysate, containing usually 10^9 plaque-forming units (PFU) per ml, was centrifuged at $4{,}000 \times g$ for 30 min to remove cell debris. To the supernatant fluid, we added 450 g of $(NH_4)_2SO_4$ per liter of lysate; the mixture was left standing overnight in the cold. The precipitate was collected by centrifugation and resuspended in a small volume of phage saline (NaCl, 5 g; $CaCl_2 \cdot 2H_2O$, 0.73 g; tris-(hydroxymethyl)aminomethane (Tris) buffer, 2 mmoles; gelatin, 10 mg; and distilled water, 1 liter; *p*H 7.2). After dialysis against phage saline, the phage suspension was treated with 2 μg of deoxyribonuclease per ml, 5 μg of ribonuclease per ml, 20 μg of trypsin per ml, and 0.002 M $MgSO_4$ for 30 min at 37 C, and then was clarified by low-speed centrifugation. The phage particles in the supernatant fluid were sedimented by another centrifugation at $36{,}000 \times g$ for 120 min. The phage was resuspended in a small volume of phage saline, and then the debris and clumps were removed by centrifugation at $4{,}000 \times g$ for 30 min. This differential centrifugation was repeated. After filtration through a membrane filter (Millipore Corp., Bedford, Mass.; type DA), the phage suspension was placed in a refrigerator at 4 C.

Preparation of phage DNA. A modification of the method of Kaiser and Hognes (6) was employed for extraction of DNA. The phage suspension, diluted with phage saline to give 10^{11} PFU/ml, was treated with 10^{-2} M ethylenediaminetetraacetate (EDTA) in the cold. An equal volume of freshly distilled phenol saturated with saline-citrate at 4 C was added to the phage suspension, and it was agitated by hand for 10

min. The two phases were separated by centrifugation, and the aqueous phase was dialyzed against saline-citrate for 24 hr at 4 C. The optical density of this dialysate was 8.7 (10-mm cuvette) at 260 mμ, and no intact phage particles could be detected by the plaque assay.

Preparation of protoplasts of streptomycetes. The streptomycetes were incubated in 30 ml of medium A (glucose, 10 g; yeast extract, 10 g; and distilled water, 1 liter; *p*H 6.8) on a reciprocating shaker at 28 C for 16 hr, and the cultured mycelium was washed with medium HB (sucrose, 28 g; glucose, 1 g; yeast extract, 1 g; NaCl, 0.5 g; and $MgSO_4 \cdot 7H_2O$, 0.173 g; in a total volume of 100 ml; *p*H 6.8). The mycelium was resuspended in 30 ml of medium HB, in a 200-ml Erlenmeyer flask, containing lysozyme (2 mg/ml) and trypsin (200 μg/ml), and the suspension was incubated on a reciprocating shaker (2-cm amplitude and 75 strokes per min) at 28 C for 5 hr. The mycelium thus treated was sedimented by centrifugation at 3,300 × *g* for 30 min. The sediment was further treated twice as above but without trypsin. The protoplasts thus obtained were washed twice with medium HB by centrifugation at 2,800 × *g* for 30 min and resuspended in medium HB. The protoplasts thus prepared can be stored in a refrigerator for 3 weeks without appreciable loss of capacity as hosts. Immediately before use, the protoplasts were washed twice with medium HA ($MgSO_3$ was omitted from medium HB) by centrifugation and suspended in 20 ml of medium PA-HB (sucrose, 28 g; glucose, 1 g; yeast extract, 1 g; NaCl, 0.88 g; 0.5 M phosphate buffer, *p*H 6.0, 10 ml; 20% bovine serum albumin fraction V, 10 ml; in a total volume of 100 ml).

Procedure for infecting protoplasts with phage DNA. Unless otherwise stated, the following procedure was employed for infection of protoplasts with phage DNA. The suspension of protoplasts was diluted with medium PA-HB to give a concentration of 4 × 10^8 protoplasts/ml. The dialyzed DNA prepared from a phage suspension containing 10^{11} PFU/ml was diluted with an equal volume of cold HS medium (sucrose, 60 g; NaCl, 0.88 g; and Tris buffer, 0.5 mmoles, *p*H 7.2; dissolved in distilled water to make 100 ml). The infection mixture consisted of 0.25 ml of the protoplast suspension (4 × 10^8 cells/ml), 0.2 ml of the twofold diluted DNA, and 0.05 ml of test solution or distilled water. This mixture was incubated on a reciprocating shaker at 28 C, and the number of phage produced was determined at 16, 20, and 24 hr. The production of mature phage was used as a measure of successful infection, since the infective centers could not be measured owing to the fact that the phage particles could not form plaques in the hypertonic agar medium supplemented with sucrose for protection of protoplasts.

Procedure for assay of phage titers. The phage produced in the infection mixture was assayed by a modification of Adam's double-layer method (1) as described in a previous paper (10). The samples were diluted with phage saline with special care to avoid osmotic shock and were plated on an agar layer with a culture of *S. kanamyceticus* At-463. Plaques were counted after 48 hr of incubation at 28 C.

RESULTS

Properties of phage DNA. The ultraviolet-absorption spectra of the purified phage particles and of their DNA are presented in Fig. 1. The phage was suspended to give 3.9 × 10^9 PFU/ml in 0.05 M phosphate buffer containing 0.15 M NaCl and 0.01 M $MgSO_4$, and its DNA was diluted to yield an optical density value of 0.6 at 260 mμ in 0.05 M phosphate buffer containing 0.15 M NaCl. The ultraviolet-absorption spectrum of the phage DNA can be expected to be similar to that of native DNA (7), since the ratios of the optical density at 260 mμ against that at 230 mμ and of the optical density at 260 mμ against that at 280 mμ are 2.0 and 1.8, respectively.

The buoyant densities of the phage particles and of their DNA were determined by equilibrium density gradient centrifugation in CsCl (8). The distribution of the phage particles and of their DNA in each fraction obtained from the tubes centrifuged was measured by plaque-count assay with *S. kanamyceticus* as an indicator and by spectrophotometry at 260 mμ, respectively. Both PK-66 phage and its DNA showed single peaks (Fig. 2) which corresponded to the densities of 1.549 and 1.695 g/cc, respectively.

Conditions for infection of protoplasts by phage DNA. The influence of salts in the infection mix-

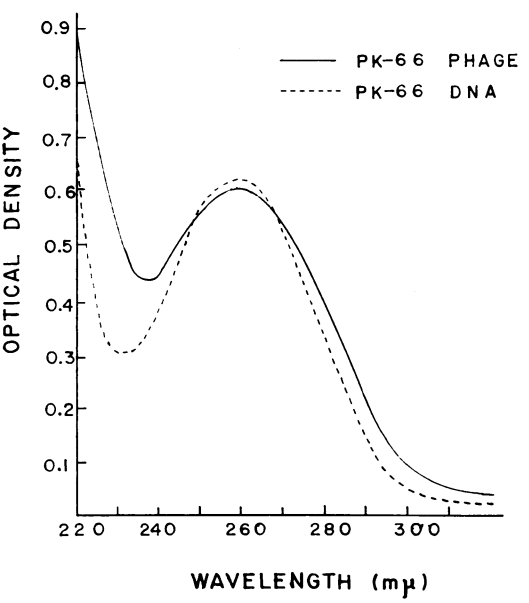

FIG. 1. *Ultraviolet-absorption spectra of the phage PK-66 and its DNA.*

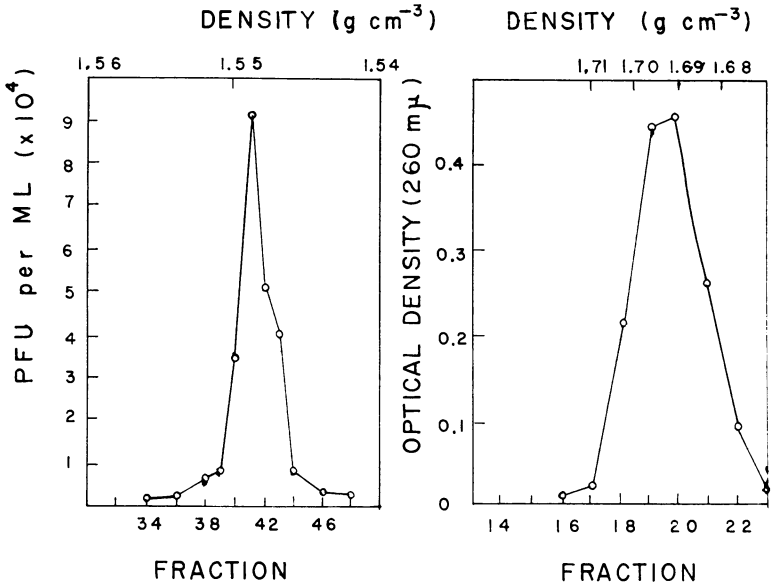

FIG. 2. *Distribution of phage PK-66 and its DNA in a CsCl density gradient.*

ture on the infectivity of the phage DNA was investigated. The infection procedure was that described above, except that NaCl was omitted and sucrose was increased to 30 g/100 ml in medium PA-HB. Phage production was assayed after 14 and 16 hr of incubation. Infectivity was stimulated in 0.15 M NaCl, 0.0005 M $CaCl_2$, or 0.003 M sodium citrate, but inhibition was observed in 0.07 M $MgSO_4$, 0.1 M KCl, 0.005 M $CaCl_2$, or 0.2% Casamino Acids (Table 1). The combination of salts which stimulated infectivity was examined. Only NaCl gave consistent results. Thus, the PA-HB medium was selected as the medium for infection of protoplasts.

Production of phage was examined at various concentrations of protoplasts in the infection mixture. Maximal production of phage was provided by the protoplasts at concentrations ranging from 7×10^7 to 4×10^8 per ml (Fig. 3).

The concentration of phage DNA in the infection mixture was studied. The dose-response curve shown in Fig. 4 resulted from a set of serial threefold dilutions of phage DNA with a fixed concentration of 4×10^8 protoplasts per ml. A proportional relationship existed between the infectivity and the DNA concentration within a range of 1:725 to 1:27. Saturation was observed at a concentration of 1:20 of the DNA suspension. These data suggest that multiplication of phage results from the interaction of a single DNA molecule with a single protoplast, though the phages produced were few as compared with the concentration of DNA.

TABLE 1. *Effect of salt composition on the infectivity of PK-66 DNA*

Salt added to infection mixture	Plaque count/ml[a]
None	690
NaCl, 150 mM	2,430
KCl, 10 mM	540
KCl, 100 mM	110
$MgSO_4$, 0.7 mM	250
$MgSO_4$, 7 mM	510
$MgSO_4$, 70 mM	50
$CaCl_2$, 0.5 mM	1,220
$CaCl_2$, 5 mM	140
Sodium citrate, 3 mM	1,900
Casamino Acids, 0.2%	140

[a] Average titers in samples taken at 14 and 16 hr of incubation.

Dilution of the infection mixture with medium PA-HB at various pH levels. The infectivity of phage DNA is markedly affected by *p*H; the best infection is obtained at *p*H 6.0 (11). This infectivity was destroyed when deoxyribonuclease was added to the mixture within 3 hr after the start of incubation. Results of the experiments on the concentration of protoplasts and on DNA described above indicated that, if the infection mixture was diluted 1:20 with medium PA-HB during the incubation, further infection of protoplasts by phage DNA almost ceased. Therefore, by diluting the incubation infection mixture with medium PA-HB of varied *p*H, the periods of

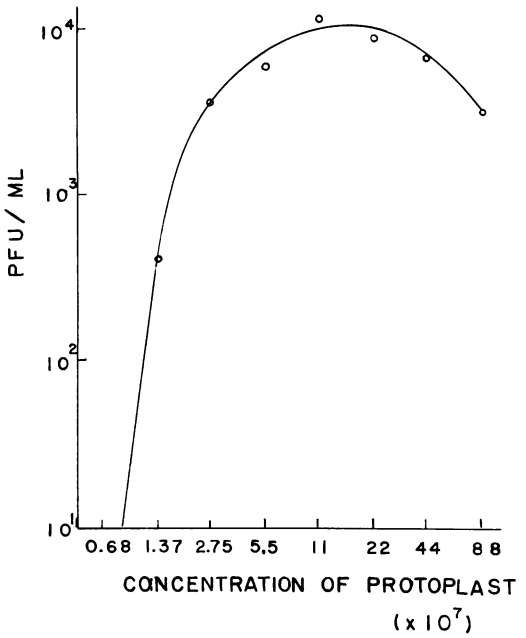

FIG. 3. *Effect of protoplast concentration on the production of intact phage.*

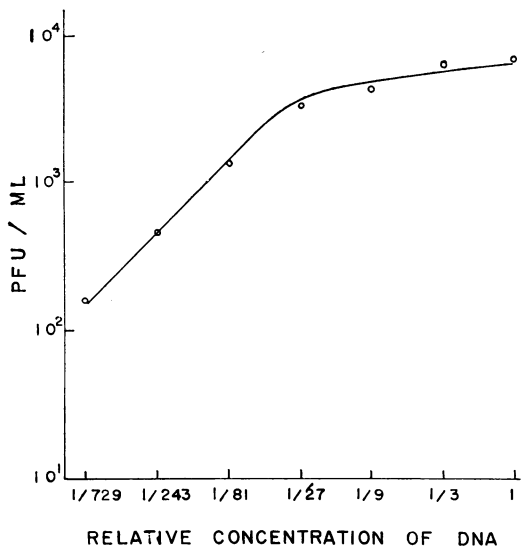

FIG. 4. *Effect of DNA concentration of PK-66 phage on the production of phage particles.*

penetration of phage DNA into protoplasts and the optimal *p*H for the production of mature phage in the infected protoplasts could be determined. The production of phage in the protoplasts infected with phage DNA was optimal at *p*H 6.0 to 6.6, irrespective of the time at which the dilution of the infection mixture was made (Table 2). The infectivity at 2 hr of incubation was much lower than that after 3 hr of incubation, but that after 3 to 6 hr of incubation was not significantly different from each other. This indicates that adsorption of phage DNA to protoplasts is practically completed within 3 hr after the start of incubation.

Time course of the multiplication of the phage in the infection mixture. The production of mature phage in the infection mixture was measured after various periods of incubation. Initial production of mature phage was apparent 14 hr after the start of incubation, and maximal production was reached after 19 hr (Fig. 5).

On the other hand, when intact mycelium and intact phage particles in medium PA-HB from which sucrose was omitted were incubated, the latent period for the production of phage was about 150 min.

Infectivity of PK-66 DNA to protoplasts prepared from streptomycetes resistant to intact phage particles. S. acidomyceticus At-85, *S. violaceoniger* At-161, and *S. erythrochromogenes* At-475 were selected as organisms resistant to infection with intact PK-66 phage particles. Their resistance was confirmed by plating them with 10^5 PFU of the phage per plate. The preparation of protoplasts and the constitution of infection mixture were the same as above.

TABLE 2. *Effect on phage production of dilution of the infection mixture with medium PA-HB adjusted to various pH levels*

Incubation period before dilution (hr)	*p*H of medium PA-HB for dilution[a]	Plaque count per ml of diluted mixture
2	6.0	60
	6.6	25
	7.2	10
	7.8	15
3	6.0	360
	6.6	165
	7.2	55
	7.8	0
4	6.0	225
	6.6	410
	7.2	20
	7.8	0
5	6.0	415
	6.6	350
	7.2	50
	7.8	0

[a] The infection mixtures were diluted 20-fold with medium PA-HB containing 0.05 M phosphate buffer of various *p*H values.

Infectivity, though it was poor, was evident in the protoplasts of *S. acidomyceticus* and *S. violaceoniger* but not in those of *S. erythrochromogenes* (Table 3). Poor infectivity was also obtained with protoplasts of *S. tanashiensis* At-97, though intact phage particles displayed high efficiency of plating against this species. These results show that the protoplasts prepared from various species of *Streptomyces* have the capacity of permitting biologically active DNA to penetrate under appropriate conditions.

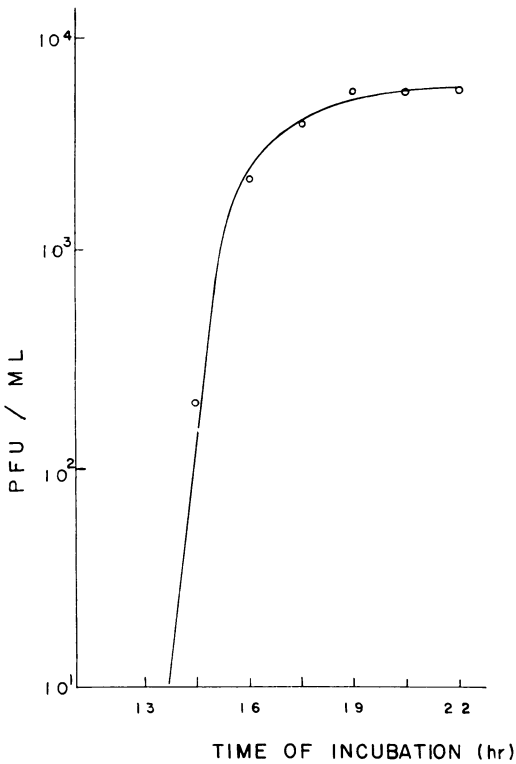

FIG. 5. *Time course of the multiplication of phage in the infection mixture.*

TABLE 3. *Infectivity of PK-66 DNA to the protoplasts prepared from streptomycetes resistant to intact phage PK-66*

Source of protoplasts	Sensitivity of mycelia to intact PK-66[a]	Plaque count/ml
S. acidomyceticus At-85	−	340
S. violaceoniger At-161	−	1,200
S. erythrochromogenes At-475	−	0
S. tanashiensis At-97	+++	110
S. kanamyceticus At-463	++	6,400

[a] The sensitivity of intact mycelia to intact phage PK-66 was confirmed by the efficiency of plating against 10^5 PFU of PK-66.

DISCUSSION

Although 1 ml of the infection mixture contained 2×10^8 protoplasts and phage DNA equivalent to 10^{10} phage particles, the number of phage produced was not more than 10^4 PFU under the optimal conditions of infection. Observation of phage particles by electron microscopy revealed that the head was 110 mμ in width and 140 mμ in length (10). If the DNA included in the phage head is assumed to be a single molecule, it is conceivable that the molecular weight of the DNA is more than 2×10^8 daltons in T-even phage. It seems that this low rate of infection to protoplasts is due to the large size of the molecule.

The system of λ DNA and *E. coli* protoplasts (9) showed that the degree of infectivity depended on the concentration of the Fraser medium in which the protoplasts and DNA were allowed to interact; a sevenfold dilution of the Fraser medium was optimal and produced the highest number of infective centers. It was also shown that restoration of Casamino Acids, gelatin, NH_4Cl, glycerol, or $CaCl_2$ in the diluted medium diminished the infectivity and that NaCl, KCl, KH_2PO_4, or Na_2HPO_4 destroyed all infectivity. In our infection system, the infectivity was enhanced by the presence of NaCl in medium PA-HB, but other inorganic salts added in the medium gave varied infectivity from one experiment to another.

Although transmission of heterologous genetic determinants to recipient strain has been carried out by conjugation between particular species of *Streptomyces* (2, 12), no method has yet been devised which is generally applicable to transmission of heterologous determinants.

The protoplasts prepared from streptomycetes naturally resistant to intact phage were susceptible to infection by phage DNA, as were those prepared from *S. kanamyceticus*. It is possible that the various species of *Streptomyces* allow biologically active DNA to penetrate into their protoplasts under appropriate conditions. Some protoplasts are able to regenerate themselves to the mycelial form under certain conditions. These infection systems, therefore, seem to offer a new method for study of the interspecies transmission of genetic materials among *Streptomyces* species.

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