INFECTION OF MYCOBACTERIUM SMEGMATIS WITH D29 PHAGE DNA*

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Specific biological activity of deoxyribonucleic acid (DNA) was first established by the experiments of Avery, MacLeod, and McCarty (1). These investigators showed that the substance responsible for the transformation of pneumococcal types was DNA. Current literature contains many examples of the biological activity of DNA including that isolated from bacteriophages. Spizizen (2) and Frazer *et al.* (3) infected protoplasts of *Escherichia coli* with subviral extracts from T_2 coliphage. Guthrie and Sinsheimer (4) infected protoplasts of *E. coli* with purified DNA from ϕX174 phage. Kaiser and Hogness (5) were able to transform *E. coli* with DNA from λdg phage, providing that nontransducing helper phages were added to the bacteria along with the DNA. More recently Romig (6) infected a transformable strain of *Bacillus subtilis* with DNA from a phage which he had isolated from soil and designated as SP3.

The experiments reported here extend these observations to include the mycobacteriophages. It was found that DNA from mycobacteriophage D29 could infect its host. Bacteria susceptible to infection with intact phage were also susceptible to phage DNA; no alteration in their physiologic state was required.

Materials and Methods

Reagents.—

Phenol: Mallinckrodt Chemical Works, St. Louis, 88 per cent, analytical reagent without preservative, saturated with water at 4°C.

2-Ethoxyethanol (ethylene glycol monoethyl ether): Matheson Coleman and Bell, Norwood, Ohio.

Buffers.—

Tris: Sigma Chemical Company, St. Louis. A 1 M stock solution adjusted to the desired pH between 7 and 9 by adding 10 N HCl and used in the final concentration of 0.05 or 0.007 M.

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Citrate-saline: A 0.15 M solution of NaCl containing 0.015 M Na citrate.

Phosphate: Standard 0.039 M at pH 7.

Chromatography.—

Paper: Whatman No. 1, 20 × 21 cm prewashed first in 0.1 N HCl and then in water.

Solvent 1: Tert-butyl alcohol, methylethyl ketone, water, ammonium hydroxide (40:30:20:10).

Solvent 2: Isopropyl alcohol, water, concentrated HCl (65:18.4:16.6).

Enzymes.—

Deoxyribonuclease (DNAase): From beef pancreas, 1 time crystallized from Worthington Biochemical Corporation, Freehold, New Jersey. Stocks made up of 1 mg/ml in distilled water and stored at 4°C for several weeks.

Ribonuclease (RNAase): From beef pancreas, 1 time crystallized from Worthington Biochemical Corporation. Stocks made up of 1 mg/ml in distilled water, boiled for 10 minutes, and stored at 4°C for several weeks.

Trypsin: From beef pancreas, A grade from California Corporation for Biochemical Research, Los Angeles. Stocks made up of 5 mg/ml in phosphate-buffered saline at pH 7.4 and stored at −40°C until used.

Snake venom: Lyophilized *Crotalus adamanteus* from Ross Allen Reptile Institute, Silver Springs, Florida.

Mycobacteriophage.—D29 was obtained originally from Dr. Seymour Froman. It was isolated by soil enrichment techniques and is active against certain strains of both virulent and saprophytic acid-fast bacilli (7). Methods for the production of high-titered lysates and for phage assay have been described (8).

Bacteria.—*Mycobacterium smegmatis* ATCC 607 (referred to hereafter as 607) was employed as host. Stock cultures were maintained, and log phase cultures were prepared as described (8).

D29 DNA.—High titered phage lysates (10^{10} to 10^{11}/ml) were prepared and stored at 4°C until used. For the preparation of DNA, the lysate was first passed through a Seitz filter and then digested with DNAase and RNAase (5 μg/ml) for 2 hours at room temperature. Phage was concentrated by differential centrifugation in a Spinco model L centrifuge, first at 20,000 *g* for 75 minutes to sediment the phage and then at 3500 *g* for 30 minutes to remove clumped cellular debris. Both the high and low speed cycles were repeated yielding a final phage suspension with a titer of approximately 9×10^{12} plaque-forming units (PFU) per ml. Phage pellets were resuspended in tris buffer at pH 7 after the first cycle of high speed centrifugation and in citrate-saline after the second. DNA was extracted by the cold phenol method of Gierer and Schramm (9). The aqueous phase phenol extract was dialyzed against 0.039 M phosphate buffer at pH 7. DNA was precipitated out of the dialysate by the addition of an equal volume of 2-ethoxyethanol (10). The fibrous precipitate was dissolved in citrate-saline and then dialyzed against the same buffer. The resultant DNA solution was stored at either 4°C or at −70°C, depending on how soon it was to be used. DNA concentration was determined by the method of Keck (11) using calf thymus DNA as standard (Worthington Biochemical Corporation).

Base Composition of DNA.—The precipitated DNA was removed from the 2-ethoxyethanol, taken up in water, and digested first with DNAase and then with snake venom. The enzymes were removed by ethanol precipitation and the resultant nucleosides were separated by 2-dimensional paper chromatography described by Fink and Fink (12). Purine nucleosides are hydrolyzed to their respective bases by the action of the second solvent (13). Individual deoxyribonucleosides or their bases visualized under ultraviolet light, were cut out and eluted from the paper in 0.1 N HCl. The eluants were analyzed in a Beckman DK automatic spec-

trophotometer. Identity and purity of the different compounds were determined by comparisons with known standard deoxyribonucleosides or bases treated under identical conditions. The amount of each base was determined from its molar extinction coefficient and ultraviolet absorption at 260 mμ wavelength. The molar base ratios were expressed as percentages of the total bases recovered.

Density Gradient Determination.—The buoyant density of D29 was determined by equilibrium density gradient centrifugation in cesium chloride with a density of 1.7 gm/ml by the method of Meselson *et al.* (14). The content of guanine plus cytosine (GC per cent) of the DNA was calculated from the values obtained (15).

RESULTS

Base Composition of Phage DNA.—Table I shows the DNA composition of D29 mycobacteriophage. The bases of D29 are paired, adenine (A) with thymine (T) and guanine (G) with cytosine (C), indicating that the DNA of D29 is double stranded. The dissymmetry ratio, that is, the ratio of A + T to G + C

TABLE I

DNA Composition of D29 Mycobacteriophage

	Base proportions*				$\frac{A+T}{G+C}$	Density, CsCl
	A	T	G	C		
	moles per cent	*moles per cent*	*moles per cent*	*moles per cent*		*g cm⁻³*
Phage	18.0	18.1	33.9	30.0	0.56	1.722

* As free bases of A and G, nucleosides of T and C.

is 0.56. The buoyant density of D29 DNA in CsCl is 1.722. The GC content of D29, calculated according to its density in CsCl (15) is 63.77 per cent, a value in good agreement with the 63.90 per cent obtained by direct measurement. With this DNA preparation, only one band was visualized in ultraviolet light, and 99 per cent of the ultraviolet absorbance at 260 mμ was recovered as deoxyribosides or their bases. When plated with 607, 1 ml of the same preparation, diluted to contain 100 μg of DNA per ml buffer, produced 200 plaques which were indistinguishable from plaques produced by intact D29.

Effect of Enzymes and Phage Antiserum on the Infectivity of D29 DNA.—Table II shows the comparative effects of DNAase, RNAase, trypsin, and D29 antiserum on intact phage and phage DNA.

Approximately 100 PFU of intact phage per ml was added to heart infusion broth containing 0.002 M $CaCl_2$ and 0.01 M $MgCl_2$, and divided into 0.5 ml samples. The samples were incubated for 30 minutes at 37°C with a specific enzyme or with phage antiserum. Duplicate samples were made in which 100 PFU of D29 DNA were substituted for intact phage. At the end of the preincubation period, the mixtures were added to soft agar seeded with 607, incubated, and observed for plaque formation.

Intact phage was not effected by any of the enzymes tested, whereas it was completely neutralized by phage antiserum. On the other hand, preincubation with phage antiserum had no effect on plaque formation by phage DNA. DNAase completely abolished the activity of DNA and in additional experiments recorded in Table III, it can be seen that a concentration as low as 10^{-3} μg of DNAase per ml is sufficient to inactivate over 90 per cent of the DNA infectivity. Treatment with RNAase resulted in a significant decrease in plaque formation; preincubation with trypsin had no effect. In preliminary experiments, testing for the effect of trypsin on D29 DNA, spurious results were ob-

TABLE II

Comparative Effects of Enzymes and Phage Antiserum on the Infectivity of Intact D29 and D29 DNA

Infectious agent	Plaques formed per ml after preincubation of reaction mixtures				
	Broth only	DNAase	RNAase	Trypsin	Antiserum
Intact phage	100	110	100	94	0
DNA	106	0	18	100	113

100 plaque-forming units of intact phage per ml were added to heart infusion broth containing 0.002 M $CaCl_2$ and 0.01 M $MgCl_2$ and divided into 0.5 ml samples. A given sample was incubated for 30 minutes at 37°C with one of the above specified enzymes (50 μg per ml) or phage antiserum. Duplicate samples were made in which 100 plaque-forming units of D29 DNA were substituted for intact phage. At the end of the preincubation period, the mixtures were added to soft agar seeded with 607, incubated, and observed for plaque formation.

TABLE III

The Effect of DNAase on D29 DNA Infectivity

DNAase	PFU/ml
$\mu g/ml$	
0	114
1	0
10^{-1}	0
10^{-2}	0
10^{-3}	6
10^{-4}	27
10^{-5}	120
50	0
0	2270

DNA was incubated with enzyme in tris buffer containing 0.02 M $MgCl_2$ at pH 7.0 for 30 minutes at 37°C; the samples were then assayed for infectivity.

tained. We soon found that the trypsin we were using was contaminated with DNAase. In fact, a given lot of trypsin which was being used as routine in our laboratory to detach from glass and to disperse tissue cell cultures, produced a 24 per cent increase in absorbance at 260 mμ of a solution of calf thymus DNA (50 μg trypsin/ml DNA).

The inactivation of DNA by RNAase was investigated further. Boiling of the RNAase to inactivate possible traces of contaminating DNAase did not remove the deleterious effect. Experiments were carried out to determine whether or not RNAase was hydrolyzing the DNA.

A solution of DNA was divided into three samples. To one sample DNAase was added (50 μg/ml); RNAase (50 μg/ml) was added to the second sample; and an equal volume of buffer was added to the third or control sample. Each sample contained $MgCl_2$ at a final concentration of 0.01 M. The mixtures were incubated at 37°C for 30 minutes and then analyzed in the Beckman spectrophotometer.

Fig. 1 shows the results. The hyperchomic effect resulting from hydrolysis of the polynucleotide chain of DNA by DNAase is lacking in the sample incubated with RNAase. As a matter of fact, the absorbance at 260 mμ is slightly lower in the sample containing RNAase than in the control sample. With a second DNA preparation there was a 10 per cent drop in absorbance at 260 mμ upon addition of RNAase. Both reactions, the one with DNAase leading to an increase in absorbance of 260 mμ and the one with RNAase leading to a decrease in absorbance, proceeded very rapidly being virtually complete within 3 or 4 minutes. These experiments suggest that RNAase is complexing with DNA in a manner analogous to that of certain other basic proteins, and that the resultant complex is not infectious.

The Effect of Calcium on the Infectivity of D29 DNA.—The influence of ionic environment on plaque formation by D29 was studied.

Bacteria were grown in heart infusion broth containing Ca^{++}; cells in the log phase (at the end of 4 hours' incubation) were centrifuged, washed in nutrient broth, and resuspended in nutrient broth only and in the same broth to which various cations had been added. Approximately 100 PFU of DNA were added per ml of culture. The cultures were placed on a wrist-action shaker and incubated at 37°C. At various times, samples were withdrawn and plated.

The results are recorded in Table IV. In nutrient broth only there is no increase with time in the number of plaques formed; at the end of 4 hours' incubation only 80 plaques are produced. In the presence of Ca^{++} there is a significant increase in the number of plaques. None of the other cations tested including Mg^{++}, shown here, Na^{+}, K^{+}, or Mn^{++} promoted an increase in plaque formation by D29 DNA; apparently only Ca^{++} has the capacity to promote the entry of DNA into the host cell where it initiates an infection with the resultant

increase, with time, in numbers of plaque formers. Plaque formation by DNA is somewhat diminished in the presence of citrate.

Rate of Reaction Between DNA and 607.—A time-course analysis of the production of infective centers by phage DNA was made.

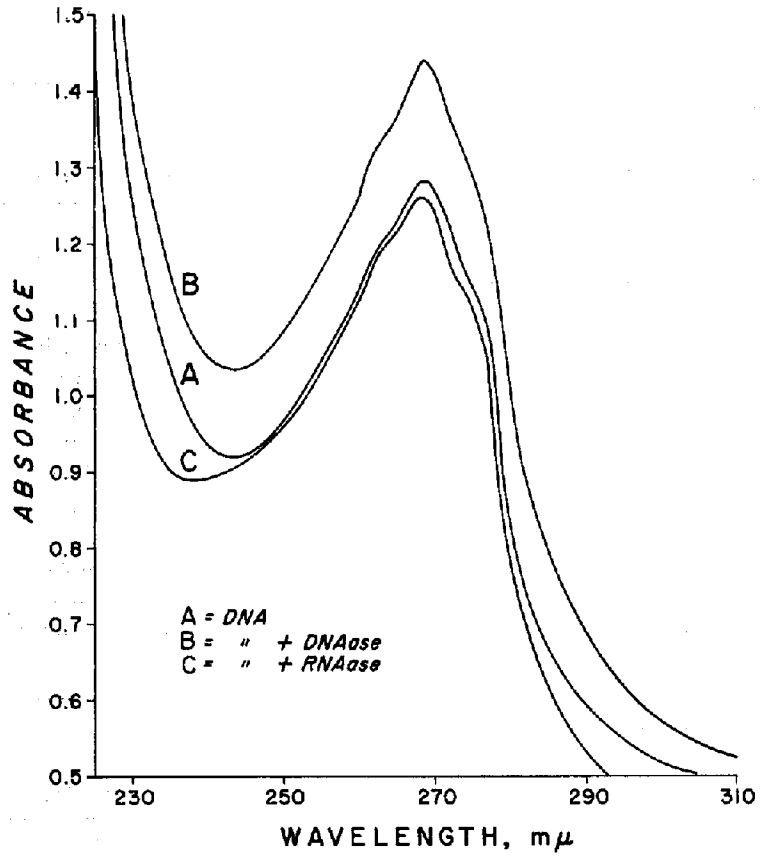

FIG. 1. D29 DNA in tris buffer at pH 7 containing 0.01 M $MgCl_2$ was divided into 3 equal samples. DNAase was added to one sample (50 μg/ml), RNAase to one (50 μg/ml), and an equal volume of buffer to the third sample. The samples were incubated at 37°C for 30 minutes and analyzed in a Beckman DK automatic spectrophotometer.

An average of 272 PFU of DNA per ml was added to a log phase culture of 607 in heart infusion broth containing 0.002 M $CaCl_2$ and incubated at 37°C on a wrist-action shaker. At intervals, a sample of the mixture was withdrawn and plated directly; an additional sample taken simultaneously, was preincubated with DNAase for 10 minutes and then plated.

Table V shows the results. In the 1st column, the number of infectious centers produced by DNA as a function of time are tabulated. There is no significant

increase in the number of plaques formed up to 150 minutes of incubation, thus, no new phage has been synthesized before 150 minutes after addition of DNA. The results in column 2 show the effect of DNAase on survival of the plaque-forming activity of the DNA. After 30 minutes' incubation 5 PFU survived DNAase treatment. Only 2 per cent of the total PFU of the input DNA had apparently entered the bacteria where it was no longer susceptible to extra-

TABLE IV

The Effect of Ionic Environment on the Production of Plaques by D29 DNA

Culture	Salt added*	Plaques formed per ml of culture during incubation at 37°C				
		10 min.	1 hr.	2 hrs.	3 hrs.	4 hrs.
Washed 607 cells in nutrient broth plus DNA	None	92	63	81	64	80
	$CaCl_2$	132	118	120	515	34,000
	$MgCl_2$	109	88	54	76	175
	Na citrate	54	74	69	72	58
	$CaCl_2$ plus DNAase‡	0				0

* 0.002 M concentration.

‡ 50 μg per ml (plus $MgCl_2$; final concentration of 0.02 M).

TABLE V

Time Course in the Production of Infective Centers by D29 DNA

Time after addition of DNA*	Plaques produced per ml of culture		
	Culture only	Culture plus DNAase‡	
		No.	Survival
min.			*per cent*
10	215	0	0
30	270	5	2
45	310	11	4
60	305	11	4
75	205	30	11
90	285	33	12
120	315	92	34
150	430	240	—
180	TNTC§	TNTC	—

* The average number of plaque-forming units of DNA added per ml of culture: 272.

‡ Samples of the culture were withdrawn at the specified times; 1 sample was plated directly and 1 was preincubated with DNAase (50 μg per ml of culture plus 0.01 M $MgCl_2$) for 10 minutes at 37°C and then plated.

§ Plaques were too numerous to count.

cellular DNAase. The per cent of DNAase survivors remained at a very low level up through 60 minutes; even after 120 minutes' incubation, only 34 per cent of the activity of the input DNA survived the action of DNAase. From these results, it would appear that the time between the addition of DNA and the appearance of phage progeny is considerably longer than the 65 minutes recorded for the appearance of progeny from intact D29 (16).

DISCUSSION

Although the results of our experiments are not definitive they indicate that the time course of a one-step growth cycle initiated by DNA is considerably longer than that produced by intact phage. The reactions between bacterial cell wall and the phage tail end-piece, culminating in DNA injection (17) must greatly accelerate the rate at which DNA reaches critical sites inside the cell. Infection produced by intact phage, where DNA is packaged in protein, is apparently a much more efficient process than that initiated by naked DNA.

The calcium requirement for infection by D29 DNA should have been anticipated. Earlier experiments had indicated that although both magnesium and calcium as well as relatively high concentrations of either sodium or potassium, could promote increased absorption of D29 phage, calcium only had the capacity to increase both absorption and productive infection (16). It was found that one of the characteristics of D29 phage–607 cell interactions was abortive infection; *i.e.*, many phage particles absorbed to the bacteria which did not produce plaques. If calcium were present in the medium then the number of plaques produced by a given number of absorbed phages was significantly increased. None of the other 8 cations tested promoted productive infection. It was suggested that calcium was functioning in the penetration of nucleic acid into the bacteria, a role for this cation which the present experiments support.

Exactly how calcium functions in the successful infection by D29 DNA is not known. In almost all enzymic reactions involving phosphorylated compounds there are specific metal ion requirements. In the majority of cases, these requirements are met by rather low concentrations of one or more divalent cations or somewhat higher concentrations of monovalent cations. In certain reactions there may be a requirement for one specific ion, and other ions in the same series are not only non-functioning but may even be inhibitory. Information regarding the mechanistic role of these ions is purely speculative. In the case of D29 one might envisage Ca^{++}, either through ionic or covalent bonding, or both, as (*a*) forming a bridge between the negatively charged DNA and host cell, (*b*) chelating with DNA and an enzyme located on the cell surface, suitably orienting both enzyme and substrate, or (*C*) as activating an enzyme concerned with getting the DNA through the cell wall barrier.

Calcium also functions in certain transformations by pneumococcal DNA (18) and, as measured by blending experiments, in the entry of T5 DNA into *E. coli* (19). In the latter, magnesium can replace calcium, though less effectively.

The efficiency of plating (eop) of D29 DNA was extremely low. A phage suspension with a titer of 9×10^{12} PFU per ml yielded a final DNA preparation with a titer of 10^{9} PFU per ml. Thus, more than 99.9 per cent of the plaque-forming activity was lost by the phenol extraction. To date, we have not made exhaustive search for methods to improve the eop of D29 DNA. However, some of our experiments are worth mentioning.

In attempts to determine the conditions most favorable for the phenol extraction of infectious D29 DNA, various procedures were tried. Extractions were carried out by 1 to 5 cycles of shaking for from 1 to 5 minutes each. Residual phenol was removed by ether extraction or, alternatively, by dialysis against tris buffer at pH 7 or against citrate-saline. DNA was precipitated out from the final aqueous phase extracts by the addition of an equal volume of 2-ethoxyethanol. The fibrous precipitate was taken up either in tris buffer or in citrate-saline and again dialyzed against the homologous diluent. In the final analysis, none of these procedures appeared to be superior to another. Paramount to the preparation of highly active DNA was a substantial amount of a high titered phage suspension (5 to 10 ml with a titer of $10^{12.5}$ PFU per ml). DNA concentration during the extraction procedure greatly influenced the activity of the final product. Mandel and Hershey (20) showed that DNA from T2 was extremely susceptible to breakage by hydrodynamic shear. The susceptibility to breakage was strongly concentration dependent, being greater the lower the concentration. Even pipetting was important in handling their DNA preparations; when diluted, they were transferred by pouring to avoid degradation by shear. These data give some indication of why the more concentrated the D29 DNA, the more stable it was with respect to length of time and numbers of extractions in phenol.

One factor which should be emphasized is the danger of intact phage surviving the phenol extraction. Careful attention must be paid to ruling out the possibility of one or two contaminating phages or more likely, clumps of phages, in each lot of DNA which is prepared. This, of course, can be done by the use of phage antiserum and DNAase treatment.

This system appears to be unique in that the host bacteria are susceptible to D29 DNA without prior treatment such as conversion to protoplasts, being made competent, or altering the cell surface with helper phages (2–6). Whether this is characteristic of 607 or of D29 DNA is not known. An investigation of the host range of D29 DNA which is underway, indicates that the interaction of the DNA with 607 leading to phage production is highly specific.

SUMMARY

DNA extracted from D29 mycobacteriophage produced plaques when plated on *Mycobacterium smegmatis* 607. The host bacterium did not require alternation such as conversion to protoplasts; cells susceptible to infection with intact phage were susceptible to DNA.

The bases found in calf thymus DNA constituted the bases of D29 DNA, adenine being paired with thymine and guanine with cytosine. The dissymmetry ratio (A + T/G + C) was 0.56, and the buoyant density in CsCl was 1.722 with a GC content of 63.77 per cent.

The efficiency of plating of the DNA is very much lower than that of intact D29, and it penetrates the host at a slower rate. As does intact phage, D29 DNA requires calcium ions for productive infection of 607.

D29 DNA is significantly inactivated by incubation with RNAase, but the inactivation probably results from a complexing with the DNA rather than from enzyme hydrolysis.

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