Deoxyribonucleic Acid Hybridization Analysis of the Defective Bacteriophage Carried by Strain 15 of *Escherichia coli*

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Evidence from several laboratories indicates that strain 15 of *Escherichia coli* is lysogenic for a defective phage. When lysates from induced cultures were centrifuged in CsCl, three bands were obtained. In order of decreasing density, these bands contained tailless particles, complete phages, and a second band of complete phages, in a ratio of 65.7:28.6:5.7. Reassociation rate measurements were used to establish that the molecular weights of the deoxyribonucleic acid (DNA) species from the phages in the first two bands are similar. A smaller genome is postulated in the complete phages from the minor band. Hybridization experiments revealed extensive homology between the DNA species from all three phage bands, thus suggesting that the complete and tailless particles are not different at the genetic level. The DNA from each phage band was also shown to hybridize almost completely with DNA from either *E. coli* 15T⁻ or a reportedly cured derivative of 15T⁻. In contrast, only about 25% of each phage DNA was able to react with DNA from *E. coli* strains B and K-12 C-600.

Strain 15 of *Escherichia coli* has been shown to be lysogenic for an apparently defective phage by several laboratories (8, 9, 11, 16, 18). Frampton and Brinkley (9) and Endo et al. (8) reported that, after induction of the phage, cell lysates contain seemingly complete phages, tailless phages, and many free phage tails. Lee et al. (15) later demonstrated that the bacteriostatic activity associated with the phages is a property of the complete viruses and not of the tailless particles.

The guanine-cytosine content of phage 15 deoxyribonucleic acid (DNA) was estimated to be 46% by Frampton and Mandel (10). These investigators also measured the molecular weight of the phage DNA by sedimentation through neutral sucrose gradients and obtained the value 3.3×10^7 . Lee et al. (15), using electron microscopy, determined that the DNA of the tailless phages has a molecular weight of 2.7×10^7 . On the basis of DNA-DNA hybridization, Cowie and Szafranski (7) showed that phage 15 and *E. coli* contain some common DNA base sequences. They found no complementarity between phage 15 DNA and that of lambda phage. Likewise, Frampton and Mandel (10) were unable to detect any homology between *E. coli*

ribosomal ribonucleic acid (RNA) and phage 15 DNA. Recently, Ikeda et al. (13) reported that DNA from the complete phages does not hybridize with that from the tailless species and consequently proposed that *E. coli* 15 is actually lysogenic for two different defective phages.

The present investigation was concerned with the following aspects of the data summarized above.

(i) Prior to the report of Ikeda et al. (13), it had been generally assumed that the complete and tailless phages were different stages of assembly of the same virus. In view of the fact that cell lysates contained many free phage tails, this assumption seemed quite reasonable. Therefore, as a first step in exploring further the possibility of double lysogeny in *E. coli* 15, it seemed necessary to determine if the method used to purify the phages has any effect on the distribution between complete and tailless particles.

(ii) To determine the extent of the genetic relationship between the complete and tailless phages, the amount of homology between their DNA species was measured in hybridization experiments. In addition, the information content and presumably the molecular weight of the phage genomes in each type of particle were

estimated on the basis of their reassociation rates.

(iii) Most, if not all, temperate bacteriophages contain genomes that exhibit extensive homology with the chromosome of the host bacteria (5). In the present case, this phenomenon was examined by determining the degree of homology between the DNA species from each type of phage and those from strains of *E. coli* which, with respect to the phage 15, were lyso-genic, reportedly cured, and nonlysogenic.

MATERIALS AND METHODS

Bacterial strains and culture media. *E. coli* 15T⁻ and its cured derivative JG151 were originally acquired from A. Yudelevich and have been maintained in our laboratory for several years. Thymine is an absolute growth requirement for both organisms. A uracil-requiring strain of *E. coli* B and a uracil-requiring strain of *E. coli* K-12 C-600 were obtained, respectively, from R. Trimble, Division of Laboratories and Research, New York State Department of Health, and D. E. Kohne, Department of Terrestrial Magnetism, Carnegie Institution of Washington. The cells were routinely grown in C minimal medium (17) supplemented with thymine (2 µg/ml) for *E. coli* 15T⁻ and JG151 and with uracil (20 µg/ml) for *E. coli* B.

Purification of bacterial DNA. Cells were grown to titers of 4×10^8 to 5×10^8 /ml, collected by centrifugation, and suspended in $2\times$ SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate). Lysis was achieved by adding sufficient 25% sodium lauryl sulfate (SLS) to give a final concentration of 1 to 2%, shaking vigorously, and then incubating for 30 min at 60 C. The lysate was chilled to 4 C, an equal volume of buffer-saturated phenol was added, and the mixture was shaken by hand for 20 min in an ice bath. The buffer consisted of 0.1 M tris(hydroxymethyl)amino-methane (Tris), 0.1 M NaCl, and 1% SLS (pH 9.0) and was added to the phenol immediately before use. The solution was centrifuged at $15,000 \times g$ for 10 min, the aqueous top layer was removed, and the DNA was precipitated by addition of twice the volume of cold 95% ethanol. The DNA was collected by spooling on a glass rod and dissolved in $0.01\times$ SSC. When the DNA was in solution, sufficient $10\times$ SSC was added to bring the concentration to $1\times$ SSC. The solution then was incubated for 1 hr at 37 C with 50 µg of pancreatic ribonuclease per ml (Worthington Biochemical Corp., Freehold, N.J.; heated at 100 C for 10 min at 1 mg/ml to destroy deoxyribonuclease before use). Finally, Pronase (Calbiochem, Los Angeles, Calif.; self-digested for 2 hr at 37 C at 5 mg/ml) was added to 100 µg/ml, and the incubation was continued for another hour. A second phenol extraction was then carried out exactly as described above. Residual phenol was removed by ether extraction. The purity of the final DNA preparation was checked by examining its absorption spectrum (Gilford model 2400 spectrophotometer) between 230 and 300 nm and by determining its hyperchromicity as a result of thermal denaturation at 260 nm in $1\times$ SSC.

The ratio of absorbances at 260 and 280 nm was 1.8 to 1.9, and hyperchromicity was always over 30%. In addition, sharp melting profiles with no measurable increase in optical density below 70 C were obtained.

Radioactive *E. coli* 15T⁻ DNA was prepared as above, except that the thymine supplement consisted of thymine-2-¹⁴C (New England Nuclear Corp., Boston, Mass.) at 51 mCi/mmol.

Induction of virus. *Escherichia coli* 15T⁻ was grown at 37 C with forced aeration to 4×10^8 to 5×10^8 cells/ml; mitomycin C (Calbiochem) was then added to 1.5 µg/ml, and incubation was continued until cell lysis was completed. Cell growth and lysis were followed at 620 nm (Bausch & Lomb, Spectronic 20 spectrophotometer). Radioactive phage DNA was prepared by growing the culture to 6×10^8 cells/ml, diluting to 3×10^8 cells/ml with an equal volume of warmed C medium containing no thymine supplement, and allowing growth to resume until the cells reached 4×10^8 to 5×10^8 /ml. Mitomycin C (1.5 µg/ml) and thymine-2-¹⁴C (51 mCi/mmol) were added, and growth and lysis followed as previously described.

When it was desired to examine the proportion of complete and tailless phages in undisturbed cell lysates, samples were removed at the completion of lysis for centrifugation in CsCl gradients. Chloroform was then added (20 ml/liter of lysate), and the culture was shaken vigorously and cooled to 4 C. The remaining cells and cell debris were removed by two cycles of slow-speed centrifugation ($10,000 \times g$ for 15 min at 4 C). Two methods of concentrating the phages from large lysates were employed. In the first, phages were collected by centrifugation ($23,500 \times g$ for 180 min at 4 C), and the pellets were suspended gently in C medium minus glucose. The second method involved precipitation of the phages with PEG 6,000 (polyethylene glycol; Matheson, Coleman, and Bell) by the procedure of Yamamoto et al. (19). PEG was used at a concentration of 10% (w/v). The phages were ultimately suspended in a small volume of $2\times$ SSC (0.5 to 1.0 ml) and then diluted $10\times$ with C medium minus glucose. Regardless of the method of concentration employed, final purification was by CsCl density gradient centrifugation.

CsCl (Henley and Co.) was added to phage suspensions to give a starting density of 1.47 g/ml. The solution was dispensed in 4.6-ml amounts into nitro-cellulose tubes, and paraffin oil was added to fill the tubes completely. Centrifugation was for 24 hr at 5 C and 34,000 rev/min in a type 40 rotor, Spinco L2-65B. Material was removed from the tubes afterward in either of the following two ways. (i) To study the composition of each phage band and the distribution between complete and tailless particles, tubes were drained from the bottom with a Buchler Universal piercing unit. (ii) When large quantities of phages were purified, the paraffin oil was carefully drawn off with a Pasteur pipette, and a 5.0-ml syringe fitted with a long cannula was used to remove each band with minimal disturbance of the gradient. Individual bands were further purified by rebanding in CsCl.

Composition of virus bands. To determine the type

of particle in the phage bands, the peak fraction of each band was dialyzed against 1% ammonium acetate, a series of dilutions was made with distilled water, and portions were added to carbon-coated collodion films. The preparations were then negatively stained with 0.5% sodium tungstate and examined with a Siemens 1 electron microscope.

The proportion of complete and tailless phages was ascertained from the distribution of radioactive DNA among the bands. To each fraction of approximately 0.07 ml, 1.0 ml of a solution containing 0.09 M $MgCl_2$ and pancreatic deoxyribonuclease I (90 $\mu g/ml$, Worthington Biochemical Corp.) was added, followed by incubation at 37 C for 1 hr. Samples were chilled to 4 C and precipitated by addition of an equal volume of cold 10% trichloroacetic acid. After the samples were held in the cold for 30 min, the precipitates were collected on filters (Whatman, GF/A) which were subsequently dried and counted. Counting was done in a Beckman LS-250 liquid scintillation spectrometer with a toluene-based cocktail containing 4.2 g of 2,5-diphenyloxazole and 106 mg of dimethyl 1,4-bis-2-(5-phenyloxazoly)-benzene per liter (both, Packard Instrument Co. Inc., Downers Grove, Ill.).

Purification of virus DNA. Each of the purified phage bands was incubated in 0.09 M $MgCl_2$ with pancreatic deoxyribonuclease (90 $\mu g/ml$) for 1 hr at 37 C. DNA was then extracted with buffer-saturated phenol as described for bacterial DNA. Neither ribonuclease nor Pronase treatment was found essential in the purification of phage DNA. Criteria of purity were as described above.

Hybridization experiments. Reassociation rate measurements of the DNA from each phage band were carried out as described by Britten and Kohne (3). The procedure was as follows.

(i) DNA in $0.01 \times SSC$ was fragmented by passage through a needle valve with a 50,000-psi pressure differential (American Instrument Co.; no. 46-13715 pump, no. 44-16106 valve). (ii) Salt concentration was adjusted by dialysis against 0.12 M phosphate buffer (PB) consisting of equimolar concentrations of Na_2HPO_4 and NaH_2PO_4 . (iii) DNA was heat-denatured, brought to 60 C, and incubated at this temperature for the duration of the experiment. (iv) To determine the extent of nonspecific binding to the column, a sample was removed at the beginning of incubation and passed over hydroxylapatite at 60 C. Additional samples were removed periodically and passed over a column of hydroxylapatite (Bio-gel HT; Bio-Rad Laboratories, Richmond, Calif.) at 60 C to determine the amount of DNA reassociated as a function of Cot (defined below). Thorough washing of the column with 0.12 M PB at 60 C removed all single-stranded DNA. Double-stranded DNA was then eluted by washing with 0.4 M PB at 60 C. (v) The percentage of the total trichloroacetic acid-precipitable radioactivity recovered in the 0.4 M PB washes was taken as a measure of the amount of DNA reassociated.

In all experiments, temperature (60 C), salt concentration (0.12 M PB), and fragment size (400 to 500 nucleotides) were controlled. The extent of reassocia-

tion for each DNA was therefore determined by the parameter Cot (DNA concentration $\times$ time, expressed as moles of nucleotides $\times$ seconds per liter), and the data are presented in terms of the fraction of DNA reassociated as a function of Cot. A Cot of 1 (mole $\times$ sec/liter) is equivalent to incubating 83 μg of DNA per ml for 1 hr (3).

To determine the extent to which there are common nucleotide sequences among the different phage bands and also between each band and several strains of *E. coli*, hybridization reactions were carried out as follows. (i) DNA was sheared as described above. (ii) To ascertain the proportion of the phage DNA from one band complementary to that from the phage of another band (or to *E. coli* DNA), a sample was prepared containing a small amount of radioactive DNA of one of the species of interest and a large quantity of unlabeled DNA of the second species. The ratio of unlabeled to labeled DNA was sufficiently large that a time for the reaction could be chosen which would allow most of the unlabeled DNA to reassociate but would permit little self-reaction of the labeled DNA. Under these conditions, the labeled DNA would appear in hybrids only if it contained some nucleotide sequences complementary to those in the bulk, unlabeled DNA. A control containing only the small quantity of labeled DNA was always incubated separately to establish the actual extent of self-reassociation of labeled DNA which occurred in each experiment. (iii) At the end of the incubation period, the samples were passed over a column of hydroxylapatite, and the column was washed with 0.12 M PB at 60 C to remove single-stranded DNA and then with 0.4 M PB at 60 C to elute double-stranded DNA. The distribution of radioactive DNA between double- and single-stranded fractions provided a measure of the amount of the labeled DNA which had reacted to form a hybrid. The amount of labeled DNA hybridized in the experimental sample, beyond that found in the control, was indicative of the proportion of the radioactive DNA species that consisted of base sequences held in common with the unlabeled DNA.

In all of the hybridization experiments, radioactivity was determined by chilling samples to 4 C, adding 25 μg of calf thymus DNA as carrier, and precipitating with cold trichloroacetic acid. The precipitates were collected on filters (Millipore HA), which were dried and counted as described previously.

RESULTS

Centrifugation in a $CsCl$ density gradient of a portion of the lysate from an induced culture of *E. coli* 15T⁻ yielded the data shown in Fig. 1. Band 1 was found to consist of tailless particles, band 2 contained complete phages, and band 3 was composed of phages morphologically identical to those in band 2. Similar data regarding both the number and composition of the phage bands were reported by Frampton and Mandel (10) for *E. coli* 15 TAU.

The proportion of the lysate represented by

each type of phage, as well as the effect of different methods of virus concentration on the distribution of phages between bands, is presented in Table 1 on the basis of the percentage of total ^{14}C -thymine counts recovered in each band. Also included is the result of passing a cell lysate through a 26-gauge hypodermic needle three times at maximum finger pressure before centrifugation. It is evident from these data that the distribution of phages between the bands is unaffected by any of the treatments employed. Consequently, there is little need for concern over the possibility of cross-contamination between bands occurring as a result of shear during the purification process.

The rate of reassociation of the DNA from each of the phages is illustrated in Fig. 2. The DNA from the tailless phages and that from the band 2 complete phages reassociate at similar rates, whereas the DNA from the band 3 complete particles reassociates somewhat faster.

FIG. 1. Distribution in a CsCl gradient, based on ^{14}C counts, of phages from a crude lysate of *E. coli* 15T⁻. (●) Phage bands, (○) CsCl density gradient.

TABLE 1. Effect of different treatments on the distribution of phage particles in CsCl gradients, based on the percentage of total ^{14}C -thymine counts recovered in each band

Treatment before centrifuging in CsCl	Total ^{14}C -thymine counts recovered in (%)		
	Band 1	Band 2	Band 3
Unconcentrated lysate..	65.7	28.6	5.7
Lysate sheared three times (no. 26 needle).	67.3	27.1	5.6
Polyethylene glycol precipitation.....	63.6	28.1	8.2
Pelleted in Spinco type 30 rotor at 20,000 rev/min.....	69.7	23.8	6.5

FIG. 2. Reassociation rates of the DNA species from the three phage bands found in CsCl gradients. In all cases, the specific activity of the DNA was approximately 40,000 counts per min per μg . Actual concentrations of DNA used for rate measurements were 7.6 $\mu\text{g}/\text{ml}$, band 1; 8.6 $\mu\text{g}/\text{ml}$, band 2; 10.2 $\mu\text{g}/\text{ml}$, band 3. The $\text{Cot } \frac{1}{2}$ was 0.056 for bands 1 and 2 and 0.038 for band 3. Solid lines represent ideal second order reaction rates calculated from the measured values of $\text{Cot } \frac{1}{2}$.

In organisms such as bacteria and viruses, which contain (for the most part) series of nucleotide sequences which occur only once per genome, the value of Cot at which one-half of the DNA has reassociated ($\text{Cot } \frac{1}{2}$) is directly related to genetic complexity (see reference 3). Therefore, the molecular weight of an unknown DNA can be calculated from its $\text{Cot } \frac{1}{2}$ if there is available for comparison a DNA whose molecular weight is known and whose $\text{Cot } \frac{1}{2}$ has been measured under conditions similar to those used for the unknown DNA. Cairns (4) has estimated the size of the *E. coli* genome to be 2.8×10^9 ; the $\text{Cot } \frac{1}{2}$ of *E. coli* DNA under the conditions employed in this investigation is 4.0 (2). Likewise, the molecular weight of simian virus 40 (SV40) DNA has been reported to be in the range 2.3×10^6 to 3.5×10^6 (1, 20) and its $\text{Cot } \frac{1}{2}$ to be 5.8×10^{-8} (12). If there is no repetition, the DNA species from the band 1 and band 2 phages ($\text{Cot } \frac{1}{2} = 0.056$) have a molecular weight of 3.92×10^7 compared to *E. coli*. When 3.0×10^6 is used for the size of the SV40 genome, a value of 2.9×10^7 for the 15 phages is obtained. Either number is in relatively good agreement with previously reported measurements (10, 15). The genome size of the band 3 phage ($\text{Cot } \frac{1}{2} = 0.038$) was found to be 2.66×10^7 relative to *E. coli* and 1.97×10^7 relative to SV40. The fact that the complete phages in bands 2 and 3 apparently contain different amounts of DNA provides a basis for the discrepancy in their buoyant densities.

Experiments to determine the extent to which the DNA species from the three phage bands

share common nucleotide sequences are summarized in Table 2. On the basis of reciprocal experiments, about 80% homology was revealed between the DNA species from the tailless band 1 and the complete band 2 viruses. A similar degree of homology was also found when labeled band 3 DNA was allowed to react with unlabeled DNA from bands 1 and 2. However, in reciprocal experiments, only 70% of the labeled DNA from the band 1 and 2 phages reacted to form hybrids with unlabeled band 3 DNA. This lack of reciprocity in comparative hybridization experiments also suggests that the phages in band 3 possess a smaller genome than do those in bands 1 and 2.

In contrast to the conclusions of Ikeda et al. (13), the data presented in Table 2 are consistent with the idea that only one kind of defective phage resides in *E. coli* 15 and that the complete and tailless particles merely represent different degrees of assembly.

The ability of the DNA from each of the phage bands to hybridize with the DNA from four strains of *E. coli* is shown in Table 3. In these experiments, the unlabeled *E. coli* DNA was incubated to $Cot = 76$ (i.e., 95% reassociation). In the case of a bacterium lysogenic for a phage, such as *E. coli* 15T⁻, unlabeled phage DNA is present as part of the large quantity of unlabeled bacterial DNA used in the hybridization reaction. If it is assumed that there is only one copy of the phage genome per host chromosome, then each phage gene will be present at the same concentration as each host gene and hence will reassociate with the same kinetics as the bulk host DNA. If the actual quantity of phage DNA in the unlabeled preparation is greater than the small amount of labeled phage DNA added, it would be expected that almost all of the labeled

TABLE 3. Sequence homology between the DNA from each of the phage 15 bands and that from four strains of *Escherichia coli*

Small amt of ¹⁴ C-labeled DNA from phage band	Labeled phage DNA retained on hydroxyapatite after incubating <i>E. coli</i> DNA to $Cot = 76$ (95% reassociation) ^a (%)			
	<i>E. coli</i> 15T ⁻	<i>E. coli</i> JG151	<i>E. coli</i> B, uracil ⁻	<i>E. coli</i> K-12 C 600
1	89	92	33	28
2	91	92	29	23
3	86	81	19	19

^a Data have been corrected for self-reaction of labeled phage DNA to the extent of 7, 5, and 9% for bands 1, 2, and 3, respectively.

DNA would react to form hybrids during the time required for the host DNA to reassociate. In the present experiments, the ratio of unlabeled host DNA to labeled phage DNA was such that the aforementioned conditions were met. Therefore, it was not surprising to find that essentially all of the DNA from each phage band was able to hybridize with the DNA from *E. coli* 15T⁻. It had not been anticipated, however, that the DNA from the phages would also be able to react almost completely with the DNA from JG151, a derivative of 15T⁻ believed cured of the phage (14). The result with JG151 focuses attention on the fact that, although the concept of curing implies a methodology for selecting a strain of bacteria that no longer carries the phage genome, almost all tests for the presence of a prophage depend on observations of known properties which require expression of one or more viral genes (inducibility, immunity to superinfection, recombination with a genetically marked superinfecting phage). When similar criteria are applied to JG151, this strain appears to be cured, as it is more resistant to thymineless death than is 15T⁻, and it produces neither phages nor an increased methylase activity characteristic of the phage when subjected to a variety of inducing treatments (14, 21). Nevertheless, the hybridization data show that JG151 still carries the phage genome and hence remains lysogenic. This result emphasizes the importance of the criteria upon which decisions regarding lysogeny are based.

Approximately 25% of the DNA from the phages was capable of forming hybrids with DNA from the nonlysogenic strains *E. coli* B and K-12 C-600, neither of which is a derivative of strain 15. Cowie and McCarthy (6) have shown that 33% of the genome of lambda phage is homologous to nonlysogenic *E. coli*. Cowie and

TABLE 2. Sequence homology between the DNA species of the three phage 15 bands

Small amt of ¹⁴ C-labeled DNA from phage band	Labeled DNA retained on hydroxyapatite after incubation with a large quantity of unlabeled DNA from the phage band ^a (%)		
	1	2	3
1		83	69
2	77		70
3	78	79	

^a Data have been corrected for self-reaction of labeled DNA to the extent of 4, 3, and 8% for bands 1, 2, and 3, respectively. In each instance, unlabeled DNA was incubated to $Cot = 0.52$, which corresponds to 90% reassociation of bands 1 and 2, and 94% reassociation of band 3.

Brenner (5) reported similar findings for the coliphages $\phi 80$ and 434 and for the salmonella phage P22. The present results are therefore consistent with the earlier data which suggest that relatively extensive homology between the genome of a temperate phage and the chromosome of its host is a characteristic of lysogeny.

To determine the amount of *E. coli* DNA complementary to that of the phage, reciprocal experiments were carried out using a small amount of radioactive DNA from *E. coli* 15T⁻ (1 μ g/ml, 22,000 ¹⁴C counts per min per μ g) and a large quantity of unlabeled phage DNA (126 μ g/ml; DNA species from three bands were pooled). It was found that 1 to 3% of the *E. coli* genome is complementary to that of the phage. The phage genome carried by *E. coli* 15T⁻ represents slightly more than 1% of the bacterial chromosome. In addition, about 25% of the phage DNA is homologous to that from nonlysogenic strains of *E. coli*. Consequently, assuming one phage genome per host chromosome, approximately 1.5% of the DNA from the lysogenic strain 15T⁻ would be expected to be homologous to phage DNA. Although the expected value is within the range determined experimentally, the possibility of two phage genomes per host chromosome cannot be excluded.

DISCUSSION

Mennigmann (16) reported that the complete phages found in lysates of *E. coli* 15T⁻ appear to be vulnerable to separation at the point where the head and tail join. Consequently, it was rather surprising to find that none of the treatments to which the phages were subjected in the present study altered the distribution between complete and tailless species. The stability of the complete particles minimizes the possibility that the purified tailless phages were unwittingly contaminated with breakdown products of the complete species during the purification process.

All of the data from the experiments described herein are consistent with the idea that *E. coli* 15 is lysogenic for only one defective phage. As acknowledged previously, this result is contrary to the report of Ikeda et al. (13) that little or no homology exists between the DNA species of the complete and tailless phages. Although the possibility exists of variation arising in subcultures of the same bacterial strain maintained for long periods of time in different laboratories, to invoke such an explanation is rarely satisfying. At the moment, however, there is no obvious alternative explanation which will permit the two sets of data to be reconciled.

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