Lack of Nucleotide Sequence Relationship Among the Temperate Bacteriophage SPO2, *Bacillus subtilis*, and Virulent Bacteriophages β 3 and β 22

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Received for publication 18 August 1969

Radiolabeled phage SPO2 fragments were tested for the ability to form interspecies deoxyribonucleic acid (DNA) duplexes with DNA from *Bacillus subtilis* and from phages β 3 and β 22. No reassociation, above control values, occurred between the DNA of phage SPO2 and that of its host or either of the virulent *B. subtilis* phages. It appears that extensive nucleotide sequence similarities between portions of host and phage DNA species are not necessary for the formation of the lysogenic state. The thermal elution profile of reassociated SPO2 DNA exhibited a normal distribution, indicating no heterogeneity in base composition. This differs from temperate enterophages whose DNA molecules contain regions of differing base composition.

Integration of temperate phage deoxyribonucleic acid (DNA) into the host chromosome at a specific site(s) suggests some type of recognition between the phage and bacterial genome at the integration site. Experiments with the use of the DNA-agar method demonstrated that at least one-third (1, 7) of the λ chromosome reassociated with DNA from *Escherichia coli*. DNA preparations from the virulent T series phages exhibited little or no base sequence relationship with *E. coli* DNA (8, 27). Further DNA studies showed that temperate phages which attack coliform bacteria are related both to each other and to *E. coli* and *Salmonella typhimurium* (1, 8). Phages λ , 434, 434hy, P22, and ϕ 80 are all at least 20% related (1). Alternatively, DNA from phage P1, which does not integrate into the host genome, exhibits little reassociation with the DNA of *E. coli* (16).

The portion of the λ genome related to *E. coli* was originally thought to exist as a contiguous segment that was necessary for integration. This attractive hypothesis was disproven by experiments showing that regions of homology with *E. coli* were dispersed throughout the λ genome (6). Nevertheless, DNA molecules of temperate enterophages are capable of significant reassociation with *E. coli* DNA.

We, therefore, examined a lysogenic system other than the temperature enterophage-*E. coli* system for sequence similarities between phage

and host DNA. The *Bacillus subtilis*-*B. subtilis* phage system was chosen because this gram-positive sporeforming bacterium is a host for both virulent and temperate viruses and is essentially unrelated to *E. coli*. SPO2 (25), a temperate phage of *B. subtilis*, and two virulent *B. subtilis* phages, β 3 and β 22 (31, 32), were selected for use in these studies.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. *B. subtilis* strains 168S^r and W23S^r were used for production of unlabeled phage preparations. Strain 168S^r served as a host for phages SPO2 and β 22, and strain W23S^r was the host for β 3. Phage SPO2_{el}, a clear plaque-forming point mutant of SPO2 that produces lytic infection of the host, was used as the source of SPO2 DNA. SPO2_{el} will hereafter be referred to as SPO2. This phage was a generous gift from W. R. Romig. *B. subtilis* strains 168 thymine⁻ (thy⁻; from L. S. Baron) and 23 histidine⁻ (from F. Rothman) were used to prepare phage DNA labeled with ¹⁴C-thymine. *E. coli* strain 1485 thy⁻ (434hy) was the source of labeled 434hy DNA.

Media. M medium (32) was used for the growth of unlabeled cells and for the preparation of lysates of unlabeled phages β 3 and β 22. Cells to be used for the preparation of labeled DNA were grown in SCM medium (11) supplemented with 0.1% casein hydrolysate, 5.5×10^{-3} M glucose, and 5×10^{-3} M CaCl₂, but lacking phosphate and sodium citrate. SPO2 lysates were prepared by infecting cells growing in NY medium (25). Meat extract was used for the basal layer of phage assay plates. M medium containing 0.6% agar was used for the soft agar overlay

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to titer phages $\beta 3$ and $\beta 22$, and NY medium plus 0.6% agar was used to titer SPO2.

Labeling procedure and DNA preparation. Thymine-requiring auxotrophs were labeled with ^{14}C -thymine (5.65 $\mu\text{C}/\mu\text{mole}$; New England Nuclear Corp., Boston, Mass.). Cells were grown in medium containing ^{14}C -thymine (1 $\mu\text{C}/\text{ml}$, 2.2 $\mu\text{g}/\text{ml}$) to early stationary phase and collected by centrifugation. The pellets were resuspended in 0.01 M tris(hydroxymethyl)aminomethane (Tris; pH 7.2)-5 $\times 10^{-3}$ M MgCl_2 , plus 15% sucrose, and lysozyme (Worthington Biochemical Corp., Freehold, N.J.), was added to the suspensions to a final concentration of 1 mg/ml. The cells were incubated at 37 C for 15 min and disrupted with 2% sodium dodecyl sulfate. The DNA was purified by extraction with water-saturated phenol, followed by the method of Marmur (21). Final precipitates were redissolved in 0.14 M sodium phosphate buffer (PB, an equimolar mixture of NaH_2PO_4 and Na_2HPO_4 , pH 6.8). Unlabeled bacterial DNA was obtained from cultures in mid-logarithmic growth phase by the methods described above.

To obtain labeled phage DNA, cells were grown to a density of $2.5 \times 10^8/\text{ml}$ in unlabeled medium supplemented with 2 $\mu\text{g}/\text{ml}$ of thymine. The cells were sedimented, suspended in 0.1 volume of un-supplemented medium and infected at a multiplicity of 5 with $\beta 3$ or $\beta 22$. After allowing 5 min for phage adsorption, the suspension was diluted to its original volume with SCM medium plus ^{14}C -thymine (1 $\mu\text{C}/\text{ml}$, 2.2 $\mu\text{g}/\text{ml}$) and allowed to lyse. Alternatively, wild-type cultures infected in the same manner were labeled with ^{32}P -orthophosphate (New England Nuclear Corp.).

Cells growing in SCM medium were unable to support the growth of SPO2. Therefore, this phage was labeled with $\text{H}_3^{32}\text{PO}_4$ in cells growing in NY broth diluted 1:4 with saline. ^3H -labeled 434hy DNA was obtained by thymineless induction of *E. coli* 1485 thy⁻ (434hy) (20).

Phage lysates were treated with 5 $\mu\text{g}/\text{ml}$ of deoxyribonuclease (5 times crystallized, Worthington Biochemical Corp.) and 5 $\mu\text{g}/\text{ml}$ of ribonuclease (bovine pancreatic, Worthington Biochemical Corp.) for 2 hr at 37 C. The lysates were then centrifuged at $10,400 \times g$ for 10 min to remove cell debris. The supernatant fluid was centrifuged at $40,248 \times g$ for 1 hr in the 21 rotor of a Spinco model L preparative ultracentrifuge. The pellets were suspended in 0.1 M Tris-5 $\times 10^{-3}$ M MgCl_2 buffer. Phage DNA was extracted by the method of Davison and Freifelder (9).

DNA preparations were subjected to mechanical shear at 50,000 psi to produce fragments 400 to 500 nucleotides in length (4). The sheared DNA was then filtered through 0.45- μm Metrical filters (Gelman Instrument Co., Ann Arbor, Mich.). Labeled DNA fragments were denatured by heating or alkali treatment and further purified by passage through a hydroxyapatite (Biogel HT, BioRad Laboratories, Richmond, Calif.) column equilibrated with 0.14 M PB and held at 60 C. Material that was bound to the column under these conditions was discarded. This procedure decreases the "zero time" binding (label

bound to the column immediately after the DNA has been denatured).

DNA reassociation reactions. The fractionation of double- and single-stranded DNA on hydroxyapatite has been described (2). Double-stranded DNA in 0.14 M PB at 60 C binds to hydroxyapatite, whereas single-stranded DNA is not bound. In 0.4 M PB no DNA binds to hydroxyapatite. In the present experiments, thermally denatured, labeled DNA fragments were incubated at 60 C with a 1,000- to 7,500-fold excess of denatured, unlabeled DNA fragments in 0.14 M PB. The length of incubation was chosen so that unlabeled DNA fragments would show maximal reassociation, whereas the reassociation of labeled DNA fragments with one another would be minimal. In time course reassociation experiments, a series of reaction mixtures were incubated at 60 C for varying periods of time to assay per cent reassociation versus time of incubation. After incubation, the samples were quickly cooled and frozen until use. Each sample was subsequently thawed and passed through hydroxyapatite at 60 C in 0.14 M PB either in a column or by a batch procedure (3). The reassociated DNA was bound to hydroxyapatite. Unreacted DNA was removed in a series of washes with 0.14 M PB at 60 C. In thermal elution experiments, the bound DNA was removed by washing the column with 0.14 M PB at increasing 2.5 C temperature increments to 100 C, followed by two to three washes with 0.4 M PB. When only the percentage of reassociated DNA fragments was of interest, the hydroxyapatite was washed with 15-ml portions of 0.14 M PB as above, followed by one wash with 0.14 M PB at 95 C, one at 100 C, and finally three washes with 0.4 M PB.

Radioactivity assay. Samples from stock solutions and hydroxyapatite experiments were precipitated in 5% cold trichloroacetic acid in the presence of approximately 100 μg of herring sperm DNA or yeast RNA carrier. The precipitates were collected on membrane filters, placed in counting vials, and dried. A 15-ml amount of scintillation fluid was added, and the samples were counted in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Eluates from hydroxyapatite experiments in which ^{32}P -labeled DNA was used were placed directly into counting vials and assayed by Cerenkov counting (5).

RESULTS

Time course DNA reassociation reactions. Experiments were designed to measure the kinetics of DNA reassociation in *B. subtilis* and in SPO2 by monitoring the amounts of DNA that bound to hydroxyapatite at various times after initiation of the reaction. Reassociation rate is dependent upon genome size, and approximates an ideal second-order reaction (4), with the rate dependent upon the number of collisions between fragments of complementary nucleotide sequence. If DNA molecules of

different molecular weight are sheared to a specific size (400 to 500 nucleotides in these experiments), the number of different fragments will depend on the original size of the molecule. For example, a microorganism whose DNA is 10^9 daltons will have 100 times as many different fragments per molecule as a microorganism whose DNA is 10^7 daltons. Therefore, under identical conditions, DNA from the larger genome will take 100 times as long to reassociate.

Figure 1 shows a DNA reassociation curve for *B. subtilis* 168. The C_0t values plotted on the abscissa are units of nucleotide pair formation which are a function of the product of DNA concentration and time:

$$C_0t = [(\text{OD}_{260}/\mu\text{g of DNA}) (\mu\text{g of DNA/ml})] / 2 \times \text{hr of incubation}$$

(4) where the optical density (OD_{260}) of denatured DNA is $0.024/\mu\text{g}$. Solutions of sheared, denatured DNA in 0.14 M PB were prepared to give a range of C_0t values from 0.012 to 182. Solutions of four unlabeled DNA concentrations (1, 10, 100, and 1,000 $\mu\text{g/ml}$), each containing $0.1 \mu\text{g/ml}$ of ^{14}C -labeled *B. subtilis* 168 DNA [5.8×10^4 counts per min per μg], were incubated for various periods of time at 60°C to cover this range of C_0t values. Possible errors introduced by changing from one concentration to another were ruled out by running reactions at

both concentrations to a similar C_0t value. Reassociation values were corrected by subtracting the "zero time" control value and normalizing the curve to 100% reassociation. The reaction appears to follow ideal second-order kinetics. The shape of the curve indicates that the *B. subtilis* chromosome is essentially unique; i.e., it does not contain significant amounts of repeated nucleotide sequences. The half C_0t value, that C_0t at which reassociation is 50% completed, is 3 for the *B. subtilis* reaction. This value is similar to the half C_0t for *E. coli* DNA (2.5 to 3.0) under similar conditions (19; A. Chiscon, *personal communication*) and corresponds to a genome size of about 3×10^9 , which agrees with published values for *B. subtilis* DNA (10, 23).

Figure 2 represents the reassociation of SPO2 DNA. Solutions at three different concentrations (0.02, 0.2, and 3 $\mu\text{g/ml}$) of labeled SPO2 DNA (1.25×10^5 counts per min per μg) were incubated at 60°C to give a C_0t range from 2.4×10^{-4} to 2.6. The data obtained from these reactions were corrected as above. The sigmoidal-shaped curve again indicates that the phage chromosome contains unique sequences. The half C_0t value of 3×10^{-2} corresponds to that of a DNA of molecular weight similar to that of λ . This agrees with the published value of 2.6×10^7 daltons for SPO2 DNA (26).

Thermal elution profiles of reassociated DNA. Thermal elution chromatography on hydroxy-

FIG. 1. Reassociation kinetics of *Bacillus subtilis* 168 DNA. Solutions, each containing $0.1 \mu\text{g/ml}$ of ^{14}C -labeled *B. subtilis* 168 DNA (5.8×10^4 cpm/ μg) plus unlabeled *B. subtilis* DNA to give final concentrations of 1, 10, 100, or 1,000 $\mu\text{g/ml}$, were diluted, loaded on hydroxyapatite, eluted, and assayed.

FIG. 2. Reassociation kinetics of SPO2 DNA. Solutions containing ^{32}P -labeled SPO2 DNA (1.25×10^5 counts per min per μg) at concentrations of 0.02, 0.2, or 3 $\mu\text{g/ml}$ were diluted, loaded on hydroxyapatite, eluted, and assayed.

apatite was used to assay both extent of reassociation and the stability of the reassociated DNA molecules. Figure 3 shows thermal elution profiles of *B. subtilis* and phage SPO2 DNA reassociation reaction products compared with those from *E. coli* and 434hy reactions. ^{14}C -labeled *B. subtilis* 168 DNA ($0.1\text{ }\mu\text{g/ml}$, 5.8×10^4 counts per min per μg) was incubated with $400\text{ }\mu\text{g}$ of unlabeled *B. subtilis* DNA for 20 hr at $60\text{ }^\circ\text{C}$ ($96\text{ }C_0t$ units). At this point reassociation was about 95% complete. ^{32}P -labeled SPO2 DNA ($0.02\text{ }\mu\text{g/ml}$, 6.0×10^4 counts per min per μg) was incubated with $100\text{ }\mu\text{g/ml}$ of unlabeled SPO2 DNA at $60\text{ }^\circ\text{C}$ for 2.5 hr to reach a C_0t of 3. This reaction showed 92% reassociation. For comparative purposes, ^{32}P -labeled *E. coli* DNA was incubated to a C_0t of 96 and phage 434hy DNA labeled with ^3H -thymine ($100\text{ }\mu\text{g/ml}$, 4400 counts per min per μg) was incubated to a C_0t of 24. The elution profiles obtained from the two bacterial reactions and the SPO2 reaction exhibited normal distributions, with thermal melting points similar to those expected for sheared DNA preparations of their base com-

position (22). The profile from 434hy DNA, however, is skewed toward the lower temperatures.

DNA base composition heterogeneity in different segments of the phage genome has been observed more dramatically by using DNA-agar or optical melting profiles. In the best studied case, λ DNA has been shown to have three discrete regions of guanine plus cytosine (GC) composition ranging from 41 to 56% (13). It appears that the base composition of SPO2 DNA is uniform throughout the molecule. To completely rule out heterogeneity in SPO2 DNA, more precise studies such as optical melting curves at multiple wavelengths (13) would be necessary.

Interspecies reassociation reactions. Interspecies reassociation reactions were carried out to detect any relatedness between SPO2 DNA and that of its host, or phages $\beta 3$ or $\beta 22$. Table 1 presents the results of control experiments to determine the extent of labeled DNA retention by hydroxyapatite at zero time and the degree of reassociation of incubated labeled fragments. The values from these "zero time" and "label only" controls (3.7 and 5.5%, respectively) are higher than the 0.5 to 2.0% control values obtained with *E. coli* DNA. It is possible that the higher values with SPO2 DNA are due to the occurrence of reassociation after denaturation, during the time when stock solutions were being cooled and frozen. The SPO2 genome is only 1% as large as the *E. coli* genome and therefore SPO2 DNA reassociates 100 times as fast as *E. coli* DNA.

Table 2 shows the reactions among labeled phage SPO2 DNA and DNA from *B. subtilis* 168, and phages $\beta 3$ and $\beta 22$ at $60\text{ }^\circ\text{C}$. We were unable to detect any significant reassociation, in excess of the control reactions, of SPO2 DNA with that of *B. subtilis*. In addition, there is no evidence for relatedness between SPO2 and either of the virulent phages tested. It was previously shown (32) that $\beta 3$ and $\beta 22$ are not related to each other or to *B. subtilis*. The ability

FIG. 3. Thermal elution profiles of DNA reassociation reaction products. Homogeneously labeled (asterisks) and unlabeled DNA fragments were incubated at $60\text{ }^\circ\text{C}$. Reaction mixtures were diluted, loaded on hydroxyapatite, eluted, and assayed.

TABLE 1. Elution of ^{32}P -labeled SPO2 fragments from hydroxyapatite

Determination	Labeled DNA			
	Total C_0t units	Counts/min unbound	Counts/min bound	Per cent bound
Zero time	0	897	34	3.7
Incubation $60\text{ }^\circ\text{C}$, 5 hr	6×10^{-4}	834	48	5.5

TABLE 2. *Extent of duplex formation among phage SPO2, Bacillus subtilis 168, and phages β 3 and β 22 at 60 C*

DNA reaction	Per cent reassociation
SPO2 ^a /SPO2	88.7
SPO2 ^a /168	0
SPO2 ^a / β 3	0.4
SPO2 ^a / β 22	0
168 ^a /168	90.7
β 3 ^a / β 3	68.8
β 22 ^a / β 22	67.2

^a Labeled fragments.

of SPO2, *B. subtilis*, β 3, and β 22 DNA preparations to reassociate in homogeneous mixtures of labeled and unlabeled DNA is shown in Table 2.

DISCUSSION

B. subtilis strains are known to carry defective phages which are inducible by ultraviolet light or mitomycin C (15, 18, 28, 29, 30). These phages have been found to contain host DNA (24). Therefore, induction of a *B. subtilis* strain lysogenic for phage SPO2 might also lead to induction of the defective particles causing the phage DNA preparation to be contaminated with bacterial DNA. In an effort to avoid this complication, a lytic point mutant strain of SPO2 was used as the source of both labeled and unlabeled DNA. In addition, the radioisotope was added to the infected culture after phage adsorption to minimize label incorporation into host DNA. The lack of reassociation between labeled SPO2 DNA and *B. subtilis* DNA attested to the absence of *Bacillus* DNA in the SPO2 DNA preparation. Furthermore, the kinetic data (Fig. 2) showing reassociation of phage DNA reveals that the DNA in this preparation reassociated at the rate expected for SPO2 DNA (100 times faster reassociation than would be expected for *B. subtilis* DNA).

Time course reassociation curves obtained from *B. subtilis* and SPO2 DNA species are characteristic of those obtained for other bacteria and phage DNA species and are indicative of molecules lacking repeated nucleotide sequences.

Romig (26) has presented evidence that SPO2 can lysogenize *B. subtilis*. Although linkage between the SPO2 prophage and bacterial marker was not demonstrated, the resistance of prophage DNA to shear and the association of transforming DNA with transfecting activity indicated that the SPO2 prophage had integrated into the host genome. Inselburg et al. (17) have recently obtained data indicating that

the SPO2 prophage is linked to the antibiotic resistance marker *ery-1* in *B. subtilis*.

Those truly temperate enterophages thus far studied are heterogeneous in DNA base composition. Their DNA molecules show a significant degree of interspecies reassociation but do not react with DNA from virulent T phages. They show at least 20% DNA relatedness to *E. coli* (18). DNA from F and RTF sex factors has also been shown to have a heterogeneous DNA base composition and be capable of significant reassociation with *E. coli* DNA (12, 14; Vee C. Jay, *personal communication*). The present experiments indicate that SPO2 DNA is not heterogeneous in base composition and is not extensively related to DNA from its *B. subtilis* host strain. Because of the relatively high control value, a small percentage of relatedness between SPO2 and *B. subtilis* DNA would not have been detected. It is clear, however, that the high extent of reassociation seen in enterophage-*E. coli* DNA reactions does not occur in SPO2-*B. subtilis* reactions. SPO2 is not related to either of the virulent *B. subtilis* phages tested. No other well-documented temperate *B. subtilis* phage was available for testing.

The amount of DNA held in common between temperate enterophages and *E. coli* is not contained in a contiguous region of the phage genome and does not appear to be necessary for integration. It is possible that relatedness between these enterophages and their host(s) is due to some severe selection pressure that prevents divergence or that the conservation of this DNA results from a continuing interchange of DNA between the host and phage. In any case, neither of these mechanisms operates between *B. subtilis* and SPO2. If these organisms were once related they are "old" enough to have diverged almost completely. The data obtained with SPO2 DNA indicate that neither heterogeneity in DNA base composition nor extensive polynucleotide sequence relatedness is a universal requirement for lysogeny.

ACKNOWLEDGMENTS

The bulk of this work was carried out while one of the authors (C. O. Y.) was in the laboratory of B. P. Doctor. We wish to thank him for the use of his laboratory facilities and for the freedom to pursue this study. We wish to thank S. Falkow and B. H. Hoyer for their interest and constructive criticisms. We are grateful to R. J. Britten for the use of his pressure cell to shear DNA samples.

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