Characterization of Temperate Bacteriophages of *Bacillus subtilis* by the Restriction Endonuclease *EcoRI*: Evidence for Three Different Temperate Bacteriophages

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Temperate bacteriophages of *Bacillus subtilis* were characterized according to host range and digestion of the bacteriophage genome by endonuclease *EcoRI*. The three bacteriophages, ϕ 3T, SPO2, and ϕ 105, were all heteroimmune, and the DNA digests showed dissimilar patterns by agarose-ethidium bromide gel electrophoresis.

The discovery of a restriction endonuclease in *Haemophilus influenzae* that was capable of cleaving DNA at sites determined by a unique sequence of nucleotides (10, 20) provided a new dimension in molecular genetics. Now for the first time fragments of DNA could be generated with specific ends, recombined, and studied in vitro and in vivo. The development of this new, powerful tool for manipulating genetic information prompted a search for similar enzymes with different specificities. Such enzymes have been isolated from *Haemophilus parainfluenzae* (7, 19), *Haemophilus aegyptius* (12), and *Escherichia coli* (8). By preparing a limit digest of DNA with these enzymes, it is possible to obtain a series of unique fragments that retain biological activity. For instance, Edgell and Hutchison (6) subjected DNA from bacteriophage ϕ X174 to this digestion and determined genetically which markers resided on the fragments. Genomes that have proven to be difficult to map by conventional means have recently been successfully investigated by fragmentation with restriction endonucleases. Morrow and Berg (13) and Mulder and Delius (14) combined the techniques of endonuclease digestion and electron microscopy to map the restriction site on simian virus 40 DNA. With the introduction of agarose-ethidium bromide gel electrophoresis (19), it became possible to rapidly visualize the products of digestion and to separate them according to size. Thus, a "fingerprint" of a genome can be obtained for taxonomic studies. It is important to emphasize the limitations of this methodology. The fingerprint will only indicate whether the genomes are broken into similar or dissimilar sizes. To determine rigorously if the fragments of the same size do indeed represent a similar region of the genome, it

would be necessary to hybridize the two fragments to show base sequence homology. Regardless of this limitation, it is doubtful if genomes that show markedly dissimilar patterns can be closely related. Therefore, we decided to apply this technique to examine the degree of relatedness between the temperate bacteriophages of *B. subtilis*.

EcoRI was isolated from *E. coli* RY13, using a procedure communicated to us by H. O. Smith and P. Geshelin. Essentially, the enzyme was purified in the following manner. Cells (124 g [wet weight]) were harvested from LB broth during the late logarithmic stage of growth and sonically treated for 30 min, and the cell debris was removed by low-speed centrifugation followed by high-speed centrifugation ($100,000 \times g$). Nucleic acids were removed by precipitation with streptomycin sulfate. The enzyme was precipitated from the supernate with ammonium sulfate (50% saturation). After dialysis, the precipitated fraction was purified by column chromatography on Whatman P11 phosphocellulose and Whatman DE-52 DEAE-cellulose. The final enzyme preparation was stored at 4 C in 50% glycerol containing 0.015% Triton X-100. The enzyme appears to be free of exonuclease activity and cleaves DNA from bacteriophage λ plac into seven fragments and polyoma DNA once (11, 13, 14). This evidence confirms that the enzyme is the *EcoRI* restriction endonuclease.

Bacteriophages were treated with pancreatic DNase and RNase and further purified by equilibrium centrifugation in CsCl. DNA was isolated from these bacteriophage preparations by treatment with 1% sodium dodecyl sulfate followed by three extractions with buffer-saturated (0.1 M Tris-hydrochloride, pH 8.0)

phenol. An equal volume of saturated NaCl was added before precipitation of the DNA with two volumes of ethanol. The preparations of DNA were added to an agarose-ethidium bromide gel and subjected to electrophoresis. The DNA from bacteriophages $\phi 105$ and SPO2 yielded a single band indicating homogeneity of the prep-

arations (Fig. 1). Both genomes have a molecular weight of $26.3 (\pm 0.3) \times 10^6$ (4).

When a digest of the DNA with *EcoRI* endonuclease was fractionated by agarose-ethidium bromide gel electrophoresis, the patterns were quite different (Fig. 1). *EcoRI* cleaved $\phi 105$ DNA into 11 fragments (8 major bands and 3

FIG. 1. DNA (1 to 5 μg) in a volume of 50 μl was added to 50 μl of TMS buffer (10 mM Tris (pH 7.5), 50 mM NaCl, 10 mM MgCl_2) and 20 μl of *EcoRI* and incubated overnight (approximately 16 h) at 30 C in a siliconized glass vial. The reaction was terminated by the addition of 10 μl of 0.1 M EDTA and 10 μl of 60% sucrose containing 1% bromophenol blue. Fifty μl of the mixture was applied to the gel, consisting of 0.7% agarose and 0.5 μg of ethidium bromide per ml. Samples were run on an E-C 470 vertical gel electrophoresis apparatus at a constant potential of 150 V (85 to 95 mA) for 3 h at 4 C. Gels were prerun for 2 h at 200 V before samples were applied. Gels were photographed under UV illumination (General Electric bulb G8T5) using Polaroid 4 $\times$ 5 type 55 film and a Vivitar red no. 25A filter. The details of this method were published previously (19). (A) $\phi 105$ DNA; (B) SPO2 DNA; (C) $\phi 3T$ DNA. A, B and C, contained only 5 μl of the DNA mixture to prevent overloading of the gel. (D) *EcoRI* digest of $\phi 105$ DNA, (E) *EcoRI* digest of SPO2 DNA; (F) *EcoRI* digest of $\phi 3T$ DNA. The diagrammatic representations of the digests (G, H, and I) were compiled from at least eight different experiments. By adjusting the DNA concentrations, it is possible to resolve the bands as indicated. The *EcoRI* digests of (G) $\phi 105$ DNA, (H) SPO2 DNA, and (I) $\phi 3T$ DNA are presented with heavy bars representing major bands and light bars representing bands that are difficult to resolve in all gels. Stippled bars represent undigested DNA.

minor low-molecular-weight bands) and SPO2 DNA into 7 fragments (the two minor bands in the middle are often difficult to observe), as indicated diagrammatically in Fig. 1. Two of the fragments have approximately the same molecular size; however, further experiments will be required to establish whether these fragments are identical.

During the course of these studies, we received another temperate bacteriophage, ϕ 3T. This phage was originally isolated and designated ϕ 3 by R. G. Tucker. To avoid confusion with the bacteriophage ϕ 3 isolated by B. E. Reilly (Ph.D. thesis, Western Reserve University, Cleveland, Ohio, 1965), we have changed the designation of the bacteriophage isolated by Tucker to ϕ 3T. Bacteriophage ϕ 3T has a guanine plus cytosine content of 35.9% (21). This guanine plus cytosine content is markedly different than the 43% obtained for bacteriophages ϕ 105 and SPO2 (1, 16). R. E. Yasbin, in this laboratory, constructed lysogenic strains in our standard isogenic strain BR151 to determine if this temperate bacteriophage interfered with transformation as did bacteriophages SPO2 and ϕ 105 (23). The efficiency of plaque formation on these lysogenic strains by the three bacteriophages indicated that ϕ 3T was heteroimmune to ϕ 105 and SPO2 (Table 1). The decreased efficiency of plating of bacteriophage ϕ 3T on *B. subtilis* lysogenic for ϕ 105 and SPO2 may be related to bacteriophage interference (15, 22). The bacteriophage ϕ 3T forms a stable association with the host, is immune to superinfection, and can be induced with mitomycin C (M. T. Williams, unpublished data) or hydrogen peroxide (21), thus indicating that the bacteriophage is temperate.

DNA from DNase-treated CsCl purified ϕ 3T was digested with *Eco*RI endonuclease and

chromatographed on agarose-ethidium bromide gel electrophoresis. There are many more *Eco*RI cleavage sites for bacteriophage ϕ 3T than were found for bacteriophages ϕ 105 or SPO2 (Fig. 1). It is difficult to resolve all of the low-molecular-weight fragments; however, it appears that 24 fragments are generated by *Eco*RI digestion.

The degree of relatedness between the temperate bacteriophages has been the subject of a number of investigations. Two of the bacteriophages, ϕ 105 and SPO2, have DNA with the same molecular weight and are morphologically similar (2, 3, 16). Bacteriophages SPO2 and ϕ 105 are certainly different in that they are heteroimmune (2), integrate at different locations in the host chromosome (9, 17), and only share approximately 14% base sequence homology as determined by electron microscopy of heteroduplex DNA molecules (4). In addition, Chow and Davidson (5) demonstrated that the SPO2 prophage is a circular permutation of the mature phage, whereas ϕ 105 prophage and mature phage are colinear. None of the mutants of bacteriophage ϕ 105 can be complemented by bacteriophage SPO2 in the genetic studies performed to date (18). This communication indicates yet another difference between the two phages. By digestion with restriction enzyme *Eco*RI, dissimilar fragments of the genome are produced, thus indicating that recognition palindromes for the enzyme are located at different sites on the genome.

The bacteriophage ϕ 3T has not been compared to the other temperate bacteriophages; however, one would expect this phage to be quite different because of its abnormally low content of guanine and cytosine (35.9%). Digestion of the ϕ 3T genome by *Eco*RI emphasizes the unrelatedness of this temperate bacteriophage. The "fingerprinting" of the genome by

TABLE 1. Efficiency of plating of temperate bacteriophages on lysogenic hosts^a

Bacteriophage	PFU/ml assayed on:			
	BR151	BR151 (ϕ 3T)	BR151 (ϕ 105)	BR151 (SPO2)
ϕ 3T	5.14×10^9 (1.00)	0	3.33×10^9 (0.65)	4.33×10^9 (0.84)
ϕ 105	3.60×10^9 (1.00)	2.90×10^9 (0.81)	0	3.23×10^9 (0.90)
SPO2	5.92×10^9 (1.00)	8.45×10^8 (0.14)	7.69×10^9 (1.30)	0

^a Bacteriophages were titered on different hosts by the semisolid overlay method described previously (23). Parentheses after the strain indicate that the host is lysogenic for the bacteriophage indicated. The efficiency of plating is given relative to strain BR151 in parentheses beneath the bacteriophage titer.

restriction endonucleases is, therefore, a rapid diagnostic tool to test relatedness between genomes in the absence of characterized mutants.

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