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Characteristics of *Bacteroides fragilis* Bacteriophages and Comparison of Their DNAs

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Absract. Although five *Bacteroides fragilis* bacteriophages isolated over a six-year period in Nebraska and Virginia had similar physical characteristics (morphology, temperature inactivation, and sensitivity to organic solvents and antisera), there were some statistically significant differences between the phages. In addition, restriction endonuclease analysis revealed that three of the five DNAs were not identical. However, the DNAs of the phages were closely related based on DNA–DNA hybridization, percent homologies, and possession of homologous regions of DNA. It appears that the five phages are strains of the same species of phage, although each phage has a unique host range spectrum.

Bacteriophages of the *Bacteroides fragilis* group have been isolated from raw sewage [1, 9, 10] and animal sera [5]. Host range studies indicate that each phage is specific for only one species of *Bacteroides* and that no two phages have the identical host spectrum [1]. An interesting observation is that, except for three phages [1], all of the more than 100 phages isolated to date worldwide for the *B. fragilis* group are essentially morphologically indistinguishable, even though their hosts can be easily speciated by phenotypic characteristics or by DNA homologies.

The purpose of the present study was to determine whether five selected *B. fragilis* phages, each having distinctly different host ranges, were different strains of the same phage species (with slight genetic variations to account for differences in host ranges) or genetically quite distinct phages (i.e., different species) with almost identical morphological features.

Materials and Methods

Bacteria and culture conditions. *Bacteroides fragilis* (84 strains) were obtained from L.V. Holdeman and W.E.C. Moore (Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia). The *Bacteroides* were grown in pre-reduced anaerobically sterilized brain–heart infusion broth-supplemented (BHI-S) [3].

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Phage isolation, growth, and purification. Sewage samples from Omaha, Nebraska, were clarified (10,000 *g*, 30 min). Phages were precipitated with 430 g/liter of ammonium sulfate and subsequent centrifugation. Phages were detected, isolated, and cloned by the agar overlay method [1]. For batch growth, phages were added to host cells in BHI-S broth at a multiplicity of infection of 0.1 and incubated anaerobically overnight at 37°C. Phages were precipitated with 8% (wt/vol) polyethylene glycol and harvested by centrifugation [12]. The phage pellets were dissolved in phage buffer (1 m*M* Tris–HCl and 10 m*M* magnesium chloride, pH 7.5), and each phage preparation was purified by three sequential CsCl density gradient centrifugations (192,000 *g*, 20 h at 18°C). The phage preparations were dialyzed against phage buffer and stored at 4°C.

DNA extraction and restriction analysis. The DNA quantitation, phenol extraction, ethanol precipitation, and butanol concentration of the five DNAs were performed as described by Maniatis et al. [6]. Each DNA (0.5 μg) was separately digested with *Eco*R I, *Bst*E II, and *Hin*d III as recommended by the supplier (Bethesda Research Laboratories, Gaithersburg, Maryland). The enzyme digests were electrophoresed in a 0.6% horizontal agarose gel in TBE buffer [6] with a current of 26 mA for 18 H.

Southern transfers and hybridizations. *Hin*d-III-digested DNA was electrophoresed, denatured (separate solutions of 0.5 *M* NaOH and 1.5 *M* NaCl), neutralized (separate solutions of 0.5 *M* Tris–HCl, pH 7.6, and 3.0 *M* NaCl), and transferred to nitrocellulose (NC) paper [11]. The NC was prehybridized in 7.0 ml of prehybridization fluid (6 × SSPE [6], 0.5% (wt/vol) sodium dodecyl sulfate (SDS), 50% deionized formamide, and 5 × Denhardt's solution [6]) for 5 h at 40°C. Denatured (boiled) probe DNA (specific activity 1.6 to 2.7 × 10^6 cpm/0.025 μg DNA) was added to the prehybridized NC and incubated for 2–3 days at 40°C. The NC was washed under stringent conditions (0.2 × SSPE and 0.1% SDS) with constant agitation for 2 h at 60°C. The washed

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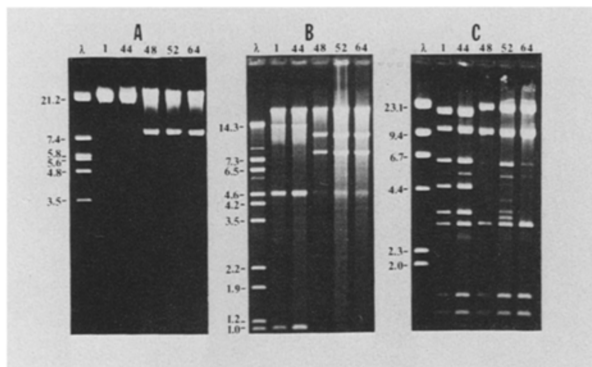

Fig. 1. Electrophoretic migration of phage DNA digested with *Eco*R I (A), *Bst*E II (B), and *Hin*d III (C). The lanes in each agarose gel, from left to right, contain DNA from phages lambda (control), Bf-1, Baf-44, Baf-48B, Bf-52, and Baf-64. The numbers to the left are the sizes (kbp) of the lambda DNA fragments.

NC was air dried, sprayed with En^{3}Hance (New England Nuclear, Boston, Massachusetts), and placed on x-ray film (X-Omat Xar-5). The film was exposed for 3–6 days at −70°C and developed in an automatic processor.

Electroelution [8] was used to isolate the 3.0- and 4.4-kbp fragments of *Hin*d-III-cleaved DNA from phage Bf-1. The hybridization procedure using the electroeluted fragments was identical to the procedure for uncleaved DNA except the fragmented DNAs had specific activities of 1.6 to 3.2 × 10^5 cpm/2.0 μg DNA and the x-ray film was exposed to the hybridized NC for 19–20 days.

Percentage homology between phage DNAs. The membrane competition method described by Johnson [4] was modified. The total required amount of Bf-1 DNA (100 μg/square) was denatured with a 0.5 vol of 0.4 *N* NaOH and diluted with 2.0 *M* ammonium acetate (volume equal to the NaOH). The DNA was spotted onto NC squares (10 × 10 mm), air dried, and baked for 18 h at 65°C. The NC squares were prehybridized as previously described. The total required amount of uncleaved Bf-1 DNA (7.5 × 10^4 cpm/7 ng/assay) was labeled with tritium by nick translation and diluted in prehybridization fluid. The probe and competitor DNAs were fragmented by sonication. Competitor DNAs (1500 ng and 3000 ng) and probe DNA were added to the prehybridize NCs. The DNAs were reannealed at 40°C for 68 h with constant agitation. Each NC square was washed under stringent conditions and air dried. The dried NC squares (in scintillation vials) were boiled for 15 min in 0.5 ml of 0.1 *M* HCl, dissolved in 1.0 ml of ethyl acetate, and mixed with Bioflour (New England Nuclear). The samples were counted for 10 min in a liquid scintillation counter.

Other procedures. Antisera to phages Bf-1 and Bf-52 were raised in rabbits as described [1]. Heat inactivation was determined by holding the phages in phage buffer at 50°C for 15 min or 65°C for 10 min. The effect of organic solvents (chloroform, ether, benzene, and toluene) on viability was determined by adding 0.2 ml of the solvent to 1.0 ml of phage, mixing vigorously for 20 s, and incubating for 30 min at 37°C. Phages on formvar and carbon-coated copper grids were negatively stained with 2% uranyl acetate, pH 4.5, prior to examination at 60 kV in a Philips 201 C electron microscope. Thermal melting points of phage DNAs were determined by Dr. J.L. Johnson (Virginia Polytechnic Institute and State University, Blacksburg, Virginia).

Table 1. Number of nucleotides and corresponding molecular weights

Phage	Nucleotide base pairs ($\times 10^3$)[a]	Molecular weights ($\times 10^6$)[b]
Bf-1	51.0[c] ± 0.5	34.7[c] ± 0.4
Baf-44	51.5[c] ± 0.7	34.0[c] ± 0.5
Baf-48B	57.6 ± 0.6	38.0 ± 0.4
Bf-52	65.3[d] ± 0.8	43.1[d] ± 0.5
Baf-64	56.7 ± 0.6	37.4 ± 0.4

[a] Mean values for three independent determinations with SEMs.
[b] Number of nucleotides multiplied by 6.6 × 10^2.
[c] Statistically lower than Baf-48B, Bf-52, or Baf-64 (p value 0.01).
[d] Statistically higher than the other phages (p value 0.01).

Results

Phage characteristics. A total of 36 *B. fragilis* phages were isolated in Nebraska. These were morphologically similar to each other and to the *Bacteroides* phages with long noncontractile tails previously described [1]. The five phages selected for detailed study were: Bf-1 and Bf-52, both isolated in Virginia [1]; and Baf-44, Baf-48B, and Baf-64, which were isolated in Nebraska. The phage heads ranged from 51.1 × 49.0 nm to 55.9 × 52.3 nm, and the tails ranged from 133 × 13.1 nm to 138 × 12.8 nm. There were statistically significant differences in some of the phage dimensions of the selected phages. While each of the phages had a unique host range pattern, the selected phages had five hosts in common. The phages were all grown in *B. fragilis* VPI 4912 to make uniform any host-controlled modifications of the phages.

There was less than a 10% decrease in phage infectivity at 50°C for the five phages. At 65°C, there was approximately a 99% decrease in infectivity for four of the phages, but only a 71% decrease for Baf-64. For all five phages, infectivity decreased at least 93% in the presence of chloroform, benzene, or toluene. However, ether did not decrease phage infectivity for any of the phages. Each of the phages was serologically related to Bf-1 and Bf-52. However, Bf-1, Baf-44, and Baf-64 were neutralized by Bf-52 antiserum statistically less efficiently than were the Bf-48B phages.

The five phages contained double-stranded

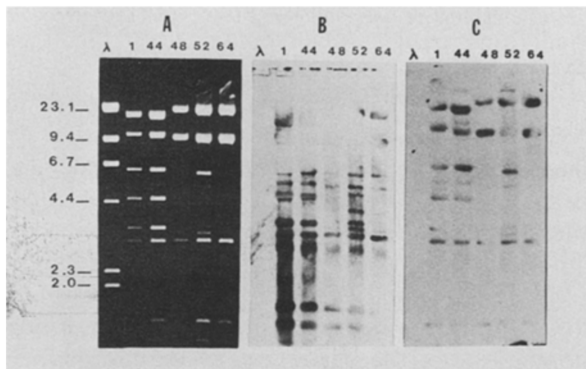

Fig. 2. Use of uncleaved DNA as radioactive probes. In each panel, the DNAs, from left to right, are lambda (control), Bf-1, Baf-44, Baf-48B, Bf-52, and Baf-64. Panel A is an agarose gel demonstrating the separation of DNAs digested with *Hin*d III. The number to the left are the sizes (kbp) of the lambda DNA fragments. Panel B is a fluorogram demonstrating the formation of radioactive duplexes with the Bf-1 uncleaved probe DNA. Panel C is a fluorogram demonstrating the formation of radioactive duplexes with the Baf-48B uncleaved probe DNA.

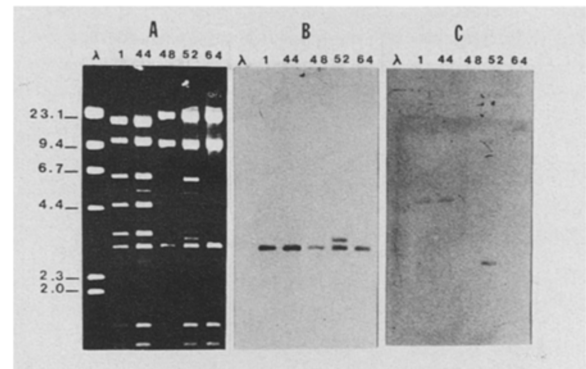

Fig. 3. Use of specific fragments of Bf-1 DNA as radioactive probes. In each panel, the DNAs, from left to right, are lambda (control), Bf-1, Bf-44, Baf-48B, Bf-52, and Baf-64. Panel A is an agarose gel demonstrating the separation of DNAs digested with *Hin*d III. The numbers to the left are the sizes (kbp) of the lambda DNA fragments. Panel B is a fluorogram demonstrating the formation of radioative duplexes with the 3.0-kilobase *Hin*d III fragment probe of Bf-1 DNA. Panel C is a fluorogram demonstrating the formation of radioactive duplexes with the 4.4-kilobase *Hin*d III fragment probe of Bf-1 DNA.

DNA as determined by the colorimetric acridine orange slide technique [2]. Their mol% G+C, calculated from thermal melting points [7], was 39.2. Molecular weights of the DNAs were calculated from *Hin*d III fragments (Fig. 1C) and ranged from approximately 34 × 10^6 for Baf-44 to 43 × 10^6 for Bf-52 (Table 1).

Restriction endonuclease analysis of phage DNA. Restriction analysis revealed three patterns of DNA cleavage: the Bf-1 and Baf-44 pattern, the Baf-48B and Baf-64 pattern, and the Bf-52 pattern (Fig. 1). While *Eco*R I produced no cleavage of Bf-1 or Baf-44 DNAs (Fig. 1A), digestion of Baf-48B, Bf-52, and Baf-64 DNAs each resulted in two comigrating fragments. The DNAs from the five phages were cleaved by *Bst*E II (Fig. 1B) and *Hin*d III (Fig. 1C). *Bst*E II digestion produced the Bf-1 and Baf-44 pattern (four comigrating fragments) and the Baf-48B, Bf-52, and Baf-64 pattern (three comigrating fragments each). *Hin*d III digestion revealed three patterns: the Bf-1 and Baf-44 pattern, the Baf-48B and Baf-64 pattern, and a unique Bf-52 pattern.

DNA–DNA homology. The molecular relatedness between the DNAs was investigated by DNA–DNA homology initially by using uncleaved DNAs as probes (Fig. 2). When Bf-1 DNA was used as a probe, all of the *Hin*d III fragments from the other four DNAs formed duplexes with the probe DNA (Fig. 2B). Similarly, all of the *Hin*d III fragments of phage DNA, with the exception of a 3.3-kb fragment of Baf-44, formed duplexes with the Baf-48B probe DNA (Fig. 2C).

In addition to the uncleaved DNA probes, a 3.0-kb fragment of *Hin*d-III-digested Bf-1 probe DNA (which comigrated with a fragment from the other four *Hin*d-III-digested DNAs) was used to determine whether or not the comigrating DNA fragments contained homologous sequences. The comigrating fragments did form duplexes with the Bf-1 DNA fragments (Fig. 3B). In addition, a 3.1-kb fragment of Bf-52 DNA formed a duplex with the 3.0-kb probe.

A 4.4-kb fragment of *Hin*d-III-digested Bf-1 probe DNA was used to ascertain if this fragment was homologous with any sequences within the other four DNAs (Fig. 3C). The 4.4-kb fragment of Bf-1 DNA had homology with the comigrating Baf-44 fragment (Fig. 3A). This fragment probe also had homology with a much smaller (approximately 2.3 kb) fragment of Bf-52 DNA. In contrast, the 4.4-kb Bf-1 DNA had no homology with any fragment of *Hin*d-III-digested Baf-48B or Baf-64 DNAs.

Percentages of homology. The percentages of homology between the five DNAs and Bf-1 DNA ranged from 57% to 95% (Table 2). The percentages of homology of Bf-1 DNA with Bf-48B (63% and

Table 2. Percentages of homology of phage DNAs to Bf-1 DNA determined by the membrane competition method

Competitor DNA[a]	Percent homology
Bf-1 low[b]	100
Bf-1 high[c]	100
Baf-44 low	95
Baf-44 high	100
Baf-48B low	63[d]
Baf-48B high	61[d]
Bf-52 low	57[d]
Bf-52 high	83
Baf-64 low	86
Baf-64 high	78

[a] Each experiment consisted of immobilized Bf-1 DNA, and in solution Bf-1 probe DNA and the indicated amount of competitor DNA.
[b] Low = 15-fold excess of competitor DNA (1500 ng).
[c] High = 30-fold excess of competitor DNA (3000 ng).
[d] Statistically higher (p value 0.01) than corresponding Bf-1 control.

61% and Bf-52 (57%) were statistically lower than with the other phages.

Discussion

We are surprised by the striking similarity among all of the *Bacteroides fragilis* group bacteriophages isolated to date. The similarities exist even though the phages were isolated from different locations over a number of years. While it seems improbable that each investigator has chosen an isolation procedure that selects for the same morphological type of phage, all but three [1] of the more than 100 *B. fragilis* group bacteriophages isolated worldwide are morphologically indistinguishable.

The phages investigated in this study were similar phenotypically, yet for every physical characteristic there were some statistically significant differences between the phages. When we investigated the genotypic characteristics of these phages, we found that when both the uncleaved DNAs and the 3.0-kb fragment of Bf-1 DNA were used as probes in the DNA–DNA hybridizations, the five DNAs were closely related. Similar findings were shown by the experiments used to determine the percentages of homology. However, restriction analysis indicated there were three different types of DNAs, and the DNA–DNA hybridizations using the 4.4-kb fragment of Bf-1 DNA as a probe revealed that this fragment was not contained within the Baf-48B or Baf-64 genomes. Therefore, this fragment was deleted from, or was never contained within, these phage genomes.

No studies were undertaken to determine the locations of any genes in the DNAs of the five phages. Therefore, it is not known what is coded for by the 4.4-kbp fragment of Bf-1 DNA, which constitutes approximately 8% of the entire phage genome. However, since this fragment is completely absent in the Baf-48B and Baf-64 genomes, it is probably not required for bacterial attachment, the general morphology of the phages, or phage reproduction and maturation.

We conclude that these five phages are probably different strains of the same phage species. However, it still seems striking that these phages are so similar phenotypically, yet there are significant differences in the genomes of Bf-48B and Baf-64.

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