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Bacteriophages Active against *Bacteroides fragilis* in Sewage-Polluted Waters

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Twelve strains of different *Bacteroides* species were tested for their efficiency of detection of bacteriophages from sewage. The host range of several isolated phages was investigated. The results indicated that there was a high degree of strain specificity. Then, by using *Bacteroides fragilis* HSP 40 as the host, which proved to be the most efficient for the detection of phages, feces from humans and several animal species and raw sewage, river water, water from lagoons, seawater, groundwater, and sediments were tested for the presence of bacteriophages that were active against *B. fragilis* HSP 40. Phages were detected in feces of 10% of the human fecal samples tested and was never detected in feces of the other animal species studied. Moreover, bacteriophages were always recovered from sewage and sewage-polluted samples of waters and sediments, but not from nonpolluted samples. The titers recovered were dependent on the degree of pollution in analyzed waters and sediments.

Over 100 different types of virus occur in domestic sewage (21). The survival and transmission of viruses in sewage-contaminated water have been recognized as potential public health problems (4, 21, 29, 30). Currently available technology for virus isolation from water has the following shortcomings. Practical methods for the detection of viruses of primary concern such as hepatitis A virus, rotavirus, and Norwalk virus are not available; and techniques for both the recovery and the identification of human enteric viruses have limited sensitivity, are time-consuming and expensive, and require highly skilled labor and sophisticated laboratory facilities (30).

Although in some situations various bacterial indicator systems have proved to be useful for assessing the virological quality or safety of a wide variety of waters (11, 24, 30), recent epidemiological and microbiological findings have raised serious concerns about the adequacy of fecal indicator bacteria for predicting the virological quality of water (6, 7, 13, 20, 22, 23, 37, 39). Therefore, indicators more closely related to enteric viruses would be preferable for the evaluation of virological water quality (5, 41). Bacteriophages may play a valuable role in this regard because their structure, composition, morphology, and size closely resemble those of enteric viruses. Several investigators have proposed the use of coliphages as virus indicators (3, 25, 35, 49–52). Replication of coliphages in estuary water during the summer months (54) and the existence of many coliphages whose natural habitats appear to be the aquatic environment (48), however, represent the serious shortcomings of coliphages as indicators of enteric viruses.

In this study the potential of bacteriophages of *Bacteroides* spp. as indicators of human enteric viruses was examined. *Bacteroides* ssp. are strict anaerobes and are a major component of human feces (19, 45). It is unlikely that they occur naturally in the environment, and consequently, the phages infecting them have the potential for being good virus indicators. The purpose of this study, therefore, was to investigate the presence of bacteriophages of *Bacteroides* spp. in human feces, sewage, and sewage-contaminated waters.

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MATERIALS AND METHODS

Bacterial strains. Twenty-five strains of *Bacteroides* were used in this study. Strains HSP 39, HSP 40, and HSP 44 were obtained from the Microbiology Service at the Hospital de Sant Pau (Barcelona, Spain). They were isolated from peritoneal exudates and were classified as *Bacteroides fragilis* according to substrate utilization, products of fermentation determined by gas-liquid chromatography, growth in 20% bile, and indole production (12). *B. fragilis* C-17898, C-13970, A-22055, C-19075, C-13700-2, C-6814, C-13182, C-1416, C-8797, C-14021, and C-13525 were isolated from clinical specimens; and strains BBE-18 and B-1 were isolated from feces and were kindly provided by García Rodriguez (Department of Microbiology, Faculty of Medicine, University of Salamanca, Salamanca, Spain). The remaining strains *B. hypermegas* DSM-1672, *B. thetaiotaomicron* DSM 2079, *B. vulgatus* DSM 1447, *B. fragilis* DSM 2151, and *B. ovatus* DSM 1896 were obtained from the German Collection of Microorganisms; *B. distasonis* ATCC 8503, *B. eggerthii* ATCC 27754, *B. uniformis* ATCC 8492, and *B. microfusus* ATCC 2972 were obtained from the American Type Culture Collection (Rockville, Md.).

Media and growth conditions. Modified blood agar base (MBAB) contained the following per liter of distilled water: 40 g of blood agar base no. 2 (Oxoid Ltd., London, England), 0.05 g of L-cysteinium chloride monohydrate (E. Merck AG, Darmstadt, Federal Republic of Germany), 0.12 g of $MgSO_4 \cdot 7H_2O$ (Merck), and 0.05 g of $CaCl_2$ (Merck). Modified brucella broth (MBB) contained the following per liter: 28 g of brucella broth (ADSA, Barcelona, Spain), 0.05 g of L-cysteinium chloride monohydrate, 0.12 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $CaCl_2$. Soft agar for the overlay technique was prepared by adding 5 g of agar per liter to MBB. MBAB-S was MBAB containing 0.05% potassium sorbate and was adjusted to pH 5.7 to avoid spore germination.

When solid media were used, cultures were incubated inside anaerobic jars (GasPak; BBL Microbiology Systems, Cockeysville, Md.). For incubation of liquid cultures, screw-cap tubes or bottles filled with MBB were used; this allowed us to avoid the use of anaerobic jars.

TABLE 1. Efficiency of different *Bacteroides* strains for the isolation of *Bacteroides* bacteriophages from sewage

Sample	MPN of the following strains/100 ml[a]:											
	B. fragilis HSP 40	*B. fragilis* HSP 44	*B. fragilis* HSP 39	*B. fragilis* DSM 2151	*B. ovatus* DSM 1896	*B. vulgatus* DSM 1447	*B. hypermegas* DSM 1672	*B. microfusus* ATCC 29728	*B. eggerthii* ATCC 27254	*B. distasonis* ATCC 8503	*B. thetaiotaomicron* DSM 2079	*B. uniformis* ATCC 8492
1	230	0	0	—	—	—	—	—	—	—	—	—
2	230	20	0	—	—	—	—	—	—	—	—	—
3	230	0	0	—	—	—	—	—	—	—	—	—
4	230	—	—	0	—	0	40	—	—	—	—	—
5	230	—	—	20	—	0	130	—		—	—	—
6	230	—	—	10	—	0	0	—	—	—	—	—
7	1,100	—	—	—	—	—	—	0	0	0	—	—
8	93	—	—	—	0	—	—	0	0	0	0	—
9	1,100	—	—	—	0	—	—	0	0	0	0	—
10	1,100	—	—	—	0	—	—	—	—	—	0	—
11	7	—	—	—	—	—	—	—	—	—	—	0
12	24	—	—	—	—	—	—	—	—	—	—	0
13	460	—	—	—	—	—	—		—	—	—	0

[a] —, Not tested; 0, values of <3.

Collection and processing of samples. Surface water samples were collected in sterile glass bottles submerged about 20 cm below the water surface. Groundwater samples were taken from 20-m-deep wells. All samples were placed at 4°C and examined within 6 h after collection.

Sediment samples were collected with a Van Veen dredge. The sediment samples were carefully deposited on a clean surface. The top layer (2 to 3 cm) of sediment was scooped into sterile plastic bags, sealed, and stored at 4°C until they were processed. Samples were tested within 12 h of collection. Samples showing layer disruption were discarded. Then, 100 g of sediment was suspended in 100 ml of buffer (Na_2HPO_4, 7 g; KH_2PO_4, 3 g; NaCl, 5 g; 0.1 M $MgSO_4$, 10 ml; 0.01 M $CaCl_2$,10 ml, distilled water to 1,000 ml) (14) for phages and vigorously shaken for 2 h at 4°C. The gross particles were allowed to sediment. Phages in the supernatant were counted by the most probable number (MPN) method, as described below for the water samples.

For feces analysis, fresh feces were collected in sterile bottles. Then, buffer for phages was added to make 0.1 g of fecal matter per ml of suspension. Then, phages were counted as described below for the water samples.

Sampling sites. Raw sewage was collected from a city sewage system, the average MPNs of fecal coliforms and fecal streptococci of which were 3.1×10^7 and 8.2×10^6, respectively. Besos and Llobregat are two highly polluted, small rivers that flow through densely populated areas north and south of Barcelona. The mean MPNs of fecal coliforms and fecal streptococci per 100 ml were 3.08×10^7 and 4.22×10^6, respectively, for the Besos River and 8.62×10^6 and 1.32×10^6, respectively, for the Llobregat River. Polluted seawater was collected from an area which receives a large amount of untreated sewage from Barcelona; the mean MPNs of fecal coliforms and fecal streptococci per 100 ml were 1.3×10^4 and 7.2×10^3, respectively. Numbers are mean values obtained twice a month over the previous 4 years. Contaminated sediments were collected either from the Besos River or from the coastal area from which seawater samples were taken.

Groundwater samples were taken from a 20-m-deep aquifer from which enteroviruses, fecal coliforms, and fecal clostridia have occasionally been isolated.

Water and sediment samples free of human pollution were obtained far (>10 km) from any human settlement in the natural park Delta de l'Ebre. Water and sediment collected in eutrophic lagoons from the park were nearly anaerobic. Other samples were taken from nonpolluted mountain streams near Manlleu, which is 70 km northeast of Barcelona.

Phage assays. Enumerations of phages by plaque counts (PFU) were conducted by the double-agar layer technique (1). Tubes containing 2.5 ml of soft MBB agar kept at 45°C were inoculated with 10^7 CFU of the indicator bacteria and a fraction not exceeding 1.0 ml of the sample to be analyzed. After mixing, the contents were poured onto a plate of MBAB and incubated inside GasPak (BBL) jars at 37°C. Plaques were counted after 18 h of incubation. When water samples were analyzed by this method, samples were filtered through membrane filters (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.) before they were mixed with the host.

Phages were also enumerated by a modification of the MPN method for coliphage described by Kott (34). Each of three screw-cap tubes (30 ml) containing 10 ml of 2.5-stretch MBB was inoculated with 10 ml of the sample and 10 ml of the host. A second set of three tubes containing 19 ml of single-strength MBB was inoculated with 10 ml of the host and 1 ml of the sample, and a third set of three tubes containing 20 ml of single-strength MBB was inoculated with 10 ml of the host and 0.1 ml of the sample. The tubes were incubated at 37°C for 36 to 48 h. Following incubation, 5 ml from each tube was centrifuged at 2,000 × *g* for 15 min, and the supernatant was either mixed with 2 ml of chloroform or filtered through a 0.22-μm-pore-size membrane filter. Fi-

TABLE 2. Host range of 27 bacteriophage isolates among 25 *Bacteroides* strains

Phage isolate	Host range among[a]:							
	B. fragilis HSP 40	*B. fragilis* HSP 44	*B. fragilis* C-17898	*B. fragilis* C-13970	*B. fragilis* A-22055	*B. fragilis* DSM 2151	*B. fragilis* (other strains)[b]	*Bacteroides* species other than *B. fragilis*[c]
B40-1	+	−	−	−	−	−	−	−
B40-2	+	−	−	−	−	−	−	−
B40-3	+	−	−	−	−	−	−	−
B40-5	+	−	−	−	−	−	−	−
B40-7	+	−	−	−	−	−	−	−
B40-8	+	−	−	+	−	+	−	−
B40-9	+	−	−	+	−	−	−	−
B40-10	+	−	−	+	−	−	−	−
B40-11	+	+	−	+	−	−	−	−
B40-12	+	−	−	+	−	−	−	−
B40-13	+	−	−	−	−	−	−	−
B40-14	+	−	−	+	−	−	−	−
B40-15	+	−	−	−	−	−	−	−
B40-16	+	−	−	−	−	−	−	−
B44-17	+	+	+	+	+	−	−	−
B44-18	+	+	+	+	+	−	−	−
B40-19	+	−	−	+	−	−	−	−
B40-20	+	−	−	−	−	−	−	−
B40-21	+	−	−	−	−	−	−	−
B40-22	+	−	−	+	−	−	−	−
B40-23	+	−	−	−	−	−	−	−
B40-24	+	−	−	−	−	−	−	−
B40-25	+	−	−	−	−	−	−	−
B40-26	+	−	−	−	−	−	−	−
B40-27	+	−	−	−	−	−	−	−
B2151-1	+	+	−	+	+	+	−	−
B2151-2	+	+	−	+	+	+	−	−

[a] +, Host supportive of phage growth; −, host not supportive of phage growth.
[b] Strains HSP 39, C-19075, C-13700-2, C-13182, C-1416, C-8797, C-14021, C-13525, BBE-18, and B-1.
[c] Strains *B. ovatus* DSM 1896, *B. vulgatus* DSM 1447, *B. hypermegas* DSM 1672, *B. microfusus* ATCC 29728, *B. eggerthii* ATCC 27754, *B. distasonis* ATCC 85023, *B. thetaiotaomicron* DSM 2079, and *B. uniformis* ATCC 8492.

nally, a loopful from each tube was spotted onto overlayed host lawns plated on either MBAB-S when the sample was treated with chloroform or MBAB when the sample was filtered. Plates were incubated anaerobically at 37°C for 16 h. The results of the tests were recorded and computed by the use of the MPN table for coliform bacteria (26). Sewage and highly polluted samples were diluted, and series of three tubes were inoculated with 1 ml of each dilution into single-strength MBB tubes.

Phage strains and preparation of phage stocks. Phages were purified by three consecutive single-plaque isolations for the preparation of phage stocks. High-titer phage stocks were prepared by the soft-agar plate method described by Adams (1). Approximately 10^7 PFU were combined with 10^8 sensitive bacteria in tubes containing 2.5 ml of soft MBB agar and then poured onto MBAB plates. Following 16 h of anaerobic incubation at 37°C, the phages were harvested by adding 3 ml of the buffer for phages (described above) to each plate. After 1 h at 4°C, the buffer was collected and centrifuged at 2,000 × *g* for 15 min. The supernatant was then filtered through a 0.22-μm-pore-size membrane and stored at 4°C.

TABLE 3. Comparison of phage counts obtained by the double-agar layer (PFU) and the MPN techniques

Sample	MPN upper limit	MPN/100 ml	MPN lower limit	PFU/100 ml
1	3.3×10^4	3.5×10^3	6.4×10^2	1.0×10^3
2	3.8×10^4	1.1×10^4	1.5×10^3	1.0×10^2
3	2.4×10^4	4.6×10^3	7.1×10^2	8.0×10^2
4	4.4×10^4	1.5×10^4	3.0×10^3	3.0×10^2
5	1.3×10^3	2.4×10^2	36	0
6	2.1×10^5	2.0×10^4	7.0×10^3	1.0×10^2
7	2.4×10^4	4.6×10^3	7.1×10^2	6.0×10^2
8	4.4×10^5	1.1×10^5	3.0×10^4	1.4×10^3
9	3.8×10^3	9.3×10^2	1.5×10^2	1.5×10^2
10	9	4	<0.5	0

Determination of host range. The *Bacteroides* strains were grown in MBB and overlayed on MBAB plates. Then, drops of phage stocks containing 10^8 to 10^9 PFU/ml were spotted onto the respective plates. The plates were incubated anaerobically at 37°C for 16 h, after which areas of phage lysis within the bacteria lawns were clearly visible.

RESULTS

Strain choice. A total of 13 sewage samples were tested for the presence of bacteriophages by using 12 different strains as indicator hosts (Table 1). In every sample, *B. fragilis* HSP 40 was the host that allowed for the greatest recovery of bacteriophages.

Host range. During this study 25 bacteriophages infecting *B. fragilis* HSP 40 were isolated, and their host range was assayed among 25 strains of different *Bacteroides* species. None of the bacteriophages were active against *Bacteroides* spp. other than *B. fragilis* (Table 2). Moreover, all of the bacteriophages had a rather restricted host range among the *B. fragilis* strains tested (Table 2).

To ascertain the identity of strain HSP 40, six bacteriophages were isolated on type strain *B. fragilis* DSM 2151 (ATCC 25285). Two of them were active against strain HSP 40. Both were added to the host range study. Moreover, one of the phages isolated on strain HSP 40 was active against strain DSM 2151.

Method optimization. Ten samples were analyzed for bacteriophages active against *B. fragilis* HSP 40 by using the double-agar layer technique to determine PFU and by the MPN method adapted to bacteriophages. The MPN method was more efficient than the double-agar layer technique

TABLE 4. Counts obtained following removal of residual bacteria by two methods after phage enrichment

Sample	MPN/100 ml by:	
	Method 1[a]	Method 2[b]
1	1.1×10^4	1.1×10^4
2	2.4×10^3	2.1×10^3
3	2.4×10^2	9.3×10^2
4	6.4×10^2	1.2×10^3
5	2.4×10^2	43
6	43	23
7	23	23
8	23	15
9	93	15
10	1.1×10^3	9.3×10^2

[a] Method 1, Treatment with chloroform and plating on MBAB-S.
[b] Method 2, Filtering through a membrane (pore size, 0.22 μm) and plating on MBAB.

TABLE 5. Bacteriophages active against *B. fragilis* HSP 40 in feces of various animals

Animal species	No. tested	No. (%) of positive tests
Human	40	4 (10)
Cow	40	0 (0)
Pig	50	0 (0)
Rabbit	21	0 (0)
Mouse	28	0 (0)
Hen	20	0 (0)
Quail	10	0 (0)

(Table 3) with respect to both the counts of phages and the percentage of samples from which phages were recovered.

Because the purified bacteriophages described in Table 2 were not inactivated by chloroform and because the filtering of a fraction from each MPN tube was expensive and time-consuming, chloroform treatment and centrifugation were used instead of filters to obtain phage suspensions that were free of bacteria. This method, however, described by Adams (1) did not work because under anaerobic conditions bacterial growth occurred in the spots, even after treatment with chloroform. Bacteria surviving chloroform treatment were found to be anaerobic spore formers. To eliminate this problem, potassium sorbate was added to MBAB medium and the pH was lowered. An optimal concentration of potassium sorbate of 0.05% at pH 5.7 (MBAB-S medium) was determined. Growth of *B. fragilis* HSP 40 was uninhibited, while spore germination was prevented. Results in Table 4 indicate that after bacteriophage enrichment, treatment with chloroform and the use of MBAB-S medium gave results similar to those obtained by filtering through a 0.22-μm-pore-size membrane.

Bacteriophages in feces. Feces from humans and several homeothermic animals, which are more likely to contribute to water pollution, were analyzed for the presence of bacteriophages active against *B. fragilis* HSP 40 (Table 5). Bacteriophages were isolated from 4 of 40 humans studied. No phages were recovered from the feces of any of the animals tested. Successive surveys of two of the humans from whom phages were found in the feces revealed a variable pattern of phage excretion. Incidence and levels of phages in the feces ranged from 24 to 2.4×10^8/g in one and from 0 to more than 2.4×10^8/g in the other.

Bacteriophages in environmental samples. A total of 163 environmental samples were tested for the presence of bacteriophages active against *B. fragilis* HSP 40. Bacteriophages were consistently found in sewage-polluted waters and sediments (Table 6). They were detected in all the samples from raw sewage and highly polluted rivers, with values that approached 10^5 MPN of bacteriophages per 100 ml and with average values of about 10^4 and a median count of 4.0×10^3. The percentage of positive isolations decreased to 77.2% in seawater near Barcelona, where the greatest counts reached 10^3 MPN per 100 ml. Mean and median values were 10^2 and 4, respectively. Phages were isolated in 100% of river and 91% of marine sediments from the rivers and coastal area studied. Also, phages were detected in 4 of 19 groundwater samples, corresponding to an aquifer from which enteroviruses have occasionally been isolated; bacteriophages were isolated from an enrichment of 100 ml of groundwater.

Bacteriophages infecting *B. fragilis* HSP 40 were never recorded from samples of waters and sediments that had not received sewage pollution (Table 6).

DISCUSSION

One of the problems in detecting bacteriophages in the environment is the lack of an universal plating indicator for particular bacterial species. Moreover, some strains consistently give higher counts than others (15, 27). Our results are in good agreement with those of previous reports because *B. fragilis* HSP 40 consistently gave higher counts than the other *Bacteriodes* strains tested. Therefore, strain HSP 40 was used for the enumeration of bacteriophages in feces and environmental samples. The percentage of human feces samples from which phages were isolated and the phage numbers in positive samples were higher than those found by other investigators (8, 32, 40, 45). Therefore, strain HSP 40 seems to be an efficient indicator strain for the detection and enumeration of bacteriophages active against *B. fragilis* in environmental samples.

The host range of phages isolated by using *B. fragilis* HSP 40 as the host was very narrow. This high specificity of bacteriophages infecting *Bacteroides* spp. has already been observed by other investigators (8, 15, 33, 40). No polyvalent phages active against *B. fragilis*, which are so frequently observed with coliphages (17, 18, 43, 53), seemed to occur in *Bacteroides* spp. This high strain specificity may be the reason for the failure to isolate phages from the feces of the nonhuman animals tested. In addition, this high specificity may indicate that the similarity among strains of *Bacteroides* infecting different animal species is very low. It can therefore be concluded that bacteriophages active against *B. fragilis* are good indicators of human fecal pollution. Moreover, according to the narrow host range shown by the majority of bacteriophages infecting *Bacteroides* spp., results of this study confirm the classification of strain HSP 40 as *B. fragilis*.

Although the recovery of *Bacteroides* phages from raw sewage has already been reported (8, 15, 40), no data have

TABLE 6. Levels of bacteriophages active against *B. fragilis* HSP 40 in water and sediments

Samples	No. of samples	% Samples positive for phages	Maximum value/100 ml	Minimum value/100 ml	Mean value/100 ml	Median value/100 ml
Sewage	33	100	1.1×10^5	7	6.2×10^3	1.1×10^3
River water[a]	22	100	1.1×10^5	93	1.6×10^4	4.6×10^3
River sediment[a]	5	100	4.6×10^5	90	1.08×10^5	4.3×10^3
Seawater[a]	22	77.2	1.1×10^3	<3	1.2×10^2	4
Marine sediment[a]	12	91.0	43	<3	13.4	9
Groundwater[a]	19	21.0	NK[c]	0		
Nonpolluted[b]	50	0				

[a] Samples from areas with sewage pollution.
[b] Water and sediments from areas without known sewage pollution.
[c] NK, Not known, because we only tested whether they were present in 100 ml.

been available on levels of recovery. By using the MPN method, a method which consistently gave values significantly higher than those by PFU counts, bacteriophages active against *B. fragilis* HSP 40 were isolated from all sewage samples tested. The values, which ranged from 7 to 1.1×10^5 MPN of phages per 100 ml, were significantly higher than those reported for human enteric viruses in sewage around the world, which have ranged from 0 to 2.0×10^3 per 100 ml (2, 9, 13, 28, 31, 36, 38, 42, 44, 46, 47, 55), and lower (about 2 log units) than those found for coliphages in sewage (16, 25, 35). Although the percentage of humans excreting bacteriophages that are active against *B. fragilis* HSP 40 is low, which is in agreement with data from other investigators (31), the amounts excreted are so high that they can account for the values recovered from sewage. Moreover, they were isolated from many different types of sewage-polluted waters and sediments, although either the percentage of positive samples or the phage counts decreased as we moved further away from the pollution source. On the contrary, phages were never detected in waters or sediments without known sewage pollution.

These data indicate that bacteriophages active against *B. fragilis* can be considered human sewage indicators, a concept that has been proposed by other investigators for coliphages (10). Further studies about the behavior of *B. fragilis* bacteriophages in the environment need to be done to ascertain whether they deserve to be considered as potential surrogate indicators of human viruses in the environment.

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