Diversity of *Bacteroides fragilis* Strains in Their Capacity To Recover Phages from Human and Animal Wastes and from Fecally Polluted Wastewater

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Great differences in capability to detect bacteriophages from urban sewage of the area of Barcelona existed among 115 strains of *Bacteroides fragilis*. The capability of six of the strains to detect phages in a variety of feces and wastewater was studied. Strains HSP40 and RYC4023 detected similar numbers of phages in urban sewage and did not detect phages in animal feces. The other four strains detected phages in the feces of different animal species and in wastewater of both human and animal origin. Strain RYC2056 recovered consistently higher counts than the other strains and also detected counts ranging from 10^1 to approximately 10^3 phages per ml in urban sewage from different geographical areas. This strain detected bacteriophages in animal feces even though their relative concentration with respect to the other fecal indicators was significantly lower in wastewater polluted with animal feces than in urban sewage.

Techniques for distinguishing between human and animal fecal pollution are necessary for assessing the overall protection of water supplies and implementing effective remediation, for epidemiological studies, and even for legal purposes when it is necessary to determine the source of environmental contamination. Chemical (24) and microbiological (11, 20, 23, 25, 26, 30, 35) methods have been proposed for such purposes. Among the microbiological methods, the detection of bacteriophages infecting strain HSP40 of *Bacteroides fragilis* has very attractive features (7, 12, 15, 21, 33–35). Strain HSP40 detects numbers of phages ranging from 10^1 to 10^2 per ml of urban sewage in some geographical areas, such as Southern Europe, South Africa, and Israel (3, 4, 9, 13, 32, 35). However, it recovers lower numbers of phages in sewage from other geographical areas, such as the United States (22). This jeopardizes its usefulness as a universal method.

The purpose of this study was to search for new host strains of *B. fragilis* that detect more phages than strain HSP40, to assess their usefulness in distinguishing phages originating in the human gut from those originating in the guts of different animal species, and to assess whether they can detect bacteriophages in a wider geographical area than strain HSP40 does.

One hundred and fourteen strains provided by the Microbiology Services of three hospitals in Barcelona and Madrid plus strain HSP40 (ATCC 51477) (34) were used as host strains for bacteriophages infecting *B. fragilis*. *Escherichia coli* HS (10), and *Salmonella typhimurium* WG49 (17) were used to enumerate F-specific bacteriophages. The double-agar layer technique (1) was used for the detection of *B. fragilis* phages as previously described (36), and for the detection of F-specific bacteriophages using either *E. coli* HS (10) or *S. typhimurium* (17) as host strains, all quantifications were done in duplicate. Fecal coliform bacteria were enumerated by standard methods (2). Very diverse samples polluted with human and animal feces were analyzed. Human fecal samples were obtained from 43 healthy volunteers. Fecal samples from cattle, pigs, poultry,

horses, and sheep were either liquid manure, lixiviates of recent solid manure, or recent solid manure. Raw urban sewage samples were collected from inflowing waters of sewage treatment plants from the geographical sites indicated below. Samples of wastewater from different slaughterhouses were obtained as samples containing a mixture of feces from many different animals. All samples were collected in sterile bottles and kept in the dark at 4°C until examination. Samples from the Barcelona area were examined within 6 h of collection. The samples from other countries were kept at 4°C or frozen (-20°C) for between 2 days and 1 week before the assay. Previous studies (data not shown) proved that numbers of phages in sewage do not decrease significantly under these conditions.

For fecal analyses, aliquots were suspended in peptone saline at a ratio of 1:10 (wt/vol). The mixture was thoroughly mixed by magnetic stirring for 1 h at 4°C. The large particles were then allowed to sediment for 5 min at 4°C, and the supernatant was decontaminated with chloroform 1/3 (vol/vol) as described elsewhere (1). To analyze fecal coliform bacteria, 1 g of feces was added to 10 ml of 1/4-strength Ringer's solution. After vigorously shaking the mixture for 10 min, 10-fold dilutions were performed, and bacteria were enumerated as indicated above. For the quantification of phages from sewage, samples were decontaminated by filtration through low-protein-binding polyvinylidene difluoride membrane filters (Millex GV; Millipore) as described elsewhere (36).

Selected strains were phenotypically characterized. The biochemical studies were done with API 20A and API 50CH kits (BioMérieux, Marcy l'Etoile, France). Tests for sensitivity to antibiotics were performed on BPRM agar with filter paper discs from BBL (Cockeysville, Md.), Neosensitabs (Taastrup, Denmark), and Oxoid (Basingstoke, England). The antibiotics tested were amoxicillin (25 μg), ampicillin (30 and 10 μg), chloramphenicol (60 μg), cephalosporin (66 μg), cefazolin (30 μg), ceftriaxone (30 μg), erythromycin (15 and 78 μg), streptomycin (10 and 100 μg), kanamycin (10, 30, and 100 μg), methicillin (5 μg), nalidixic acid (30 μg), neomycin (30 and 120 μg), nitrofurantoin (260 μg), novobiocin (30 μg), oxacillin (1 μg), oxolinic acid (10 μg), penicillin G (5 IU), polymyxin (30

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TABLE 1. Levels of bacteriophages recovered in urban sewage from the Barcelona area using different *B. fragilis* host strains

No. of strains	Range of PFU/ml[a]	Ratio of range of PFUs to numbers of bacteriophages detected by strain HSP40[b]
49	0	NC[c]
49	0–10	<0.5
14	10–100	0.5–2.0
3	>100	>2.0

[a] Average value of at least two tests performed in duplicate.
[b] Phages infecting *B. fragilis* HSP40 were enumerated in all samples.
[c] NC, not calculable.

TABLE 2. Cross-reactivity of *B. fragilis* phages isolated with different strains from urban sewage

Strain for which sensitivity was tested	Cross-reactivity (%) for *B. fragilis* strains with which phages were isolated[a]					
	HSP40	RYC4023	RYC3234	RYC2056	RYC3317	RYC3318[b]
HSP40		100	0	0	0	0 (0.15)
RYC4023	88		0	0	0	2 (0.30)
RYC3234	1	1		25	28	19
RYC2056	0	0	18		59	63
RYC3317	0	0	30	73		54
RYC3318	0	0	13	87	33	

[a] These percentages indicate the sensitivity of a strain to detect phages isolated with the other. At least 100 plaques were tested as indicated in the text.
[b] Numbers in parentheses are the percentages of sensitivity of strains HSP40 and RYC4023 to 600 plaques isolated on RYC3318 from abattoir sewage.

IU), tetracycline (80 μg), tobramycin (10 μg), and netilmicin (30 μg).

To test the capacity of phages isolated on a given strain of *B. fragilis* to infect other strains, a number of randomly chosen plaques on a determined host were inoculated with a sterile toothpick on a monolayer of the strain to test for sensitivity. After 18 h of incubation at 37°C under anaerobic conditions, an area of lysis could be seen on the monolayer if the host was sensitive to the bacteriophage.

Great variability in the capability of the 115 *B. fragilis* strains studied to recover bacteriophages from urban sewage from the Barcelona area was observed (Table 1). Only 66 strains recovered bacteriophages from 10-ml samples of sewage, and some strains recovered significantly greater numbers than others. Levels recovered by HSP40, previously shown to be very consistent in urban sewage in the Barcelona area (3), were tested in all samples to guarantee the similarity of the fecal load of the samples. Strain RYC2056 detected the highest numbers, which exceeded by a factor of 1,000 the numbers detected by other strains. This difference is greater than that observed for other fecal bacteria, such as *E. coli*, in which differences reach values of 10 to 100 (13, 16, 19, 28, 31). However, due to the high numbers of strains already tested, it is not foreseeable that more efficient natural strains will be found for the detection of phages that infect *B. fragilis*.

Strains RYC2056, RYC3317, RYC3318, RYC3234, and RYC4023, which gave consistently higher recoveries than strain HSP40 in the preliminary study, plus strain HSP40 were studied further. First, they were characterized phenotypically. From 34 biochemical tests assayed, the maximum differences observed were in the fermentation of inulin, amygdalin, and glycogen. Differences in sensitivity to antibiotics were observed only for ceftriaxone and amoxicillin. Strains HSP40 and RYC4023 presented a high linkage, whereas strain RYC3234 was the most differentiated in a dendrogram (Fig. 1) drawn by the application of hierarchical cluster analysis (SPSS Inc., Chicago, Ill.). However, despite the great phenotypic similarity, major differences in sensitivity to bacteriophage isolates were observed (Table 2). Nearly all the phages isolated with HSP40 could infect RYC4023 and vice versa. However, very few of the phages isolated with the other strains could replicate onto either HSP40 or RYC4023. These data show that strains HSP40 and RYC4023, which do not detect phages in animal feces, as shown below, can support the replication of a very small fraction (less than 1%) of phages present in animal feces. The other four strains showed varied degrees of cross-sensitivity to phages, with percentages of cross-susceptibility ranging from 13 to 87%. Some agreement between the phenotypic characteristics of the different strains and the range of phages infecting them was observed. Thus, strains HSP40 and RYC4023, which are almost identical phenotypically, showed similar patterns regarding sensitivity to bacteriophages, whereas RYC3234 was the most different in terms of both biochemical characteristics and sensitivity to bacteriophages. The great differences in sensitivity to phages of strains of *B. fragilis* and the narrow host range of phages infecting *B. fragilis* are in agreement with previous reports for species of *Bacteroides* (6, 8) and strains of *B. fragilis* (34).

Results of detection of bacteriophages infecting strains HSP40, RYC4023, RYC2056, RYC3317, RYC3318, and RYC3234 in animal fecal samples are shown in Table 3. Some, for example RYC2056, detected phages in feces of a range of animals in a percentage of samples similar to the percentage of samples from which F-specific bacteriophages were isolated, and which do not differ significantly from data reported elsewhere regarding the presence in feces of somatic coliphages and F-specific bacteriophages (15, 18, 30). Other strains, such as HSP40 (15, 34) and RYC4023, did not detect phages from animal feces. For human feces, only the presence of bacteriophages infecting RYC2056 was analyzed, and they were isolated in 28% of the samples, which is clearly higher than the
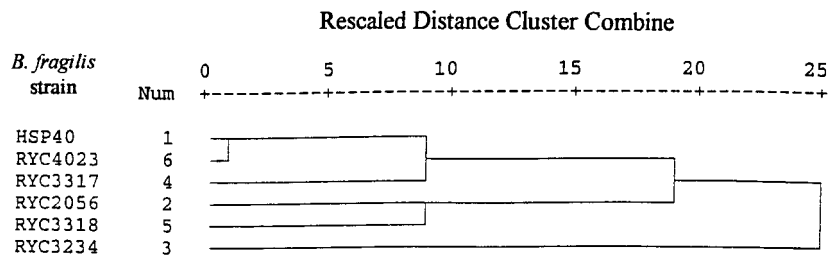

FIG. 1. This phenotype-based dendrogram reflects the relationship between the different strains of *B. fragilis*. The dendrogram was drawn using average linkage (between groups). Analysis was based on the euclidian distance square.

TABLE 3. Percentage of recovery of bacteriophages of *B. fragilis* from feces of various animal species using different host strains, F-specific phages, and levels of fecal coliforms

Host strain	% Positive samples from:				
	Pigs ($n = 13$)	Poultry ($n = 14$)	Cows ($n = 11$)	Sheep and horses ($n = 7$)	Humans ($n = 43$)
B. fragilis HSP40	0	0	0	0	10–13[a]
B. fragilis RYC4023	0	0	0	0	ND[c]
B. fragilis RYC3234	31	0	9	0	ND[c]
B. fragilis RYC2056	31	29	0	0	28
B. fragilis RYC3317	54	29	18	0	ND[c]
B. fragilis RYC3318	46	14	0	14	ND[c]
E. coli HS(pFamp)R[b]	46	50	9	29	ND[c]
Fecal coliforms	100	100	100	100	ND[c]

[a] Data obtained from references 15 and 34.
[b] Host strain for F-specific phages.
[c] ND, not done.

percentage described for phages infecting HSP40 (15, 34). Probably due to the great diversity indicated above regarding sensitivity to bacteriophages, the different strains of *B. fragilis* differ in their capability to support replication of phages present in feces of different animals. The presence of phages in feces depends on the presence in the gut of host strains which are similar in terms of receptors and modification-restriction enzymes to the tested strain and which as a result are able to support phage replication. The great variability in infectivity of bacteriophages infecting *B. fragilis* is then a consequence of the variability between strains of *B. fragilis* present in the guts of different animal species. *Bacteroides* species have evolved in confined environments and have differentiated in accordance with nutrition and other characteristics of the guts of different animal species. The data presented here suggest that phages infecting *Bacteroides* may be a valuable tool in studying the variability of the *Bacteroides* present in the microflora of human and animal communities, which have been shown to vary depending on diet, age, etc. (5, 27).

In an extended study of urban sewage of the area of Barcelona (Table 4), the six strains recovered phages from all the samples. Strain RYC2056 recovered significantly (Student's *t* test; $P < 0.01$) higher numbers of bacteriophages than strains HSP40 and RYC4023 in all samples studied. The other strains recovered significantly (Student's *t* test; $P < 0.01$) higher numbers of phages than strain HSP40 in most of the samples.

Average values of F-specific bacteriophages detected in the samples outnumbered those of *B. fragilis* by factors ranging from 40 to 100 for HSP40 and from 10 to 20 for RYC2056. The average number of fecal coliform bacteria was about 20-fold the number of F-specific coliphages, which is the normal ratio in sewage from different geographical areas (13, 17, 29, 35).

The fecal loads of slaughterhouse wastewater studied were higher than those of urban sewage in terms of the values of F-specific bacteriophages and fecal coliforms, which were on average 10 times greater than those of urban sewage (Table 4). In these samples, the ratio of F-specific bacteriophages to fecal coliforms is very similar to the ratio in urban sewage. Of the *B. fragilis* bacteriophages, only strains RYC2056, RYC3317, and RYC3318 recovered bacteriophages from all samples analyzed. Once again, strain RYC2056 detected numbers significantly higher than the numbers detected by the other strains (Student's *t* test; $P < 0.01$). However, in this case the average values of F-specific bacteriophages outnumbered the average values of phages detected by strain RYC2056 by a factor greater than 10^3. Strains HSP40 and RYC4023 recovered very low numbers of phages in some of the samples from slaughterhouse sewage, whereas they were not detected in animal feces. But, in this case the average values of F-specific bacteriophages exceeded the average values of phages detected by strain HSP40 by a factor greater than 10^4.

Since strain RYC2056 recovered more bacteriophages than the others in the area of Barcelona, studies to determine its capability to recover phages from urban (Table 5) and abattoir (Table 6) wastewater samples from different geographical areas were undertaken. Those samples were analyzed for the presence of F-specific bacteriophages and phages infecting *B. fragilis* HSP40 and RYC2056. Bacterial densities were not determined, since bacterial densities could not be conserved during transportation of the samples. Levels of F-specific phages ranged from 1.2×10^3 to 5.9×10^4 PFU per ml, which are the usual values for urban sewage of developed countries (3, 13, 17, 29). These numbers confirm the properness of the method used for sample transportation. Numbers of phages detected by HSP40 ranged from 0 to 4.5×10^2 PFU per ml. Strain RYC2056 recovered significantly higher (Students' *t* test; $P < 0.01$) and more constant numbers than HSP40, ranging from 2.2×10^1 PFU per ml up to 8.1×10^2 PFU per ml (Table 5). The analyses of bacteriophages from slaughterhouse wastewater of the same geographical areas showed the presence of phages in a few samples when the host used was RYC2056, but no phages were found when HSP40 (Table 6) was the host. F-specific phages were detected in all the samples

TABLE 4. Bacteriophages infecting different *B. fragilis* strains, F-specific phages, and fecal coliforms in different types of sewage from Barcelona area (PFU per ml or CFU per ml)

Host strain	Bacteriophage result for urban sewage ($n = 12$)				Bacteriophage result for slaughterhouse wastewater ($n = 18$)			
	Range (minimum–maximum)	% Positive samples	Arithmetic mean	Median	Range (minimum–maximum)	% Positive samples	Arithmetic mean	Median
B. fragilis HSP40	3.2×10^1–1.9×10^2	100	8.2×10^1	7.4×10^1	0–7.5×10^0	39	1×10^0	0
B. fragilis RYC4023	3.3×10^1–1.8×10^2	100	9.3×10^1	9.6×10^1	0–1.6×10^1	28	1.4×10^0	0
B. fragilis RYC3234	4.6×10^1–1.9×10^2	100	9.4×10^1	8.8×10^1	0–2.3×10^1	94	5.9×10^0	3.9×10^0
B. fragilis RYC2056	8.2×10^1–4.4×10^2	100	2.4×10^2	2.1×10^2	2.9–2.4×10^2	100	3.7×10^1	2.0×10^1
B. fragilis RYC3317	5.9×10^1–1.9×10^2	100	1.5×10^2	1.4×10^2	1.4–2.0×10^2	100	2.2×10^{1b}	9.4×10^0
B. fragilis RYC3318	4.2×10^1–3.3×10^2	100	1.7×10^2	1.5×10^2	0.2–7.8×10^1	100	1.5×10^1	7×10^0
E. coli HS(pFamp)R[a]	1.4×10^3–7.6×10^3	100	4.1×10^3	3.6×10^3	1.6×10^2–1.7×10^5	100	3.7×10^4	4.0×10^3
Fecal coliforms	7.9×10^3–1.9×10^5	100	6.3×10^4	6.0×10^4	2.0×10^4–6.8×10^6	100	9.6×10^5	3.0×10^5

[a] Host strain for F-specific phages.
[b] $n = 17$.

TABLE 5. Levels of bacteriophages of *B. fragilis* and F-specific phages in urban sewage of different countries

Country	Bacteriophage level (PFU/ml) of:		
	B. fragilis HSP40	*B. fragilis* RYC2056	*S. typhimurium* WG49[a]
The Netherlands	1	4.6×10^2	5.1×10^3
The Netherlands	2.6	7.7×10^2	7.6×10^3
Ireland	1.4	3.0×10^2	4.6×10^3
Ireland	1.6	4.4×10^2	6.9×10^3
Austria	8.5	8.1×10^2	1.6×10^3
Austria	0.5	6.1×10^2	2.2×10^3
Portugal	0.4	1.8×10^2	1.8×10^4
Portugal	0.1	1.0×10^2	3.8×10^4
Portugal	0	1.0×10^2	5.5×10^3
Germany	2.2	7.8×10^2	4.8×10^3
Germany	1.3	6.0×10^2	2.2×10^3
Sweden	0.9	2.2×10^1	1.9×10^3
France	3.1×10^1	2.3×10^2	1.2×10^3
South Africa	1.1×10^2	1.8×10^2	1.2×10^4
South Africa	4.5×10^2	ND[b]	5.9×10^4
South Africa	1.2×10^2	5.4×10^2	1.7×10^4
South Africa	2.3×10^2	5.0×10^2	2.4×10^4

[a] Host strain for F-specific bacteriophages.
[b] ND, not done.

analyzed, although the numbers were more variable than those of urban sewage. Strain RYC2056 detected a significant number of phages in more areas than HSP40 did.

When all the quantitative data belonging to samples (mainly polluted with human feces) and those belonging to samples of animal feces and abattoir sewage were grouped, Students' *t* test results indicated that the data for F-specific bacteriophages did not differ significantly ($P < 0.01$) between the two groups of samples, whereas numbers of phages infecting HSP40 and RYC2056 did ($P < 0.01$). To minimize the effect of the fecal load of the samples studied, the ratios of F-specific bacteriophages to phages infecting HSP40 and F-specific bacteriophages to phages infecting RYC2056 from each sample were calculated. Both ratios significantly (Student's *t* test; $P < 0.01$) differentiated the samples with pollution of human origin from those with pollution of animal origin.

In spite of the fact that strain RYC2056 detects bacteriophages in nonhuman feces, it presents some attractive features to be further assayed as a potential host strain for the detection of bacteriophages infecting *B. fragilis*, which are a good model of human viruses in the environment. Indeed, it detects good numbers of bacteriophages in a wide range of geographic areas and, from the results of the statistical analysis of the data set

TABLE 6. Levels of bacteriophages of *B. fragilis* and F-specific phages in wastewater from animal origin of different countries

Country	Bacteriophage level (PFU/ml) of:		
	B. fragilis HSP40	*B. fragilis* RYC2056	*S. typhimurium* WG49[a]
The Netherlands	0	3.4×10^3	3.0×10^4
The Netherlands	0	8.6×10^1	3.8×10^3
Ireland	0	0	4.8×10^2
Denmark	0	0	1.0×10^1
Portugal	0	0.8	2.2×10^2
Germany	0	1.3	5.7×10^3
South Africa	0	0	2.0×10^2
South Africa	0	0	5.0×10^3

[a] Host strain for F-specific bacteriophages.

presented herein, it may be more useful than other indicators in differentiating human from animal fecal pollution.

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