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Method for Isolation of *Bacteroides* Bacteriophage Host Strains Suitable for Tracking Sources of Fecal Pollution in Water

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Bacteriophages infecting *Bacteroides* are potentially a good tool for fecal source tracking, but different *Bacteroides* host strains are needed for different geographic areas. A feasible method for isolating *Bacteroides* host strains for phages present in human fecal material is described. Useful strains were identified for application in Spain and the United Kingdom. One strain, GA-17, identified as *Bacteroides thetaiotaomicron*, was tested in several locations in Europe with excellent performance in Southern Europe.

Microbial source tracking methods are designed to enable researchers to uncover the sources of fecal pollution in a water body (19). Bacteriophages infecting *Bacteroides* are potential tools for microbial source tracking (4, 13, 22, 24, 26, 29). However, it is well documented that *Bacteroides* host strains vary in their ability to discriminate between phages of different sources but also that phage detection by a given host strain varies geographically. Thus, *Bacteroides fragilis* strain HSP40 detects good numbers of phages in different areas of the Mediterranean region (4, 9, 10, 28, 29, 30) and in South Africa (12), but it fails to detect significant numbers of phages in Northern Europe (22) and the United States (15). In contrast, other strains, such as RYC 2056, detect similar numbers of phages in different geographical areas but do not discriminate between the sources of fecal pollution (5, 7, 18, 22). Strains tested in the United States to date appear to behave like RYC 2056 (15).

Limitations of existing source tracking methods (19, 24, 25, 26, 27), combined with the good source tracking performance of strain HSP40 in certain geographical areas (4, 9, 12, 28, 30), along with increasing information about the specificity between the animal host and the bacteria of the *Bacteroides* group (11, 32) and the narrow host ranges reported for phages infecting *Bacteroides* (6, 8, 16, 22, 30), prompted our search for new *Bacteroides* host strains.

We describe here a rapid method for isolating and further testing *Bacteroides* host strains potentially useful for source tracking.

Isolation of new hosts for phages infecting *Bacteroides*. Four trials for isolation of *Bacteroides* strains from raw municipal sewage from Spain (two trials), Colombia (one trial), and the United Kingdom (one trial) were carried out by two independent operators.

Decimal dilutions of sewage samples were plated onto *Bacteroides* bile esculine agar (17) and incubated at 36°C (±2°C) for 44 (±4) h in anaerobic jars. Anaerobiosis was achieved with commercial anaerobic generators (Merck KGaA, Darmstadt, Germany). Black colonies with a black or dark halo (17) were picked and plated for pure culture on *Bacteroides* bile esculine agar plates incubated under aerobic and anaerobic conditions (anaerobic jars). Gram staining of isolates growing only under anaerobic conditions was carried out. Gram-negative obligate anaerobic rods isolated at this stage (level 1 isolates) (Table 1) were further processed. They were grown in BPRM broth at 36°C (±2°C) for 18 (±2) h in anaerobic conditions. *Bacteroides* phage recovery medium (BPRM) is the medium used for bacteriophage enumeration. Isolates showing good growth after overnight incubation were used as host strains for the enumeration of phages present in reference phage suspensions.

Phages infecting all isolates as well as *Bacteroides fragilis* strain RYC 2056 (ATCC 700786) were enumerated by the double-agar-layer method on BPRM agar and incubated in anaerobic jars according to the ISO standard method (2, 3). Reference suspensions of phages naturally occurring in fecally contaminated samples stored in multiple vials, with high intra- and intervial homogeneity and stability, containing between 100 and 300 PFU on *B. fragilis* RYC 2056 were used to determine the isolates' efficiency for detecting phages. These suspensions were prepared as previously described (20). These suspensions were adjusted with phages infecting *B. fragilis* RYC 2056 (ATCC 700786), since fairly constant numbers are found in municipal raw sewage and in animal fecal wastes (5, 7, 18, 22). These reference suspensions were prepared in advance so that level 1 isolates could be rapidly and accurately tested for the presence of phages.

Five hundred microliters of a reference suspension of

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TABLE 1. Isolates obtained in four independent trials using municipal wastewater from Spain, Colombia, and the United Kingdom

Trial	Origin	No. of samples	No. of isolates	No. of isolates in:			Strain designation
				Level 1[a]	Level 2[b]	Level 3[c]	
1	Spain	8	259	113 (43.6)	7 (2.7)	1 (0.4)	GA-17
2	Spain	13	374	250 (66.8)	10 (2.7)	2 (0.5)	H1-8 and H16-10
3	Colombia	1	30	15 (50.0)	1 (3.3)	1 (3.3)	HB-13
4	U.K.[d]	3	540	336 (62.2)	53 (9.8)	1 (0.2)	GB-124

[a] Level 1, gram-negative rods growing in strict anaerobic conditions. Percentages of positive colonies are given in parentheses.
[b] Level 2, level 1 isolates demonstrating good growth on BPRM and phage counts of >0 PFU/ml of the wastewater reference containing around 100 PFU/ml using RYC 2056 as a host strain. Percentages of positive colonies are given in parentheses.
[c] Level 3, level 2 isolates showing specificity for municipal wastewater and showing counts similar to or higher than those of RYC 2056. Percentages of positive colonies are given in parentheses.
[d] U.K., United Kingdom.

phages was tested on monolayers of the level 1 isolates. Isolates giving plaque counts similar to or higher than those of RYC 2056 were processed for further analysis (level 2 isolates). The next step was to test phage reference suspensions obtained as described above from animal fecal sources (slaughterhouses and farms of cattle, pigs, and poultry wastes) on these level 2 isolates. Only isolates giving zero or very low counts with reference samples of animal origin were selected as potential new hosts for fecal source tracking (level 3 strains). Results regarding the number of colonies screened in each trial, successful isolations, and the names of the isolated strains are shown in Table 1.

Discriminatory capability was assessed by using the strains as host strains to test reference bacteriophage suspensions prepared from municipal wastewaters from Spain and the United Kingdom and with reference bacteriophage suspensions from different animal sources (Table 2). The five isolates detected numbers of bacteriophages in municipal wastewater from Spain similar to or higher than those detected by strain RYC 2056. The numbers of phages detected in municipal wastewater from the United Kingdom by strains GA-17 and GB-124 were about five times lower than values detected using strain RYC 2056, and the numbers detected by strain HB-13 were about 50 times lower than values given by strain RYC 2056. There are therefore geographical differences. Again the five strains detected either zero or very low numbers of phages in fecal material of animal origin. With regard to the plaques observed with the five host strains, all demonstrated clear plaques with the exception of strain GA-17, which gave a percentage of turbid plaques. However, in most samples tested during this phase, the presence of turbid plaques did not impair the interpretation of results. Strains GA-17, HB-13, and GB-124 emerged as the most promising strains for future source tracking studies. Taking into consideration the results of this phase, strain GA-17 was tested in different geographical locations.

At least one host strain was isolated per attempt. Therefore, the technique is feasible for the isolation of potential hosts of *Bacteroides* for source tracking purposes, allowing up to 500

TABLE 2. Discriminatory ability of the newly isolated host strains to detect phages present in reference suspensions of different fecal origins

Strain or category	No. of bacteriophages for[a,b]:				
	Human 1 (*n*)	Human 2 (*n*)	Cattle (*n*)	Pigs (*n*)	Poultry (*n*)
RYC 2056	3.8×10^2[c] (12)	1.4×10^2 (10)	4.0×10^2 (10)	3.7×10^2 (10)	1.8×10^2 (10)
GA-17	1.2×10^3 (12)	3.2×10^1[d] (10)	0 (10)	1.2×10^0 (10)	0 (10)
GA-17 > RYC[e]	10/12	1/10	0/10	0/10	0/10
RYC 2056	1.6×10^2 (14)	ND[f]	4.2×10^2 (7)	3.4×10^2 (8)	1.9×10^2 (7)
H1-8	8.6×10^1 (14)	ND	0 (7)	0 (8)	2.5×10^1 (7)
H1-8 > RYC[e]	11/14		0/7	0/8	0/7
RYC 2056	1.1×10^2 (6)	ND	1.6×10^2 (6)	3.4×10^2 (6)	2.6×10^2 (6)
H16-10	1.0×10^2 (6)	ND	0 (6)	0 (6)	6.9×10^1 (6)
H16-10 > RYC[e]	3/6		0/6	0/6	0/6
RYC 2056	8.1×10^1 (15)	1.3×10^2 (4)	1.8×10^2 (7)	3.4×10^2 (7)	1.8×10^2 (6)
HB-13	6.5×10^2 (15)	2.2×10^0[d] (4)	0 (7)	0 (7)	0.6 (6)
HB-13 > RYC[e]	15/15	0/4	0/7	0/7	0/6
RYC 2056	1.1×10^2 (12)	1.0×10^2 (9)	2.6×10^2 (7)	6.1×10^2 (8)	2.2×10^2 (8)
GB-124	5.0×10^2 (12)	2.5×10^1 (9)	0.17 (7)	0.33 (8)	0 (8)
GB-124 > RYC[e]	11/12	3/9	0/7	0/8	0/8

[a] Bacteriophage enumeration values (PFU/ml) are the averages for *n* experiments.
[b] Human 1 corresponds to reference municipal wastewater from Spain (Barcelona area), and Human 2 is reference municipal wastewater from the United Kingdom (Brighton). *n,* no. of samples.
[c] All samples of reference material were prepared as described in Materials and Methods. Values are the average for *n* samples tested.
[d] These samples showed very turbid plaques that were difficult to visualize.
[e] Number of samples showing higher numbers of PFU/ml with the tested strain than with strain RYC 2056.
[f] ND, not done.

TABLE 3. Results of 16s RNA characterization of the five isolates

Category	Relevant information for isolate:				
	GA-17	H1-8	H16-10	HB-13	GB-124
Size of amplicon (bp)	1,488	1,488	1,488	1,488	1,488
Size sequenced (bp)	1,407	1,390	1,410	1,401	1,385
Most homologous strain	*B. thetaiotaomicron* ATCC 29148	*B. fragilis*	*B. thetaiotaomicron* ATCC 29148	*B. thetaiotaomicron*	*B. ovatus* ATCC 8483T
Accession no.	L16489	X83940	L16489	AE016937	X83952
% Sequence identity	99	99	98	98.5	96.5
Reference[a]	21	23	21	32	23

[a] Reference for the most homologous strain.

colonies to be analyzed. It is also possible to isolate strains useful in different geographic areas. The total cost associated with each attempt to isolate new strains did not exceed 1,000 euros inclusive of consumables and labor (approximately 1 month). The technique has low development costs in comparison with emerging library-dependent source tracking techniques (19).

Identification of the isolates. The five selected strains underwent presumptive identification by amplification and sequencing of the 16S rRNA gene. As a template for the PCR, one microliter of bacterial lysate obtained after treatment at 95°C for 15 min as described elsewhere (14) was used. PCR amplification of the 16S rRNA gene was performed according to standard procedures using the Amplitaq DNA polymerase kit (Perkin-Elmer, Applied Biosystems). Primers used were Upper Bact16S (GCTACCTTCTTACGACTT) and Lower Bact16S (GAGTTTGATCCTGGCTC) (31) with an annealing temperature of 42°C. A 5-μl volume of each PCR product was analyzed by agarose (1%) gel electrophoresis, and bands were visualized by staining with ethidium bromide. The 1,488-kb PCR products obtained were used directly for sequencing. Sequencing was performed with the ABI PRISM BigDye 3.1 terminator cycle sequencing ready reaction kit in an ABI PRISM 3700 DNA analyzer (both from Perkin-Elmer; Applied Biosystems, Barcelona, Spain) according to the manufacturer's instructions. All sequences were performed in duplicate. Searches for homologous DNA sequences in the EMBL and GenBank database libraries were performed with the BLAST tool (http://www.ncbi.nlm.nih.gov).

Results of the genotypic characterization are summarized in Table 3. Three of the strains (GA-17, H16-10, and HB-13) showed high homology with *Bacteroides thetaiotaomicron*, one with *Bacteroides fragilis* (H1-8), and one with *Bacteroides ovatus* (GB-124). These results were in accordance with the results of phenotypical characterization using the commercial kit API 20A (BioMérieux, Marcy l'Etoile, France).

Evaluation of strain GA-17 in different European countries. Samples from municipal and hospital wastewaters from Cyprus, France, Spain, Sweden, and the United Kingdom and samples with fecal contamination of animal origin (wastewa-

TABLE 4. Values of bacteriophages of *Bacteroides* and fecal coliforms present in wastewaters with human fecal pollution and fecal material of animal origin from different European countries

Country	Origin	*n*[d]	Phages of RYC 2056[e]		Phages of GA-17[e]		FC[f]
			Avg no.	% Positive	Avg no.	% Positive	
Spain	Human	22	4.3×10^4	100	5.9×10^4	100	1.3×10^7
	Cattle	7	1.5×10^4	57	<50[a]	0	1.0×10^7
	Pig	9	1.2×10^5	100	2.5×10^2	50	5.0×10^7
	Poultry	8	5.7×10^4	100	<50	0	1.3×10^7
France	Human	14	2.6×10^4	100	2.4×10^4	100	4.0×10^6
	Cattle[b]	10	1.2×10^3	70	<50	0	3.2×10^6
Sweden	Human	10	2.2×10^3	90	3.6×10^3	80	2.0×10^6
	Cattle	8	1.4×10^2	25	<50	0	1.3×10^5
	Poultry	1	5.0×10^1	100	<50	0	3.2×10^2
United Kingdom	Human	10	1.1×10^4	100	2.9×10^3[c]	100	5.0×10^6
	Cattle	4	2.0×10^2	25	<50	0	2.5×10^7
	Pig	3	8.1×10^4	100	<50	0	1.6×10^8
	Poultry	3	1.4×10^2	66	<50	0	2.7×10^3
Cyprus	Human	10	3.3×10^4	100	1.2×10^4	100	2.5×10^7
	Pig	5	2.3×10^4	100	<50	0	1.0×10^7
	Poultry	5	1.1×10^3	100	<50	0	6.3×10^7

[a] Values under the detection limit of the experiment.
[b] Samples predominantly from cattle, although a few of them could have received inputs from pig and sheep.
[c] Very turbid plaques.
[d] *n*, no. of samples.
[e] No. of phages is expressed as PFU/100 ml.
[f] No. of fecal coliforms (CFU/100 ml).

ters from slaughterhouses or liquid slurries of cattle, pig, and poultry) were used to determine the discriminatory ability of one of the isolated strains (GA-17) in different geographical areas. Levels of fecal contamination in the samples were determined by enumerating fecal coliforms according to standard methods (1).

Results are shown in Table 4. The numbers of phages detected by strain GA-17 in municipal or hospital wastewaters were similar to or slightly higher than the number detected by strain RYC 2056, with average values ranging from 3.7×10^3 PFU per 100 ml in the United Kingdom to 1.1×10^5 PFU per 100 ml in Spain. These numbers have some numerical relationship with the density of fecal coliforms found in the wastewater samples tested. However, in samples from the United Kingdom, high percentages (>90%) of turbid plaques were reported, which were hardly visible to the untrained eye. The results reported herein include the turbid plaques detected by an experienced operator. This aside, the plaques obtained in samples from other locations were mostly clear and were counted by less-experienced operators. Therefore, we can conclude that as a result of geographical differences, it will be necessary to determine suitable host strains for use within specific regions. In contrast, phage counts in samples of animal origin were either zero or very low in all locations. Thus, the results with regard to strain GA-17 are extremely promising for the future of source tracking in Southern Europe and to a lesser extent in Sweden. In contrast, data for the United Kingdom were not as promising, and strains such as GA-17 or others that may be isolated with the method described here will have to be used.

In conclusion, we think that the detection and enumeration of phages infecting *Bacteroides* is an efficient tool to add to the "toolbox" needed for source tracking and that the method described here makes feasible the isolation of *Bacteroides* host strains for both different geographical areas and different fecal sources.

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