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Comparative Resistance of Phage Isolates of Four Genotypes of F-Specific RNA Bacteriophages to Various Inactivation Processes

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The effect of natural inactivation in freshwater, chlorination, ammonia, extreme pHs, temperature, and salt content on phage inactivation was evaluated on mixtures of F-specific RNA bacteriophage isolates belonging to genotypes I, II, III, and IV. The bacteriophages studied were previously but recently isolated from natural samples, characterized as F-specific RNA bacteriophages and genotyped by plaque hybridization with genotype-specific probes. Natural inactivation in river water was modeled by in situ incubation of bacteriophages inside submerged dialysis tubes. After several days bacteriophages of genotype I showed the highest persistence, which was significantly different from that of bacteriophages of genotype II, IV, or III. The pattern of resistance of phages belonging to the various genotypes to extreme pHs, ammonia, temperature, salt concentration, and chlorination was similar. In all cases, phages of genotype I showed the highest persistence, followed by the phages of genotypes II, III, and IV. The phages of genotypes III and IV were the least resistant to all treatments, and resistance of genotypes III and IV to the treatments was similar. Bacteriophages of genotype II showed intermediate resistance to some of the treatments. The resistance of four phages of genotype I to natural inactivation and chlorination did not differ significantly. These results indicate that genotypes III and IV are much more sensitive to environmental stresses and to treatments than the other genotypes, especially than genotype I. This should be taken into consideration in future studies aimed at using genotypes of F-specific RNA bacteriophages to fingerprint the origin of fecal pollution.

Fecal contamination of aquatic environments can hinder contact recreation and shellfish growing water and spoil drinking water, since it may include pathogenic microorganisms. There is a general belief that exposure to waters polluted with human feces is more hazardous than exposure to waters polluted with animal feces, although no sound epidemiological studies support this view. However, regardless of the health risks involved, management of fecal contamination of water can only improve if the sources of pollution are identified by water analysis and thus tools to distinguish between human and animal fecal sources are needed. Chemical and microbiological methods had been proposed for tracking the origin of fecal contaminants. Fecal sterols of human and animal origin have been used for this purpose (22). Microbiological indicators include the following: ratios of fecal coliforms to fecal streptococci (13); the presence of *Rhodococcus coprophilus* (23); the presence of some phenotypes (24) and ribotypes (25) of *Bifidobacterium* species; the presence of some phenotypes of *Bacteroides* species (21); the distribution patterns of ribotypes of *Escherichia coli* (7, 29); the distribution patterns of repetitive DNA sequences of *E. coli* (8); antibiotic resistance patterns (15); bacteriophages specific for various strains of *Bacteroides fragilis* (36); phage typing of *Staphylococcus aureus* (40); and the distribution pattern of serogroups of F-specific RNA phages.

F-RNA bacteriophages are divided into four main subgroups that can be recognized by serotyping. The structure and size of virions of all F-specific RNA bacteriophages are similar, as well as the genome size (12). Some good conservation of the nucleotide sequences between phages of serotypes I and II and between phages of serotypes III and IV exists (12). The main reported difference among the four groups of phages is that the genome of serotypes I and II lacks the readthrough protein. Studies with a number of phage isolates indicate that serotypes II and III are mainly isolated from human feces, whereas serotypes I and IV are usually found in animal feces (10–12, 17, 19, 28). More recently, it has been shown that the subgroups can also be grouped in four main genotypes that, with few exceptions, show overall comparability with serotypes (6, 18, 19). Probes for each genotype allow direct plaque hybridization (6, 14, 31), and then it is relatively easy to study the distribution of subgroups in water samples. Available data, which are limited, indicate that subgroups II and III predominate in water samples contaminated with human pollution and subgroups I and IV in those impacted with animal fecal wastes (6, 18, 19). This predominance is, however, clearer in recently polluted water than downstream of the original source of pollution. Indeed, effluents of water treatment plants and river water samples seem to show a displacement to predominance of genotypes I and II (9, 32, 37). Moreover, several observations suggest differences in the stability of different F-specific RNA bacteriophages and indicate a low stability or persistence of phages of genotype IV (G. M. Brion, J. S. Meschke, and M. D. Sobsey, Poster HRMP-A49, 10th Health-Related Wat. Microbiol. Symp., 2000; A. Havelaar, unpublished data).

Studying the distribution of genotypes of F-specific RNA bacteriophages for tracking the origin of fecal pollution only

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can be considered seriously if the members of the four genotypes show a similar pattern of resistance to an array of natural inactivating conditions, as well as to different disinfecting treatments. Otherwise, they will only be useful for tracking recent pollution. The resistance of the four genotypes of F-RNA bacteriophages to natural inactivation and to various treatments should then be examined.

We studied the response of various recent bacteriophage isolates belonging to the four genotypes to natural inactivation, to disinfecting processes such as chlorination, and to distinct conditions such as pH, ammonia, water temperature, and salinity, which may condition their survival in the environment.

MATERIALS AND METHODS

Bacteriophages. Recent bacteriophage isolates were used for the experiments described herein. They were selected from plaques belonging to different samples to avoid the selection of phages of the same clone. After plaque purification, they were partially characterized by plaquing them in F-plus and F-minus hosts to determine whether they were F specific, assaying their sensitivity to RNase to determine whether they were RNA phages and by plaque hybridization with the probes of the four genotypes to assign them to a genotype. Bacteriophages MS2, GA, Qβ, and FI were used as reference controls in all of these experiments. In any case, all of the bacteriophage isolates examined here were identified as F-specific RNA bacteriophages of the assigned genotype when the distributions of serotypes of F-specific RNA phages in environmental samples were studied.

Four phage isolates of each genotype were selected and included in the study presented herein.

Phages FR11, FR12, FR13, and F14 were isolated from bird feces, urban sewage, and the sewage of hospital samples and assigned to genotype I. Phages FR21, FR22, FR23, and FR24 were isolated from various very polluted river water samples and were assigned to genotype II. FR31, FR32, FR33, and FR34 were isolated from urban and hospital wastewater samples and assigned to genotype III. FR41, FR42, FR43, and FR44 were isolated from bird feces and assigned to genotype IV.

Bacteriophage suspensions. F-specific RNA bacteriophage suspensions were obtained by enrichment on *Salmonella enterica* serovar Typhimurium WG49 (NCTC 12484) as described by the International Organisation for Standardisation (ISO) (4). Dilutions to obtain "stock suspensions" with a given numbers of bacteriophages were prepared with peptone-saline solution as indicated by ISO (4) and stored at 4°C for short storage. Five percent sterile glycerol was added before freezing them at −70°C in the case of long storage according to the method of Schijven et al. (33).

The mixtures FR11+FR12+FR13+FR14 [referred to here as M(I)], FR21+FR22+FR23+FR24 [M(II)], FR31+FR32+FR33+FR34 [M(III)], and FR41+FR42+FR43+FR44 [M(IV)] were prepared with equivalent numbers of each one of the bacteriophages.

All of the mixtures and suspensions of individual phages to be used for a given experiment or a set of identical experiments were prepared with exactly equivalent dilutions of "stock phage suspensions" to avoid the possible influence of organic material (i.e., culture medium rests, peptone saline, and glycerol) on the various inactivation processes studied.

Bacteriophages detection and enumeration. F-specific RNA bacteriophages were detected and enumerated by the double-layer technique (1) according to the ISO standard (4). Briefly, serovar Typhimurium strain WG49 (NCTC 12484) (16) was used as the host strain. Serovar Typhimurium WG49 was grown in tryptone-yeast extract-glucose, and the phages were quantified by determination of PFU with the double agar layer by using the media described above. The incubation temperature was always 37°C.

Plaque hybridization. Plaque transfer and fixation onto membranes were carried out as described elsewhere (31). Briefly, fresh plates with 20 to 100 plaques were kept at 4°C for 1 h and then covered with a nylon membrane (Amersham International) to adsorb phages from zones of lysis for 1 min. Four subsequent transfers were obtained from one plate by increasing the time of adsorption by 1 min for every additional transfer. After plaque transfer, nylon membranes were placed for 5 min on a filter paper soaked with a solution of 0.1 mol of NaOH liter^{-1} for the release and denaturation of bacteriophage nucleic acid. Hybond-N^{+} membranes were then transferred for 1 min to a filter paper soaked with 0.1 mol of sodium acetate (pH 6.0; neutralization) liter^{-1}, dried, and illuminated for 5 min with UV light to fix the denatured nucleic acid. Membranes were stored at 4°C until the hybridization procedure.

The specific oligonucleotides used to identify F-specific RNA bacteriophages as members of one of the subgroups were as described elsewhere (6).

Plaques were hybridized as previously described (31). Briefly, fixed membranes were prehybridized for 10 min at room temperature in a buffer containing 6× SSC (0.9 M NaCl plus 0.09 M sodium citrate, pH 7), 0.1% sodium dodecyl sulfate, 1× Denhardt solution, and 0.1 mg of salmon sperm DNA ml^{-1} (30).

Hybridization was performed in the same solution by adding 2.5 pmol of digoxigenin-labeled probe ml^{-1} for 1 h at 37°C. The nylon membranes were washed and then treated for chemiluminescent detection with Lumigen-CSPD according to the manufacturer's guidelines (Roche Diagnostics). Hybridized plaques appeared as dark or black circular spots on the X-ray film.

Inactivation by ammonia (pH 10) and by extreme pH values. To analyze the effect of ammonia and extreme pH values, the bacteriophage suspensions were inoculated to a final concentration of 10^6 PFU ml^{-1} in tubes containing 10 ml of one of the following solutions: (i) 0.1 M Tris-HCl (pH 2), (ii) 0.1 M Tris-HCl (pH 10), and (iii) 40 mg of NH_3 (pH 10) liter^{-1}. At this pH, >80% of ammonia is in the uncharged state, which has been described as having a highly inactivating effect on poliovirus (38).

In all cases, the tubes were then incubated at 20°C and at the times indicated in Results, the pH was brought to neutrality and the bacteriophages were enumerated as described above.

Effect of temperature on phage inactivation. The effect of temperature on bacteriophages was tested on the four mixtures of the four genotypes. To this end, the bacteriophages were inoculated to a final concentration of 10^6 PFU ml^{-1} in 10-ml tubes containing a phosphate buffer (0.05 M $NaHPO_4$, 0.02 M KH_2PO_4, 0.09 M NaCl, 10^{-3} M $MgSO_4$, 10^{-4} M $CaCl_2$) used to prepare bacteriophage suspensions (1). The tubes were wrapped with aluminum foil to avoid the effect of light and placed at 4, 20, and 37°C. An aliquot of 1 ml was removed before treatment and after 4, 7, 11, and 30 days. Bacteriophages were then enumerated as indicated above.

Effect of salt concentration on phage inactivation. Salts (artificial seawater; ADSA-MICRO, Barcelona, Spain) were added to mineral water with a very low salt content (24.8 mg of Ca^{2+}, 4.4 mg of Mg^{2+}, 9.6 mg of Na^{+}, 97 mg of HCO_3^{-}, 4.9 mg of Cl^{-}, and 10.7 mg of SO_4^{2-} liter^{-1}) to obtain artificial seawater samples with salinities of 21 g liter^{-1}, corresponding to some estuarine and brackish waters, and of 42 g liter^{-1}, corresponding to oceanic waters. Mineral water is recorded as having a salinity of zero in Table 3. Aliquots of the four mixtures of phages were added to tubes containing 10 ml of water corresponding to the three salinities at a final concentration of 10^4 PFU ml^{-1}. The tubes were wrapped with aluminum foil to avoid light and placed at 20 ± 2°C. Then, 1 ml of each tube was removed at 4, 7, 11, and 30 days, and the phages were enumerated.

Inactivation by chlorination. The effect of chlorine was tested both on the bacteriophage mixtures and on individual suspensions of bacteriophages of genotype I.

Aliquots (50 ml) of mineral water with a low mineral content (the same reported in the previous paragraph) were spiked with 500 μl of each mixture of bacteriophages to a concentration of 10^6 PFU ml^{-1}. Then, 1.2 mg of chlorine liter^{-1} was supplied as hypochlorite, and the mixtures were incubated for 5 and 10 min at room temperature (21 ± 2°C). Residual chlorine was neutralized by the addition of sodium thiosulfate, and the bacteriophages were enumerated.

The same experiment was performed with individual suspensions of the bacteriophages FR11, FR12, FR13, and FR14. A total of 500 μl of each bacteriophage was inoculated in 50 ml of mineral water to a final concentration of 10^6 PFU ml^{-1}. As above, 1.2 mg of chlorine liter^{-1} supplied as hypochlorite was added, and the mixtures were incubated for 5 and 10 min at room temperature (21 ± 2°C). Residual chlorine was neutralized by the addition of sodium thiosulfate, and the bacteriophages were enumerated.

In situ natural inactivation. Natural inactivation was tested both on the bacteriophage mixtures and on individual suspensions of bacteriophages of genotype I.

In situ inactivation experiments were performed as follow. The mixtures were inoculated to a final concentration of 10^3 to 10^4 PFU ml^{-1} in the *Llobregat* river water from the site where the inactivation experiment was performed. The four mixtures were then placed in dialysis tubes (one tube for each mixture), with a cutoff of 14,000 Da, which were sealed and placed in the river at a 25-cm depth in the same site where the river water used for the mixture was collected. Samples were taken after 3 and 7 days and F-specific RNA bacteriophages were enumerated as described above. These experiments were performed in September 1999. Water characteristics were measured between 10 and 11 h a.m. The temperature ranged from 21.0 to 22.9°C, the pH ranged from 7.8 to 7.9, and the turbidity ranged from 18.0 to 84.0 nephelometric turbidity units.

TABLE 1. Log_{10} reduction of the numbers of bacteriophages in the mixtures of phages of the four genotypes after inactivation by ammonia (pH 10) and by extreme pH values[a]

Exptl condition (pH)	Time (h)	Log_{10} reduction (SD) in phage numbers in genotype mixture:			
		M(I)	M(II)	M(III)	M(IV)
0.1 M Tris-HCl (2)	4	1.0 (0.2)	1.3 (0.2)	2.5 (0.1)	2.9 (0.1)
0.1 M Tris-HCl (10)	8	0.3 (0.2)	1.7 (0.8)	1.8 (0.7)	2.1 (0.9)
NH_3 (40 mg liter^{-1}) (10)	8	0.2 (0.3)	1.8 (0.8)	1.9 (0.7)	2.3 (0.7)

[a] Values are the arithmetic means of the results of four experiments performed in duplicate.

The same in situ inactivation experiments were performed with the bacteriophages FR11, FR12, FR13, and F14 belonging to genotype I, in October 1999, with fewer daily hours of sunshine. Water characteristics were measured between 10 and 11 h a.m. Temperatures ranged from 16.9 to 17.4°C, the pH ranged from 7.4 to 7.8, and the turbidity ranged from 14.0 to 20.2 nephelometric turbidity units.

Data processing and statistical analysis. The inactivation of bacteriophages is reported as a log_{10} reduction, which was calculated as the decrease in log_{10} units ($\log N_0/N$) of phage numbers before and after inactivation. Statistical computations and tests were performed by using the SAS statistical program (3). Differences were considered significant when P was <0.05, as determined by analysis of variance (ANOVA).

RESULTS

Inactivation by ammonia and extreme pH values. The results of inactivation by extreme pH values and by ammonium at pH 10 are shown in Table 1.

Bacteriophages of mixtures M(III) and M(IV) showed the greatest log_{10} reductions at pH 2, with no significant difference between them (ANOVA, $P > 0.05$). These reductions of 2.5 and 2.9 log_{10} were significantly greater (ANOVA, $P < 0.05$) than those corresponding to mixtures M(I) and M(II) which were similar (ANOVA, $P > 0.05$) and averaged 1.0 and 1.3 logs.

The log_{10} reduction after treatment with NH_3 at pH 10 did not differ significantly from the reduction observed after treatment with Tris-HCl at pH 10 alone. In both cases, M(II), M(III), and M(IV) showed the highest log_{10} reductions. These log_{10} reductions, ranging from 1.7 to 2.1 and from 1.8 to 2.3, respectively, did not differ significantly (ANOVA, $P > 0.05$), but they were significantly greater (ANOVA, $P < 0.05$) than the log_{10} reduction of mixture M(I), which with values of 0.2 and 0.3, respectively, showed the highest resistance.

Effect of temperature on phage inactivation. The log_{10} reductions of the four mixtures of the four genotypes of F-specific RNA bacteriophages was clearly dependent on temperature as shown by the differences of inactivation at 4, 20, and 37°C (Table 2). The log_{10} reductions of the four mixtures at 37°C differed significantly (ANOVA, $P < 0.05$) from their log_{10} reductions at 4 and 20°C, whereas the log_{10} reductions experienced by the four mixtures at 4 and 20°C did not differ significantly (ANOVA, $P > 0.05$). This finding shows that temperature plays an important role in F-specific RNA bacteriophage inactivation.

Phages of mixture M(I) were relatively stable after storage at 37°C, with a log_{10} reduction significantly (ANOVA, $P < 0.05$) smaller than those of the other mixtures of bacteriophages, which did not differ significantly (ANOVA, $P > 0.05$) between them. Thus, the log_{10} reduction of the mixture of phage isolates of genotype IV, the most inactivated, was almost 4 log_{10} units greater than the reduction of phages of mixture I.

TABLE 2. Log_{10} reduction of the numbers of several mixtures of bacteriophages stored at 37, 20, and 4°C[a]

Incubation temp (°C) and duration (days)	Log_{10} reduction (SD) in phage numbers in genotype mixture:			
	M(I)	M(II)	M(III)	M(IV)
37				
4	0.2 (0.1)	0.3 (0.2)	0.4 (0.5)	0.6 (0.2)
7	0.2 (0.1)	0.7 (0.3)	1.1 (0.2)	1.3 (0.3)
11	0.3 (0.1)	1.2 (0.3)	1.6 (0.5)	2.0 (0.7)
30	0.7 (0.1)	3.0 (0.6)	3.2 (0.9)	4.6 (0.4)
20				
4	<0.1	<0.1	<0.1	<0.1
7	<0.1	<0.1	<0.1	<0.1
11	<0.1	<0.1	<0.1	<0.1
30	0.1 (0.06)	0.4 (0.1)	0.3 (0.1)	0.5 (0.2)
4				
4	<0.1	<0.1	<0.1	<0.1
7	<0.1	<0.1	<0.1	<0.1
11	<0.1	<0.1	<0.1	<0.1
30	<0.1	0.1 (0.05)	0.1 (0.02)	0.3 (0.03)

[a] See Table 1, footnote *a*.

Inactivation of the various mixtures at 20°C and 4°C did not differ significantly (ANOVA, $P > 0.05$), with the only exception of the log_{10} reductions observed between M(I) and M(IV) after 30 days of incubation at 20°C, which differed significantly (ANOVA, $P < 0.05$).

Effect of salt concentration on phage inactivation. Table 3 shows the log_{10} reductions of the four mixtures after storage in mineral water, salinity recorded as 0, and in mineral water

TABLE 3. Log_{10} reduction of the numbers of several mixtures of bacteriophages stored in artificial seawater at 42 and 21 g liter^{-1} and in mineral water at 20°C[a]

Expt condition and duration (days)	Log_{10} reduction (SD) in phage numbers in genotype mixture:			
	M(I)	M(II)	M(III)	M(IV)
Artificial seawater at 42 g liter^{-1}				
4	0.3 (0.1)	0.7 (0.2)	0.9 (0.4)	0.9 (0.5)
7	0.7 (0.3)	1.3 (0.5)	1.5 (0.4)	2.0 (0.9)
11	0.9 (0.4)	2.4 (0.8)	3.1 (0.1)	3.4 (0.1)
30	2.4 (1.0)	>4.5[b]	>4.5[b]	>4.5[b]
Artificial seawater at 21 g liter^{-1}				
4	0.6 (0.4)	0.9 (0.3)	1.0 (0.3)	0.9 (0.2)
7	0.7 (0.2)	1.9 (0.5)	1.8 (0.5)	2.6 (0.9)
11	1.0 (0.5)	2.7 (0.5)	3.2 (0.1)	3.4 (0.1)
30	1.7 (0.3)	>4.5[b]	>4.5[b]	>4.5[b]
Mineral water				
4	0.3 (0.1)	1.1 (0.4)	1.8 (0.2)	1 (0.7)
7	0.6 (0.1)	1.9 (0.5)	2.6 (0.5)	2.5 (1.0)
11	0.7 (0.1)	3.0 (0.2)	2.7 (0.1)	3.4 (0.1)
30	1.7 (0.6)	>4.5[b]	>4.5[b]	>4.5[b]

[a] See Table 1, footnote *a*.
[b] Phages of M(II), M(III), and M(IV) were below the detection limit at day 30.

TABLE 4. Log_{10} reduction of the numbers of bacteriophages in the mixtures of phages of the four genotypes after chlorination[a]

Time (min)	Log_{10} reduction (SD) in phage numbers in genotype mixture:			
	M(I)	M(II)	M(III)	M(IV)
5	0.6 (0.3)	1.2 (0.7)	2.4 (1.0)	4.3 (0.5)
10	3.7 (1.4)	5.2 (1.8)	5.9 (1.3)	6.2 (1.1)

[a] See Table 1, footnote *a*.

TABLE 5. Log_{10} reduction of the number of each bacteriophage belonging to genotype I after chlorination[a]

Time (min)	Log_{10} reduction (SD) of the number of bacteriophages			
	FR11	FR12	FR13	FR14
5	0.5 (0.4)	<0.1	0.2 (0.1)	<0.1
10	2.6 (0.4)	2.0 (0.4)	3.6 (0.1)	2.8 (0.4)

[a] Values are the arithmetic means of the results of three experiments performed in duplicate.

containing 21 and 42 g of salts liter^{-1}. The log_{10} reductions suffered by the different mixtures from day 0 to day 11 at the various salt concentrations did not differ significantly (ANOVA, $P > 0.05$). Therefore, salt concentration does not seem to influence phage inactivation at the short term. On day 30, no bacteriophages from mixtures M(II), M(III), and M(IV) were detected; thus, inactivation could not be quantified, nor could any effect of the salt concentration on these bacteriophages be detected. In contrast, phages of mixture M(I) were still present at all salt concentrations, and their log_{10} reduction in artificial seawater at 42 g of salts liter^{-1} was significantly different (ANOVA, $P < 0.05$) from that measured in mineral water, at salinity 0, and in artificial seawater at 21 g of salts liter^{-1}. Thus, it may be considered that salt content has some influence on the survival of these phages after long periods of time.

Again, differences between the inactivation of the four mixtures were observed. At all salinities, the log_{10} reduction of M(I) was significantly (ANOVA, $P < 0.05$) lower than the log_{10} reductions of the mixtures M(II), M(III), and M(IV). Inactivation of these three groups did not differ significantly (ANOVA, $P > 0.05$).

Inactivation by chlorination. The effect of chlorination on the four mixtures of F-specific RNA bacteriophages is shown in Table 4. The log_{10} reductions after 10 min differed significantly from those after 5 min (ANOVA, $P < 0.05$).

After 5 min of contact with chlorine, the bacteriophages of mixtures M(III) and M(IV) showed the greatest log_{10} reductions, which with values of 2.4 and 4.3 were significantly different (ANOVA, $P < 0.05$). These reductions were significantly different (ANOVA, $P < 0.05$) from those of mixtures M(I) and M(II), which with values of 0.6 and 1.2 were not significantly different (ANOVA, $P > 0.05$).

After 10 min of chlorination the bacteriophages of mixtures M(II), M(III), and M(IV) showed the greatest log_{10} reductions that did not differ between them (ANOVA, $P > 0.05$) but were significantly higher (ANOVA, $P < 0.05$) than the log_{10} reduction of bacteriophages of M(I).

In contrast, the log_{10} reductions of the four bacteriophages FR11, FR12, FR13, and FR14 belonging to genotype I did not differ significantly (ANOVA, $P > 0.05$) after 5 and 10 min of chlorination (Table 5). When we compared the log_{10} reduction of each bacteriophage with the reduction of the mixture, only the inactivation of FR12, which was the most resistant, differed significantly (ANOVA, $P < 0.05$) from the inactivation of the mixture. However, we can estimate the inactivation of the most resistant phage in the mixture as follows. First, the number of the most resistant phage in the mixture is one fourth of the total numbers of bacteriophages, and in log_{10} this represents a decrease of 0.6 log in its initial number. Second, at the end of the inactivation treatment it may be predominant because the inactivation differences recorded among the four phages are >0.6 log (Table 5). Therefore, we can suppose that the log_{10} reduction of the most resistant phage after 10 min was 3.1 instead of the 3.7 logs recorded for the mixture. In this case the inactivation of phage FR12 when studied alone does not differ significantly (ANOVA, $P > 0.5$) from its inactivation in the mixture.

Summarizing, the four phages studied of genotype I do not differ significantly regarding their resistance to chlorine. In any case, the most susceptible to chlorine of the four phage isolates of genotype I was more resistant than the most resistant phage isolate of all of the other groups.

Natural in situ inactivation. Results of natural inactivation in situ are shown in Table 6. All of the mixtures of bacteriophages were gradually inactivated. M(I) showed the highest persistence. Its log_{10} reductions at 3 and 7 days were significantly (ANOVA, $P < 0.05$) lower than those of mixtures M(II), M(III), and M(IV), which did not differ significantly from one another (ANOVA, $P > 0.05$). Although the differences were not significant, the most affected bacteriophages were those of mixture M(III), followed by those of mixtures M(IV) and M(II).

The results of natural in situ inactivation experiments of the four bacteriophage isolates of genotype I are shown in Table 7. At 3 days, bacteriophage FR13 showed the greatest inactivation. Its log_{10} reduction was significantly (ANOVA, $P < 0.05$) higher that those of bacteriophages FR12, FR13, and FR14, which did not differ significantly from one another (ANOVA, $P > 0.05$). However, at 7 days, the decimal reduction of the four bacteriophages did not differ significantly (ANOVA, $P > 0.05$).

Log_{10} reductions at both times were significantly greater (ANOVA, $P < 0.05$) for the mixture M(I) than for the four bacteriophages FR11, FR12, FR13, and FR14. Because of the

TABLE 6. Log_{10} reduction of the numbers of bacteriophages in the mixtures of phages of the four genotypes calculated from the beginning of the natural inactivation experiment at different sampling times[a]

Duration (days) of inactivation in situ	Log_{10} reduction (SD) in phage numbers of genotype mixture:			
	M(I)	M(II)	M(III)	M(IV)
3	2.2 (0.1)	3.0 (0.6)	3.5 (0.1)	3.2 (0.1)
7	2.5 (0.1)	3.5 (0.4)	4.5 (0.1)	4.0 (0.4)

[a] See Table 4, footnote *a*.

TABLE 7. Log_{10} reduction of the number of each bacteriophage belonging to genotype I calculated from the beginning of the natural inactivation experiment at different sampling times[a]

Duration (days) of inactivation in situ	Log_{10} reduction (SD) in the number of bacteriophages			
	FR11	FR12	FR13	FR14
3	1.3 (0.1)	1.0 (0.1)	1.7 (0.1)	1.0 (0.1)
7	1.6 (0.3)	1.7 (0.1)	1.9 (0.1)	1.8 (0.1)

[a] See Table 5, footnote *a*.

small differences, ranging from 0.1 to 0.3, between the log_{10} reductions of the four bacteriophages, the approach taken with the results of the chlorination experiments cannot be used. However, we can consider here that the minor inactivation of phages studied alone may be due to the lower temperatures, the pH, and the daily hours of sunshine recorded during the "in situ" inactivation experiments of the individual bacteriophages as indicated in Materials and Methods. To summarize these findings, the four phage genotypes studied do not differ significantly with regard to resistance to natural inactivation in freshwater and, in any case, the most susceptible to natural inactivation of the four phage isolates of genotype I was more resistant than the most resistant phage isolate of all of the other groups.

DISCUSSION

Using the distribution of genotypes of F-specific RNA bacteriophages for fingerprinting the origin of fecal contamination in natural samples requires previous knowledge of the comparative resistance of the four genotypes to inactivation by various factors. Data on stability in response to different treatments and conditions of phages MS2 and f2, which belong to genotype I, are relatively abundant (2, 5, 20, 35, 39), and data on Qβ, which belongs to genotype III, are also available (27), but comparative data are lacking. The present study was carried out to obtain comparative data on the susceptibility of bacteriophages of each of the four genotypes of F-specific RNA bacteriophages to various inactivating factors. The results of this study show that the pattern of resistance to inactivation varies according to the genotype.

As the experiments were designed, in the case of great differences of susceptibility to inactivating factors among the four bacteriophage isolates of a given genotype, only the inactivation of the most resistant phage of the group will be detected. Thus, the probability that only the differences among the most resistant phage of each genotype were determined in the experiments presented herein exists. However, in the worst case the approach followed here provided more information on the stability of phages of different genotypes than an approach based in the study of only one bacteriophage per genotype. Moreover, the susceptibility of the four phage isolates of the more resistant genotype, i.e., genotype I, to the two more inactivating treatments, chlorination and "in situ" natural inactivation, was studied. The results indicated that at least the four phage isolates studied here, if they differ to some extent, do not differ among them as much as they differ from the phages of the other genotypes. The inactivation experiments could also have been done with the most representative bacteriophage of each group (MS2, GA, Qβ, and FI), but this possibility was discarded to avoid potential effects of long-term subcultivation on the resistance of those bacteriophages. Therefore, it may be considered that results presented here are rather representative of what happens in nature regarding the differences in stability of F-specific RNA bacteriophages to various inactivating factors.

The phage isolates of genotype I were those that survived more successfully to all treatments tested, with very significant differences with respect to phages of the other genotypes in all cases. They were more resistant to all inactivating conditions than bacteriophages of genotypes III and IV and also more resistant than phages of genotype II to most inactivating treatments and conditions. Bacteriophages of genotype II kept an intermediate position regarding resistance, although they were more similar to genotypes III and IV than to genotype I. The susceptibilities to inactivation of the phage isolates of genotypes III and IV studied were similar in most inactivating conditions studied. Only chlorination at 5 min showed a significant difference, with phages of genotype IV being more sensitive. However, although in many cases the differences were not significant, genotype II usually showed greater persistence than genotypes III and IV. Therefore, we can conclude with the bacteriophages studied here that the order of resistance to different inactivating factors from greatest to smallest is as follows: genotype I $>$ genotype II $>$ genotype III $>$ genotype IV. This view confirms previous observations (Brion et al., 10th Health-Related Wat. Microbiol. Symp.; A. Havelaar, unpublished data) regarding the low resistance of phages of genotype IV.

Temperature and pH (26) and indirect photo-oxidation (34) seem to be among the more important factors in inactivating F-specific RNA bacteriophages in freshwaters. The experiments presented here on the effects of temperature and pH on phage stability coincide with the inactivation observed in the in situ inactivation experiments regarding the different genotypes. These results will explain the trend observed under natural conditions with a certain shift in the predominant genotypes from water recently polluted with fecal wastes and waters downstream of the original source of pollution (32, 37).

The data presented here constitute an important piece of information regarding the variability of resistance to different inactivating factors of different F-specific RNA bacteriophages, frequently seen as a homogeneous group of bacteriophages. The differences observed among phages of the four genotypes to different inactivating conditions jeopardize the use of the distribution of genotypes of F-specific RNA bacteriophages for tracking the origin of fecal pollution. However, more bacteriophages belonging to the different genotypes will have to be studied and more inactivation experiments will have to be performed before this potentially useful indicator system for identifying human and animal fecal contamination can be discarded.

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