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Evaluation of an *Escherichia coli* Host Strain for Enumeration of F Male-Specific Bacteriophages

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A method was developed for the selective enumeration of F male-specific bacteriophages in samples of environmental waters. The host strain for the phages, *Escherichia coli* HS(pFamp)R, has three antibiotic resistance markers, ampicillin on the Famp plasmid, which codes for pilus production, and streptomycin and nalidixic acid on the chromosome. The strain is resistant to coliphages T2 to T7 and ϕX174. More than 95% of the phages from environmental samples which plaqued on the host strain were F male specific. The host bacterium had a higher plaquing efficiency than *E. coli* K-12 Hfr for F-specific phages in stock suspensions and sewage effluents. The F male-specific phage levels in prechlorinated, secondary-treated sewage effluents generally were about 10^3 to 10^4 PFU/100 ml. The levels in the influents to the sewage treatment plants and in septic tank contents were about 10^5 PFU/100 ml. RNA-containing phages composed about 90% of the total F-specific phage population in sewage effluents.

The most prevalent and important sewage-related waterborne diseases in developed countries are acute gastroenteritis and infectious hepatitis (6, 7). The most frequently identified etiologic agents for these diseases are the Norwalk-like and hepatitis A viruses, respectively (7, 9). Coliform indicator systems generally have been used to classify environmental waters against the risk of waterborne infectious disease by their users. The effectiveness of these systems in assessing the risk of viral disease, especially when chlorinated wastewater effluents contaminate the water, has been questioned by a number of investigators (4, 5, 13). The basis for their concern is numerous reports that certain culturable human enteric viruses (e.g., poliovirus, coxsackievirus, etc.), which are found in some wastewater effluents (14), generally survive better than the coliform indicators during wastewater chlorination and residence in environmental waters (4, 10, 13, 29). In addition, coliform levels in environmental waters are poorly correlated to levels of these enteroviruses (2, 4). Because of these observations, routine monitoring for these viruses have been proposed by some of these investigators (24).

Although it is generally accepted that the culturable enteroviruses usually are more resistant to environmental stress than the coliform indicators, there are two major objections to routine monitoring of the culturable enteroviruses in environmental waters. The conceptual one is the virtual absence of epidemiological evidence demonstrating their importance as agents of waterborne disease when the source of these agents is municipal wastewater discharges (7, 11). The practical objection is that enumeration of the enteroviruses is too laborious, expensive, and time-consuming for routine monitoring of environmental waters.

Certain bacterial viruses, somatic coliphages, which are both easily enumerated and indigenous to sewage effluents have been suggested as alternatives to the coliforms as water quality indicators (17, 19, 20, 30). These coliphages, which initiate their infectious process by adsorbing to receptors on the bacterial envelope and whose host cells are *Escherichia coli* strains such as B, C, and K-12 F^-, are found consistently in sewage at levels considerably greater than those of the enteroviruses (20). Nevertheless, these coliphages have not found acceptance as water quality indicators, even by those individuals who recommended enterovirus standards. The objections to their use have been their inconsistent presence in human feces (26), the possibility that they could encounter an appropriate host and be replicated in environmental waters, and the greater sensitivity of some of these somatic coliphages to chlorination than some of the enteroviruses (25, 27).

A more homogeneous group of bacteriophages, including f2 and MS2 (19, 20), was examined several years ago as a possible alternative to the coliphages as water quality indicators. These are single-stranded RNA viruses. Along with a group of filamentous, single-stranded DNA, fd-like viruses, they compose the F male-specific bacteriophages. These phages attach to the sex pilus (conjugation tube) coded by the F gene on the chromosome or a plasmid. F pili are produced by a very limited number of *E. coli* strains found in environmental waters (21, 28).

The F male-specific phages, especially the fd-like component, are extremely resistant to the viricidal effect of combined chlorine (21a). However, the most compelling support for the use of F male-specific phages as models for the environmental behavior of the important viral pathogens, at least with regard to chlorination, was reported by Keswick et al. (18). They found that, of all the viruses examined, only the f2 phage was as resistant as the Norwalk virus to the cidal effects of chlorination.

E. coli K-12 Hfr strains have been used as the host strains for F male-specific phages for some time (12, 20), but they also plaque the somatic T coliphages. A *Salmonella typhimurium* host has been developed by Havelaar and Hogeboom (16), but an *E. coli* host would be preferable. In this report, we present evaluation data for an assay using such a host strain.

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MATERIALS AND METHODS

Host strains and phage stocks. The *E. coli* host strain, HS(pFamp)R, for the F male-specific bacteriophages is a mutant of HS(pFamp)R which was selected for resistance to the somatic coliphages T2 and T4 and streptomycin. Its parent was received indirectly from James B. Kaper. HS(pFamp)R was cured of the Famp plasmid (8) to yield a host (HS-RC) which plaques only somatic coliphages. *E. coli* B, C, K-12 F^-, and K-12 Hfr, the F male-specific phages f2 and fd, and the somatic coliphages T2 to T7 and φX174 were obtained from various sources. The f2 and fd phage stocks were propagated on *E. coli* K-12 Hfr.

Bacteriophage assays. The agar overlay method of Adams (1) was used to assay and enumerate the bacteriophages. Bottom base agar contained 10.0 g of Tryptone (Difco), 1.0 g of dextrose, 5.0 g of sodium chloride, and 15.0 g of agar in 1,000 ml of deionized water. The medium was autoclaved for 15 min at 121°C, cooled to 55°C, supplemented when appropriate with 0.015 g each of ampicillin and streptomycin (Sigma Chemical Co.) from filter-sterilized solutions, and poured into sterile petri dishes (150 by 15 mm). Double-strength soft agar contained 20 g of tryptone, 2 g of dextrose, 10 g of sodium chloride, 10 g of yeast extract (Difco), 0.15 g of $CaCl_2 \cdot 2H_2)$, and 15 g of agar in 1,000 ml of deionized water. The medium was brought to a boil with continuous stirring to dissolve the agar, dispensed into test tubes (16 by 150 mm), autoclaved at 121°C for 15 min, and cooled to 47°C in a water bath.

Duplicate 4-ml quantities of each sample or appropriate dilutions thereof were assayed as follows. A 0.25-ml portion of a mid-log-phase, 37°C, tryptone broth culture of the host strain and 4 ml of the sample were added to a tube of the soft agar medium. The contents of the tube were mixed rapidly and poured immediately into a petri dish containing the hard base agar. After the soft agar overlay solidified, the plates were inverted and incubated aerobically at 37°C. Plaques were counted within 24 h.

Sewage samples. Samples were collected from pre- and postchlorinated effluents from both primary and secondary municipal sewage treatment plants (STPs) within the Rhode Island and New Jersey areas. Contact times exclusive of those during transport in the lines to the outfalls varied from 20 to 30 min, and total chlorine residual levels ranged from 0.5 to 4.3 mg/liter. Samples were collected in sterile polypropylene bottles, iced immediately, and assayed within 6 h of collection. When needed, 10-fold serial dilutions of the samples were prepared in sterile phosphate-buffered saline (3). An appropriate quantity of a 10% solution of sodium thiosulfate was added to the postchlorinated samples to neutralize the chlorine remaining therein.

Samples also were collected on eight separate occasions from trucks which clean household septic tank systems. Thus, each sample was potentially a composite containing sewage from more than one septic tank.

Chlorination of wastewater effluents. Samples from prechlorinated, secondary-treated effluents at two wastewater treatment plants (STPs) were assayed, chlorinated for varying periods, dechlorinated with a 10% sodium thiosulfate solution, and reassayed. The acid-washed glassware used in these experiments was prepared as described in *Standard Methods for the Examination of Water and Wastewater* (3). Household bleach (Clorox Co., Oakland, Calif.), diluted 1:100 in deionized water, was used to obtain total chlorine residual levels in the sewage samples. The amount of bleach needed to yield the desired chlorine residual level was determined by pretitrating the sewage effluent with chlorine and measuring residual levels after a 20-min contact time. Total chlorine levels were determined by the *N*,*N*-diethyl-*p*-phenylenediamine method by using a commercial kit (Hach Co., Loveland, Colo.).

TABLE 1. Specificity of the host strain, *E. coli* HS(pFamp)R, for F male-specific bacteriophages as seen from direct plating of sewage effluents[a]

Total Cl^{2-} residual (mg/liter)	% of plaques on HS(pFamp)R at 37°C	
	HS(pFamp)R (25°C)	HS-RC (37°C)
0	19.0	1.2*
1.1–1.4	0.9	0.5[b]†
2.2–2.7	<0.9[b]†	<0.6[b]

[a] There were five samples in each experiment.
[b] When somatic phage could not be detected, the sensitivity limit was used in the analysis.
* Significantly less than the percentage for HS(pFamp)R incubated at 25°C at $P < 0.05$ by a two-tailed Student's *t* test. Normality was assumed.
† Significantly less than the percentage for the samples before chlorination at $P < 0.05$ by a two-tailed Student's *t* test. Normality was assumed.

Host specificity for F male-specific bacteriophages. Two types of experiments were conducted, direct and indirect. In the first type, sewage samples before and after chlorination in the laboratory were plaqued on the host strain, HS(pFamp)R, incubated at 37°C (HS Famp-37°C) and 25°C (HS Famp-25°C) and on the host strain cured of its plasmid (HS-RC-37°C). Incubation at 25°C was used because strains of *E. coli* which contain an F factor on the chromosome (K-12 Hfr) or on a plasmid (pHB11) do not express sex pili, the attachments sites for the F-specific phages, at temperatures ≤25°C (22). In the indirect experiments, pre- and postchlorinated effluents from several STPs were plaqued on HS(pFamp)R incubated aerobically overnight at 37°C. Virions from the resulting plaques were transferred with sterile toothpicks onto petri dishes with freshly poured soft agar overlays (31) containing HS-RC-37°C, HS Famp-25°C, and HS Famp-37°C. Phages which, when transferred, produced plaques on either HS-RC-37°C or HS Famp-25°C as well as HS Famp-37°C were scored as somatic.

RNase (Sigma) at a concentration of 100 μg per tube was added to the soft agar overlay to determine the percentages of RNA- and DNA-containing F phages (1) and their sensitivities to chlorination.

RESULTS

Specificity of *E. coli* HS(pFamp)R for F male-specific bacteriophages. Prior to the examination of *E. coli* HS(pFamp)R for its sensitivity to the range of somatic (wall) phages found in sewage, this host strain was screened for the availability of receptors for the somatic coliphages T2 to T7 and φX174 and the F male-specific bacteriophages f2 and fd. This was accomplished by spotting drops of the concentrated phage suspensions (≥10^9 PFU/ml) on lawns of the test organisms. *E. coli* HS-RC (the host strain cured of its plasmid), K-12 Hfr, K-12 F^-, B, and C were used as controls. HS(pFamp)R was lysed by the F male-specific phages but not the somatic phages, while the strain cured of its plasmid (HS-RC) was not lysed by any of the phages. The T phages spotted *E. coli* B, K12 F^-, and K-12 Hfr; φX174 spotted *E. coli* C; and the F male-specific phages spotted K-12 Hfr as well as HS(pFamp)R.

When prechlorinated sewage effluents were examined,

TABLE 2. Specificity of the host strain, *E. coli* HS(pFamp)R, for F male-specific bacteriophages as seen from the spotting of picked plaques on test strains

Chlorination stage[a]	Plaques from HS pFamp-37°C which spot on[b]:	
	HS pFamp-25°C (no. positive/total) (%) (4[c])	HS-RC-37°C (no. positive/total) (%) (6[c])
Prechlorination	36/864 (4.5)	57/1,521 (3.9)
Postchlorination	15/815 (2.0)*	30/1,568 (2.1)*

[a] Chlorination at the STPs.
[b] Plaques were toothpicked to lawns of test strains incubated at the temperatures indicated.
[c] Number of STPs.
* Significantly different from values for prechlorinated effluent at $P < 0.05$ by chi-square analysis.

TABLE 4. Comparative plaquing efficiency of F-specific phages in sewage effluents on *E. coli* HS(pFamp)R and K-12 Hfr

Total Cl^{2-} residual (mg/liter)	Geometric mean[a] of F phages (10^3)		a/b[b]
	HS(pFamp)R[c] (a)	K-12 Hfr[d] (b)	
0	3.44*	2.07	1.71 ± 0.44
1.1–1.4	>3.38*	2.06	1.65 ± 0.21
2.2–2.7	2.89*	1.75	1.66 ± 0.25

[a] Effluents were sampled five times each.
[b] Means of the individual ratios ± one standard deviation.
[c] Obtained by subtracting the PFU on HS-RC from those on HS(pFamp)R.
[d] Obtained by subtracting the PFU on K-12 F^- from those on K-12 Hfr.
* Significantly different from K-12 Hfr at $P < 0.05$ by Student's t test. Normality was assumed.

only 1.2% of the phages which plaqued on the host strain were somatic as seen from the comparison of the number of plaques on this host strain before [HS(pFamp)R] and after (HS-RC) it was cured of its plasmid (Table 1). The number of plaques on the host strain incubated at 25°C, however, was about 19% of that when the incubation temperature was 37°C. If, as reported by Novotny and Lavin (22), F pili are not produced at 25°C, there must be somatic phages in sewage which are plaqued on HS(pFamp)R at 25°C but not at 37°C. This was demonstrated directly when the virions from an appreciable number of the plaques picked from HS(pFamp)R incubated at 25°C did not produce lysis at 37°C (data not shown). The somatic phages were much more sensitive to chlorination than the F phages as seen from the lower percentages of plaques on HS Famp-25°C and HS-RC-37°C relative to HS Famp-37°C when the effluents were chlorinated to total residual chlorine levels as low as 1.1 mg/liter (Table 1).

About 4% of the sewage-derived phages which plaqued on the host strain incubated at 37°C were shown to be somatic in experiments in which the plaques on HS Famp-37°C from prechlorinated effluents from four to six STPs were picked to HS Famp-25°C and HS-RC-37°C (Table 2). Comparable estimates were obtained with both host-incubation temperature combinations in contrast to the differences obtained from direct plating. These results also indicate that the 19% value (Table 1) was inflated by somatic phages which replicate on the host at 25°C but not at 37°C. The somatic phages again were shown to be more sensitive than the F phages to wastewater chlorination (Table 2).

An attempt was made to increase the specificity of the host strain for F male-specific bacteriophages by selecting mutants which were resistant to a predominant somatic phage as seen by plaque morphology. This phage (T-HS) produced a plaque with a minute clear center surrounded by a hazy halo. It had an icosahedral head and a tail and did not spot or plaque *E. coli* B or K-12 F^-. Phage T-HS was picked, purified, and propagated, and about 200 mutants resistant to T-HS as observed by spotting and plaquing were obtained by the method of Adams (1). When sewage was plaqued on these mutants at 25 and 37°C, T-HS type plaques were no longer observed with any of the mutants at either incubation temperature. However, with all the mutants examined, a new plaque type was obtained from sewage at both incubation temperatures. Further attempts to increase the specificity of the host strain to the F male-specific phages were discontinued.

The plaquing efficiency of the host strain HS(pFamp)R was compared with that of *E. coli* K-12 Hfr by using pure stocks of the F male-specific phages f2 and fd and the F male-specific phages found in sewage. The mean recoveries of both f2 and fd on HS(pFamp)R were significantly and appreciably higher than those on K-12 Hfr (Table 3). Essentially the same results were obtained for F male-specific phage recoveries from sewage effluents before and after chlorination (Table 4).

RNA-containing phages composed about 90% of the total F-specific phage population in the effluents from the two treatment facilities examined. The percentage of these phages in the F-specific phage population did not change appreciably following chlorination to levels of ≤3.2 mg/liter.

The F male-specific phage levels in the prechlorinated effluents generally were about 10^4/100 ml. They were appreciably higher in the effluents from the plants where treatment was only carried to the primary stage than in those in which there was treatment to the secondary level (Table 5).

The high phage levels in the STP influents and their infrequent presence in human feces suggested that the phage propagated on occasionally found host cells in the sewage lines or that they were introduced from sources (e.g., storm water) other than from human fecal wastes. The F-specific phage levels in septic tank effluents were then examined to address the possibility that storm water, industrial inputs, or water infiltrating the sewage lines was the source of these phages. High levels of the F-specific phages (log mean 4.36

TABLE 3. Comparative plaquing efficiency of *E. coli* HS(pFamp)R and K-12 Hfr with F-specific phage stocks[a]

Phage	Mean of PFU on:		a/b
	HS(pFamp)R (a)	K-12 Hfr (b)	
f2	229*	121	1.88
fd	391*	239	1.64

[a] Each experiment had nine replicate plates.
* Significantly different from K-12 Hfr at $P < 0.001$ by a two-tailed Student's t test. Normality was assumed.

TABLE 5. Prechlorination levels of F male-specific bacteriophages

Type of treatment	N[a]	Statistic	Log_{10} PFU/100 ml
Primary	6	$\bar{X}$	4.806
		SD	0.505
Secondary	14	$\bar{X}$	3.940
		SD	0.640

[a] Number of plants sampled.

± 0.76) were found in the eight pools of sewage taken from septic tanks.

DISCUSSION

The host strain, *E. coli* HS(pFamp)R, as modified in our laboratory, satisfies most of the requirements for the enumeration of F male-specific bacteriophages in environmental waters. More than 95% of the phages from sewage samples which plaque on it are F male specific, and it has a significantly higher plaquing efficiency than *E. coli* K-12 Hfr for the F male-specific phages when stock suspensions or sewage effluents are examined. The host strain is resistant to three antibiotics which can be included in the plaquing medium to prevent overgrowth by bacteria present in the sample. Resistance to one of the antibiotics is encoded by the F plasmid, and its inclusion in stock cultures selects against any variants which may have lost the plasmid. Finally, the host strain is easily grown and is not derived from a pathogen.

F male-specific bacteriophages at levels of 10^3 to 10^5/100 ml were found in the final postchlorinated effluents from all the primary and secondary STPs examined and in all the composite samples from all septic systems. The source of these phages, however, remains obscure. F male-specific phages are infrequently found in human feces and then only at low levels (15, 23). Thus, the consistent presence and relatively high numbers of these phages in sewage cannot be readily accounted for solely by human fecal inputs. A possible explanation is that the F-specific phages are replicated in the sewage system from originally low inocula. However, the mechanism of F-specific phage multiplication in the sewage system remains unexplained. The phages in sewage could derive from lower animal fecal wastes carried into the sewage via storm water or infiltration of the sewage lines, but this explanation does not account for the high phage levels in the contents of septic tanks.

The F phages cannot be considered fecal indicators in the conventional sense since they are not consistently present at high levels in human fecal wastes. However, they are consistently and abundantly present in sewage and sewage-polluted waters. The presence of these phages in a water sample could be used as an index of sewage contamination, making them a valuable tool for the assessment of water quality. It remains to be seen whether the F phage levels in lower animal fecal wastes (15) will preclude this use. The F phages also could be used as simulants for the environmental behavior of the Norwalk virus, at least with regard to chlorination, as suggested from data of Keswick et al. (18). The broader application of the F male-specific bacteriophage indicator system as a basis for water quality guidelines and standards should be examined in the course of prospective epidemiological studies to determine the extent to which F phage levels in bathing, shellfish-growing, and drinking water are correlated to illness via the three respective transmission routes.

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