Synthesis of Indicator Strains and Density of Ribonucleic Acid-Containing Coliphages in Sewage

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Escherichia coli strains freshly isolated from natural sources are inefficient indicators of coliphages present in sewage. Four E. coli strains recently isolated from clinical specimens were mutagenized to obtain lac" mutants. Such mutants were infected with an F"lac" sex factor of E. coli K-12. Pairs of isogenic lac" and lac"/F"lac" strains were used as indicators of coliphages present in sewage, and it was found that such strains can be effectively used for a direct and almost selective enumeration of F-specific coliphage contents of sewage samples. Serological tests were applied to a number of F-specific phages isolated. All the isolates that were tested fell into two distinguishable antigenic classes: members of one class being related to ribonucleic acid (RNA) phage MS2 and those of the other being related to another RNA phage, namely, Qφ. MS2-related phages have been found to be more widely distributed than the Qφ related phages. Most habitats sampled were found to yield only one or the other kind of phage. Single-stranded deoxyribonucleic acid-containing F-specific phages were not detectable by the methods employed by us.

Sewage is well known to contain a great variety of bacteriophages active on many species of Enterobacteriaceae (1, 5). Most bacterial strains which have undergone prolonged culturing on laboratory media are excellent indicators for different sorts of phages present in sewage (5). This property, however, renders such bacteria unsuitable for the detection of specific types of phages. We have found that Escherichia coli strains recently isolated from natural sources are very inefficient indicators for coliphages present in sewage (our unpublished observations). We reasoned that, if the genotypes of such bacteria are suitably altered so as to make them sensitive to a specific group of phages, such genetically modified bacterial strains may prove useful for the detection and enumeration of specific kinds of phages in the natural habitats. With this in view, lactose-negative mutants of a number of E. coli strains isolated from clinical samples were F-duced with the F"lac" sex factor of E. coli K-12. These strains were then used for detection and quantitative enumeration of F-specific coliphages in sewage. We find the ribonucleic acid (RNA)-containing F-specific coliphages to be widespread in sewage ranging in density from zero to $5 \times 10^3$ plaque-forming units (PFU) per ml of sewage. Single-stranded deoxyribonucleic acid (ssDNA)-containing, F-specific phages were not detectable by the methods employed by us. Pertinent observations are recorded in this communication.

MATERIALS AND METHODS

Bacterial and phage strains. The bacterial and phage strains used are listed in Table 1. Strain A'327 was the primary source of F"lac" factor which was transferred to other strains by mating. E. coli strains marked M were isolated from clinical specimens in the Microbiology Department of the University of Hong Kong and were supplied to us by C. T. Huang and C. H. Chan-Teoh.

Bacteriophage KC is probably identical to RNA phage MS2; anti-KC serum inactivates both phages at the same rate. Phage HK102 was isolated from local sewage as an F-specific phase (5), and it is easily distinguishable from KC and MS2 by serological tests. This phage, however, is antigenically very closely related to RNA phage Qφ, if not identical to it (see footnote to Table 1). Phages MS2 and Qφ have been shown to belong to two distinct antigenic classes (9, 13) showing only slight cross-reaction in serological tests (9).

Media, diluent, and antibiotics. Bacterial cultures were grown in tryptone yeast extract broth (TYB). Tryptone broth (TB) was used as the diluent. Phage assays were made in tryptone broth agar (TBA) containing $10^{-2}$ M CaCl₂ and $10^{-2}$ M MgCl₂. Composition of TYB, TB, and TBA has been published elsewhere (4, 6).
**Table 1. Bacterial and phage strains**

| Strain no. | Pertinent properties* | Source |
|------------|-----------------------|--------|
| K-12 bacterial strains | | |
| TD55 | lac⁻/F⁺lac⁺ | W. Hayes |
| TD23 | met str F⁻ | F. H. Symons |
| TD121 | met str F⁻ | F⁺ cured derivative of TD23 |
| TD134 | met str lac⁻ F⁻ | NG induced lac⁻ mutant of TD121 |
| TD192 | met str lac⁻/F⁺lac⁺ | TD134 infected with F⁺lac⁺ of TD55 |
| TD342 | prototrophic, lac⁻ F⁻ | |
| Wild-type M strains of *E. coli* | | |
| M42 | CPST; sensitive to T5 | C. T. Huang and C. H. Chan-Teoh |
| M49 | CPST; partially sensitive to T6 | C. T. Huang and C. H. Chan-Teoh |
| M56 | Sensitive to T6 and partially sensitive to T4 | C. T. Huang and C. H. Chan-Teoh |
| M78 | S; sensitive to T2 and partially sensitive to T1 | C. T. Huang and C. H. Chan-Teoh |
| M81 | PT; sensitive to T3, T6, T7 and partially sensitive to λ. | C. T. Huang and C. H. Chan-Teoh |
| M105 | PST; sensitive to T1 | C. T. Huang and C. H. Chan-Teoh |
| M117 | P; sensitive to T4. | C. T. Huang and C. H. Chan-Teoh |
| lac⁻/F⁺lac⁺ derivatives of M strains | | |
| M49-3 | | By transferring F⁺lac⁺ from TD192 |
| M81-4 | | into NG induced lac⁻ mutants of parental strains of similar numerical designation. |
| M105-5-2 | | |
| M117-3 | | |
| Phage strains | | |
| T1 to T7 | | |
| KC⁺ | F-specific phage antigenically related to MS2 | A. D. Hershey |
| HK102⁺ | RNA-containing, F-specific phage antigenically related to Qβ. | Dhillon et al. (5) |
| Qβ⁺ | RNA-containing, F-specific phage | Anonymous reviewer of this manuscript. |
| (9, 13) | | |
| AE2 | ssDNA-containing, filamentous, F-specific phage (14) | F. H. Symons |

*Explanation of symbols: met stands for methionine requirement; lac⁻ and lac⁺ denote inability and ability to ferment lactose, respectively; and str denotes resistance to high concentrations of streptomycin (1,000 μg/ml), C, P, S, and T denote the phenotypes of resistance to chloramphenicol, penicillin, low concentration (50 μg/ml) of streptomycin, and tetracycline, respectively. Concentrations of antibiotics used are described in Materials and Methods.

+ lac⁻ and lac⁻/F⁺lac⁺ derivatives of M strains were tested for antibiotic resistance and T-phage sensitivity patterns and were found to resemble the parental strains.

+ RNA-containing, F-specific phages are of two basic antigenic types, namely, MS2 and Qβ (9, 13). Anti-KC serum inactivates phages KC and MS2 at the same rate. Anti-HK102 serum inactivates phages HK102 and Qβ at nearly the same rate. K values for the two phages being 86 and 74, respectively.

Lactose fermentation was tested either on complete medium (EMB agar purchased from Difco Co.) or on minimal eosine yellow methylene blue agar (EMS medium of Lederberg described in reference 3).

Antibiotic resistance was tested by incorporating sterile filtered solutions of antibiotics into nutrient agar (NA) medium purchased from Difco Co. The following concentrations of antibiotics were used (micrograms per milliliter final concentration): chloramphenicol (C), 25 μg; benzylpenicillin (P), 40 μg; tetracycline (T), 20 μg. Two concentrations of streptomycin sulfate (S) were used; a lower concentration of 50 μg/ml and a higher concentration of 1,000 μg/ml. Genetic nomenclature used for drug resistance is explained in one of the footnotes of Table 1.

**Methods.** General methods of bacteriophage technology were those described by Adams (1). Methods used for collection, preparation, and assay of sewage...
samples have been described elsewhere (5). Special methods pertinent to this investigation are described below.

**Synthesis of F' strain.** lac- mutants were induced by N-methyl-N'-nitro-N-nitrosoguanidine (NG; Aldrich Chemical Co., Milwaukee, Wis.) treatment. NG was added to aerated, log-phase bacterial cultures to give a final concentration of 50 μg/ml and incubation continued for 60 min. NG treatment was effectively terminated by making 1:100 dilutions in TYB in culture tubes which were static incubated for 14 to 24 h and thereafter diluted 10⁻³, and 0.05-ml portions were spread on EMB plates. lac- clones were detectable after incubation of plates for 48 h at 37 C. Presumptive lac- clones were purified by two successive single colony isolations; their lac phenotype were confirmed and stored on NA slants at room temperature.

For F' infection, 0.05 ml of static, 14- to 24-h-old TYB cultures of parental strains (F'lac- and lac'/F'lac⁺) was mixed in 5 ml of TYB in a culture tube, and such mating mixtures were static incubated for 14 to 24 h. Mating mixtures involving lac- mutants of M strains (prototrophic) as recipients and strain TD192 as donor were plated on unsupplemented EMS medium counterselecting the met⁺ donor strain. Presumptive recombinants were identified by their lac⁺ phenotype after 48 h. These were purified by two successive single colony isolations on EMS medium selecting for lac⁺ phenotype. Finally two to four lac⁺ colonies showing lac⁻ sectors were pooled and stored on NA slants.

Both lac⁻ and lac'/F'lac⁺ derivatives of M strains were tested and were found to show the antibiotic resistance patterns of their respective, wild-type progenitors.

For sex factor (F' or F⁺) curing, Hirota's method was used (8). Turbid bacterial cultures were diluted 10⁻⁴ in TYB, pH 7.6, containing acridine orange (E. Merck) at the final concentration of 15 μg/ml. After 48 h of static incubation, suitably diluted portions were plated on EMB agar plates. F'lac⁺ curing was assessed both by counting the number of lac⁻ segregants and by determining the resistance of a variable number of such segregants to standard F-specific phages, KC (RNA-containing) and AE2 (ssDNA-containing). F⁺ curing was assessed by determining the resistance of a variable number of single-colony isolates to the standard F-specific phages, KC and AE2.

All incubations were at 37 C.

**Description of habitats.** All sewage samples were of high-population-density, urban origin except samples XII and XV which were taken from open ditches of two semi-rural localities. Samples II to VII were taken from a single locale at weekly intervals, except samples IV and V which were collected on the same day at an interval of 5 h.

**PFU assays.** Sewage samples were collected, processed, and treated with chloroform as described elsewhere (5). Portions (0.1 ml) were plated in duplicate on F' lac⁻ and lac'/F'lac⁺ derivatives of M strains. Presumed F-specific (PFS) phage estimates were made in the following manner. We found that standard F-specific phages (Table 1) produce only faint plaques on F' derivatives of M strains. Thus, if a sample produced no faint plaques on the F' indicator but did yield such plaques on its F⁺ derivative, these plaques were presumed to be due to F-specific phages and their counts were used to determine PFS phages. In some instances this method was not applicable because faint plaques of similar morphology were observed both on the F' strain and its F⁺ derivative. In cases like this, PFS estimates were obtained by subtracting PFU registered by the F' strain from the PFU registered by the F' derivative. The precise method used for determining this parameter is mentioned for each such datum of Table 4.

**Purification and host range determination of PFS phages.** A number of PFS isolates were purified by two or three successive single-plaque isolations on the original indicator strain (F' derivatives of M strains). Finally, an isolated plaque was stabbed with a needle which was shaken in 1.5 ml of TB containing three drops of chloroform. Loopfuls of such phage suspensions (low titer suspensions) when spotted on suitable indicators usually gave 50 to 500 plaques. Selected isolates were numbered consecutively from 01 to 96. Precise host range of these isolates was determined by spotting loopfuls on four indicator strains. Possible responses of these tester indicator strains and the resultant inferences are given in Table 2.

It will be shown that the PFU classified as F-specific are antigenically related to the standard F-specific phages and thus adsorb to the F-pili present on F' or F⁺ cells. More than one explanation can be given to explain the host range of PFU classified as host range M (HRM), but we are inclined to favor the following. We believe that these phages do not adsorb to the F-pili and the receptor sites for their adsorption are not produced by cells of strains TD23, TD121, and the F' M strains. The F'lac⁺ sex factor used by us probably carries the genetic information for the production of receptor sites for HRM phages and this information is borne on that part of the episome which is of bacterial origin. Hence, the F' derivative of M strains are resistant, but their F⁺ derivatives are sensitive to the HRM phages. In view of the above, HRM class PFU will not be counted as F-specific.

Original PFS estimates were corrected in view of the results of the diagnostic test described above to

| Table 2. Scheme for further classification of PFS phage isolates |
|---------------------------------------------------------------|
| Host strains and responses*                                    |
|                                                              |
| **M strains**                                                 |
|                                                              |
| **K-12 strains**                                              |
|                                                              |
| **Host range**                                               |
|                                                              |
| **Inference**                                                |
|                                                              |
| F' lac⁻ | lac'/F' lac⁺ | TD121 (F⁺) | TD23 (F⁺) | Sex indifferent | Host range M | F-specific |
| +      | +            | +           | +         |                 |              |            |
| -      | +            | -           | -         |                 |              |            |
| -      | +            | -           | +         |                 |              |            |
| a +, indicates lysis; --, indicates no lysis.                 |

Note: +, indicates lysis; -, indicates no lysis.
Serological identification of F-specific isolates. Antisera against three F-specific phages were prepared according to the methods described (1). K (inactivation rate constant) values of antisera obtained are as follows: anti-AE2, 4,000; anti-KC, 6,000; and anti-HK102, 1,400.

Antisera were prepared against phage TD192, employed in the characterization of isolates from sewage. Serological homology of 51 F-specific isolates was determined by the following qualitative test. A 0.1-ml portion of diluted antisera (anti-HK102, 1:40; anti-AE2, 1:100; and anti-KC, 1:500) and F* indicator strain TD23 were incorporated in the top agar layer of separate plates, and these were spotted with loopfuls of low-titer suspensions of the three antigen phages against which the sera were prepared, as well as F-specific phages isolated during this study. The three antigen phages showed no lysis of indicator cells in the plates containing their respective antisera. Antigenic homology of newly isolated F-specific phage isolates was inferred from the antisera plates showing no lysis of indicator cells, or showing a significantly reduced number of plaques.

The validity of qualitative tests was confirmed by subjecting six isolates to quantitative tests (see Table 8). Since phage KC is indistinguishable from RNA phage MS2 on the one hand, and HK102 and QS show a very close resemblance on the other, F-specific isolates resembling KC in the serological tests will be referred to as "MS2 related" and those resembling HK102 in the serological tests will be referred to as "QS related".

RESULTS

F'lac+ recombinants and their properties. As a consequence of NG treatment, stable lac- mutants of six M strains were obtained. M78, despite repeated attempts, gave only unstable lac- mutants and was thus excluded from further work. One lac- mutant of each of the six M strains was crossed with the donor strain TD192 as described in Materials and Methods. On the whole, such crosses were less fertile than crosses involving both parents of K-12 derivation. Under the experimental conditions employed, cross TD134 × TD342 showed 50 to 100% transfer of the F'lac+ factor to the recipient strain cells. However, in crosses, TD134 × F- M strains, the maximum frequency of lac+ F-ductants observed was 2% for strain M105 and no such recombinants were obtained from crosses involving lac- mutants of recipient strains M42 and M56. F'lac+ transfer frequencies for the other three strains were: M49, 0.02%; M81, 0.01%; and M117, 0.04%. Five or more presumed F'lac+ recombinants were tested for sensitivity to F-specific phages KC and AE2. Each clone was found to be sensitive to either one or the other and, more often, to both of these phages.

The frequencies with which pure lac- F- segregants arise in the cultures of F'lac+ recombinants of M strains were found to be higher than those observed for K-12 strains of similar genotype. It was 10% for one culture of M105-5-2 and ranged from 20% to over 90% for some cultures of M117-3. Almost 100% curing of the F' factor was achieved by growing M105-5-2 in pH 7.6 broth containing 15 μg of acridine orange per ml. lac- F- recombinants of spontaneous origin were found from cultures of strain M105-5-2 and rather more efficiently after acridine orange treatment but not from cultures of strain M117-3.

Since F'lac+ recombinants of M strains were found to segregate F- lac- segregants at a high rate, the efficiency of plating (EOP) of standard F-specific phages on these strains was tested. The EOP values relative to a K-12 lac-/F'lac+ strain, TD192, are presented in Table 3. It can be seen that the only M strain on which F-specific phages plate with an efficiency equaling that of the K-12 F' strain is M105-5-2.

The RNA phage KC produces clearer plaques than the ssDNA phage AE2 on K-12 indicators as well as F'lac+ derivatives of M strains. One notable exception is strain M81-4 on which the RNA phage KC is unable to form plaques (EOP less than 10^-4). On the whole, a given F-specific phage has been observed to produce clearer plaques on K-12 strains than on M strain derivatives. This is probably due to a higher rate of spontaneous loss of the F' sex factor from the latter strains.

For the assay of F-specific phages in sewage, four F'lac+ strains were selected, namely, M49-3, M105-5-2, M117-3, and M81-4. M81-4 was selected because it was felt that, since it permits plaque formation only by AE2, it may allow direct and independent estimation of ssDNA phages of the AE2 type.

Suitability of indicator strains and estimates of F-specific phages. Table 4 shows the PFU per ml of sewage when plated on lac- and lac+ F'lac+ derivatives of M strains. M49 deriv-

| Indicator | Phage relative EOP: |
|-----------|---------------------|
|           | KC                  | AE2                 |
| TD192     | 1.0                 | 1.0                 |
| M49-3     | 2 × 10^-4           | 3 × 10^-4           |
| M81-4     | <10^-4              | 10^-4               |
| M105-5-2  | 9 × 10^-1           | 1.0                 |
| M117-3    | 2 × 10^-1           | 3 × 10^-2           |
atives (both lac^-F^- and lac^-/F'laclac^+) are the least efficient indicators of phages present in sewage. However, excepting sample II, the F' derivative of strain M49 (Table 4, column a) registered more PFU than the F^- strain (Table 4, column b). The difference of a-b is the estimate for PFU per ml of the presumed F-specific (PFS) phages. Fourteen plaques of such PFS phages were tested further (5 from sample III and 9 from sample XI) according to the classification scheme described in Materials and Methods. They grew only on the F' strain M49-3 and failed to grow on M49 F^-, K-12 F^+, or F^-, and were thus not F-specific phages. In Table 5, therefore, they are listed as HRM phages (see Materials and Methods). We thus conclude that M49-3 is not suited for the purpose of detecting F-specific phages in nature. This is not surprising in view of the low EOP of both RNA- and ssDNA-containing standard F-specific phages on this strain.

The F' derivative of strain M81 also turned out to be unsatisfactory for the present purpose. As can be seen from Table 4, the F^- strain on the whole tended to register more PFU than the F' strain. Sample VII when plated on the F' strain produced turbid plaques which were absent from the F^- strain, and the titer of such PFS phages was 20 PFU/ml. However, when two such plaques were tested further, they turned out to be of HRM class rather than true F-specific type (Table 5). Thus, M81 derivatives also are unsuited for quantitative estimation of F-specific phages in sewage.

Derivatives of the other two M strains, namely, M105 and M117, proved satisfactory for the estimation of RNA-containing F-specific phages. Plating on both male and female derivatives of these two strains indicated the presence of PFS phages either because PFU estimates registered by F' indicators were in excess of PFU estimates of corresponding F^- clones, or because more or less turbid plaques (typical of F-specific phages) were present on the F' strain and absent from the corresponding F^- clones. In most cases and as might be expected, F' strains registered more PFU and at the same time showed turbid plaques that were absent from plates of F^- strains. Numbers of PFS phage per ml of sewage determined either by subtraction or estimated from numbers of turbid plaques are given in Table 4. The PFS estimates of

| Sample | Indicator strain^a and PFU/ml |
|--------|-------------------------------|
|        | M49          | M81          | M105         | M117         |
|        | a  b         | a  b         | a  b         | a  b         |
| II     | Total PFS    | 10  20       | 40  20       | 40  120      |
| III    | Total PFS    | 120 0        | 7,850 1,650  | 1,600 500   |
|        | a-b = 120    | a-b = 6,200  | a-b = 1,100  |
| IV     | Total PFS    | 0 0          | 1,000 500   | 6,250 1,140  |
|        | a-b = 120    | a-b = 500    | a-b = 5,110  |
| V      | Total PFS    | 0 0          | 1,600 910   | 2,290 890   |
|        | a-b = 340    | a-b = 1,400  |
| VI     | Total PFS    | 0 0          | 5,800 6,030 | 1,720 310   |
|        | a-b = 20     | a-b = 430    | a-b = 10    |
| VII    | Total PFS    | 0 0          | 2,360 2,770 | 420 150     |
|        | a-b = 190    | a-b = 250    |
| IX     | Total PFS    | 0 0          | 1,180 6,990 | 190         |
|        | a-b = 320    | a-b = 270    |
| X      | Total PFS    | 10 0         | 6,690 5,920 | 930 120     |
|        | a-b = 80     | a-b = 360    |
| XI     | Total PFS    | 80 0         | 7,930 540   | 380 280     |
|        | a-b = 350    | a-b = 250    |
| XII    | Total PFS    | 0 0          | 2,770 910  | 440 660     |
|        | a-b = 40     | a-b = 50     |
| XIII   | Total PFS    | 10 0         | 1,120 1,940 | 340 30      |
|        | a-b = 380    | a-b = 280    |
| XIV    | Total PFS    | 0 0          | 1,200 1,940 | 380 250     |

^a lac^-/F'laclac^+ derivative of M strain; b = F^- lac^- derivative of M strain.

Numbers centered in columns are PFS phage estimates when these were determined by direct counting of faint plaques appearing on the F' indicator and not by the subtraction a-b.

^TMTC, Too many to count.
Table 5. Characterization of single plaque isolates

| Sample no. | Original F indicator | No. of isolates tested | Characteristics of selected single plaque isolates |
|------------|----------------------|------------------------|--------------------------------------------------|
|            |                      |                        | Selection criterion | Host range M | Sex in different | F-specific infective PFU | F-specific homologs |
|            |                      |                        |                    |              |                 |                        | KC | HK102 |
| II         | M117-3               | 2                      | PFS                | 2            | 0                | 0                       | 0  | 0     |
| III        | M49-3                | 5                      | PFS                | 5            | 3                | 0                       | 3  | 0     |
| IV         | M105-5-2             | 5                      | PFS                | 2            | 0                | 3                       | 0  | 5     |
| V          | M117-3               | 5                      | PFS                | 0            | 5                | 0                       | 0  | 5     |
| VI         | M105-5-2             | 5                      | PFS                | 3            | 0                | 2                       | 0  | 2     |
| VII        | M81-4                | 2                      | PFS                | 2            | 0                | 0                       | 0  | 0     |
|            | M105-5-2             | 5                      | PFS                | 1            | 4                | 0                       | 0  | 0     |
| IX         | M105-5-2             | 1                      | PFS                | 1            | 0                | 0                       | 0  | 0     |
| X          | M105-5-2             | 5                      | PFS                | 0            | 0                | 5                       | 3  | 2     |
| XI         | M105-5-2             | 4                      | PFS                | 0            | 0                | 4                       | 2  | 2     |
| XII        | M117-3               | 5                      | PFS                | 0            | 0                | 5                       | 5  | 0     |
| XIII       | M105-5-2             | 1                      | PFS                | 0            | 0                | 0                       | 0  | 0     |
| XIV        | M105-5-2             | 6                      | PFS                | 0            | 2                | 4                       | 4  | 0     |
| XV         | M105-5-2             | 3                      | PFS                | 0            | 0                | 3                       | 3  | 0     |
|            | M117-3               | 5                      | PFS                | 0            | 0                | 5                       | 5  | 0     |
| Total      | 67                   | 67                     | PFS                | 17           | 7                | 43                      | 36 | 7     |

Table 4 were corrected on the basis of final recovery of true F-specific phages among the PFS isolates subjected to the final diagnostic test; these data are given in Table 6. The method of derivation of these data may be clarified by the following examples.

Sample IV when plated on M105 derivatives was found to contain 6,200 PFS phages per ml (Table 4). Five plaques from the F'-seeded plate were selected and were subjected to the diagnostic host range test. Only three phages isolated turned out to be F-specific and, in view of this, the actual number of F-specific phages was calculated to be $6,200 \times 3/5 = 3,720$ per ml (Table 6). Another example involving a different method of estimation is given by sample XIII plated on M105 derivatives. Although the number of PFU registered by the F- strain in this case exceeded the PFU registered by the F' strain, turbid plaques were found only on the latter strain and these were presumed to be F-specific phages. The datum 340 PFU/ml of PFS phages of Table 4 is based on this plaque count. Six PFS phage plaques were subjected to the host range test and only four were found to be truly F-specific. Thus, the corrected estimate of male-specific phages was calculated to be $340 \times 4/6 = 226$ PFU per ml, and this is recorded in Table 6. In those cases where all the PFS phage plaques tested turned out to be F-specific (e.g., sample XI on M117 derivatives), no correction was indicated and so the PFS estimates of Table 4 were taken as correct estimates of F-specific phages and entered in Table 6, unaltered.

The data given in Table 6 clearly show that the derivatives of M105 and M117 can be used for a more or less direct titration of F-specific phages in sewage. Both strains have some merits and some demerits. Strain M117-3 does not register HRM phages present in sewage (Table 7). However, as has been mentioned before, the EOP of F-specific phages on this strain is low, and thus this strain is likely to have revealed only a fraction of such phages.
Hence, estimates based on M117 derivatives are likely to be underestimates. M105-5-2 is an efficient indicator for F-specific phages, but it also registers the HRM class of phages. Also, some sex-indifferent phages present in sewage produce plaques on this strain which are indistinguishable from plaques of F-specific phages (e.g., samples VI and VII, in Tables 4, 5, and 7). Therefore PFS phage estimates obtained from the use of M105 derivatives are subject to substantial corrections based on final host range determinations of a sample of representative plaques. Nevertheless, bearing these limitations in mind, these bacterial strains are the only host strains available which can be used for a more or less direct and quantitative enumeration of F-specific phages present in sewage.

The data given in Table 6 clearly show the wide distribution of F-specific phages. Only three samples out of ten tested were found to be free from F-specific phages. When detected, the frequency of such phages was always in excess of 10^2 PFU/ml. Samples IV, VI, and VII were collected from a single site at weekly intervals. Differences in PFU per milliliter show that the numbers of such phages fluctuate widely over a period of time. It can be concluded from the estimates for samples IV and V that such fluctuations are not too abrupt and PFU contents may well be moderately stable over very short time intervals. These two samples were collected from the same site on the same day at an interval of 5 h, and their PFU contents do not differ from one another as much as they differ from other samples.

**Serological identification of F-specific phages.** The data presented in Tables 5 and 8 show that F-specific phages detected by our methods are antigenically related to one or the other of the standard RNA phages. On the whole, RNA phages antigenically homologous with phage KC (and hence MS2 related) are more widely distributed than those homologous with HK102 (and hence Q5 related) (Table 5). With the exception of samples X and XI, all other samples yielded only one or the other kind of phage but not both. Samples II to VII were taken from a single locality at weekly time intervals. None of the six samples taken from this single source ever yielded F-specific phages serologically related to phage KC. In contrast to the wide distribution and high density of RNA coliphages in sewage, we have failed to detect the presence of ssDNA, F-specific coliphages.

**DISCUSSION**

In an earlier study, qualitative observations were presented on the presence of F-specific phages in sewage (5). In the present study, quantitative data have been obtained which show that F-specific RNA phages are not only widespread in nature but occur in substantial titers (more than 10^2 to 10^4 PFU per ml) in sewage. Considering the relatively low EOP of some of the indicator strains used, the actual numbers may be more than those observed by us. At any rate, the widespread distribution of such phages at high densities in nature would imply that host cells capable of supporting growth of such phages must also be abundant in

| Sample no. | M105-5-2 | M117-3 |
|------------|----------|--------|
|            | a        | b      |
| VI         | 5        | 2      |
| VII        | 5        | 0      |
| IX         | 1        | 0      |
| X          | 5        | 5      |
| XI         | 4        | 4      |
| XII        | 1        | 0      |
| XIII       | 6        | 4      |
| XV         | 11       | 10     |
| Total      | 38       | 25     |

* a, number of presumed F-specific (PFS) isolates tested; b, number of isolates confirmed to be F-specific.

Table 7. Comparison of two F'/lac+/lac- strains as indicators for F-specific phages

| F-specific phage | Percent inactivation |
|------------------|----------------------|
| Serum:           | anti-KC | anti-HK102 |
| Expt 1:          |         |            |
| #19               | 99.90   |            |
| #56               | 99.90   |            |
| #85               | 99.90   |            |
| #93               | 99.78   |            |
| #26               | 99.93   |            |
| #71               | 98.30   |            |
| KC                | 99.83   | 20.00     |
| HK102             | 11.00   | 99.97     |
| Expt 2:          |         |            |
| KC                | 96.2    | ND*       |
| HK102             | ND      | 98.0      |
| Q5                | ND      | 97.8      |

* Reaction time was 6 min at 37 C. Serum concentrations were: anti-KC = 1/5,000; anti-HK102 = 1/500. Experiment 2 was carried out at least 3 years after experiment 1. The evident drop in the titer of the sera may explain some of the discrepancies between the two experiments.

* ND*. No detectable inactivation.

Table 8. Percent inactivation of F-specific phage isolates by anti-KC and anti-HK102 sera

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nature. Under laboratory conditions such phages are often propagated on strains harboring the F' or F' episomes. However, it has been shown that E. coli strains harboring the F-related plasmids such as fi+R factors do not permit plaque formation by MS2 phage, but permit propagation of MS2 in liquid cultures (10, 11). During recent years an extensive spread of such plasmids has occurred in the wild enterobacteria, especially E. coli (7, 12). Our results on the very wide distribution of RNA phages in nature may be a reflection of the existence of populations of such bacteria in the habitats sampled by us.

The present study is an extension of some of our earlier unpublished observations. We had observed that E. coli strains recently isolated from natural sources tend to be resistant to a majority of phages present in the natural habitats, e.g., sewage. Genetic modification of such strains should enable one to synthesize bacterial strains selective for enumeration of particular classes of phages. The practicability of this approach has been demonstrated for efficient and selective enumeration of RNA phages in their natural habitats in this communication.

Our inability to recover ssDNA, F-specific coliphages may in part be due to the low EOP of such phages on some of the indicator strains employed. However, their inability to register on strain M105-5-2 on which ssDNA phage AE2 plates with an EOP of 1.0 relative to K-12 F' strains necessitates alternative considerations. ssDNA molecules are known to be subject to host-induced modification and restriction (2). It is possible that the ssDNA phages, assuming that they were represented in our samples, might have been carrying a modification which caused their restriction by the indicator strain M105-5-2. Alternatively, such phages might have been absent from the samples studied by us.

The inability of male strain M81-4 to permit plaque formation by RNA phages is an unexpected and as yet unexplained observation. Experiments are in progress to find out if the low EOP of RNA phages on M49-3 is due to failure of phage adsorption or to interference with phage multiplication occurring subsequent to adsorption.

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