Distribution of Coliphages in Hong Kong Sewage

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Coliphage content of sewage collected from 11 different localities in Hong Kong was determined. The number of plaque-forming units (PFU) ranged from $0.036 \times 10^8$ to $15.9 \times 10^8$ per ml. In general, urban sewage tended to be richer than rural sewage both in PFU count as well as plaque morphological variation. Seventy-seven isolates were subjected to a host range study. Fifty per cent of these were able to grow on Escherichia coli K-12 as well as E. coli B. Approximately 32% were found to be male specific, and the remaining 18% were K-12 specific although sex-indifferent.

Abundant occurrence of coliphages in sewage is generally recognized. However, no quantitative data on the density of coliphages in sewage appear to be available. Such information being of obvious importance for an understanding of bacteriophage ecology, we undertook to determine the density of coliphages in sewage from diverse sources in Hong Kong. Our findings form the subject of this communication.

MATERIALS AND METHODS

Organisms. The bacterial and phage strains used are described in Table 1.

Media. Liquid bacterial cultures were raised in tryptone broth which contained, in 1 liter of distilled water, 10 g of tryptone (Difco) and 5 g of sodium chloride (pH 7.2 before autoclaving). For agar overlay plates, bottom and top agar were prepared by the addition of 1.2 and 0.65 g, respectively, of agar (Difco) to 100 ml of tryptone broth. Streptomycin, when used, was added to give a final concentration of 200 µg per ml of medium. Tryptone broth was used as the diluent for sewage samples and for phage lysates.

Methods. General bacteriophage techniques were those described by Adams (1). Sewage samples were collected from both rural and urban areas (Table 2). Special methods pertinent to this study follow.

Collection and assay of sewage samples. Samples (50 to 100 ml) of liquid were collected from each site in sterile glass flasks. Coarse particulate materials were removed by passing the sample through four layers of sterile cheese cloth. Filtrate was centrifuged at 4,000 × g for 30 min, and the pellet was discarded. The supernatant fraction was either assayed directly or treated with chloroform and then assayed. Chloroform treatment was given by vigorous agitation of 10 ml of supernatant and 0.5 ml of chloroform. The mixture was refrigerated for 30 min. The upper aqueous layer was plated by agar overlay method to determine the plaque-forming unit (PFU) content of the sample. A 1.25-ml portion of sample was mixed with 10.75 ml of molten soft agar at 45 C and 0.5 ml of a log phase culture of indicator cells. Samples (2.5 ml) of the mixture were spread on four bottom agar plates. Undiluted sample and its three serial 10-fold dilutions were plated by this method. Plates were incubated at 37 C for 16 to 24 hr; those showing the largest countable number of plaques were selected for further observations. All PFU assays were made with agar media containing streptomycin and seeded with the streptomycin-resistant indicator strain 58-161.

Plaque morphology observations. Plaque morphology classes were established on the basis of size (very small, small, medium, large), clarity (turbid, clear, intermediate), and edges (sharp or diffuse) of plaques. Isolated plaques on plates of each sample were examined, and the maximum number of plaque morphological classes produced by the sample was determined.

Purification and preparation of phage stocks. Plaques intended for further study were purified, and their stocks were prepared by the following method. The center of a selected plaque was stabbed with a BSA (gaue 26) platinum needle, and the needle was shaken in 2.0 ml of broth. Ten-fold serial dilutions of this suspension were plated on indicator cells. An isolated plaque from the resulting plates was selected. After two or more such successive single-plaque isolations, an isolated plaque was cut out of the plate and transferred into a 12 by 100-mm culture tube containing about 10^8 host cells (F+ strain, 58-161) in 1.0 ml of broth. After 8 hr of incubation at 37 C, 1.0 ml of 0.65% agar in tryptone broth was added. The tube was plugged with a sterile cork stopper, sealed by dipping in liquid paraffin, and stored at 4 C.

Spot tests for host range. Phage suspensions for spot tests were prepared by stabbing the stock cultures with a thick inoculating needle (BSA, gauge 22) and shaking the needle in 2.0 ml of broth containing 2 to 3 drops of chloroform. The suspension was agitated vigorously to kill bacterial cells. Supernatants of such preparations generally assayed 10^4 to 10^5 PFU per ml. Loopfuls of the above containing 10^5 to 10^6 PFU were placed on agar overlay plates seeded with indicator bacteria. Cell-free suspensions of 58-161 containing 10^5 to 10^6 PFU of λ-K, when spotted on lawns of Escherichia coli B, produced no observable lysis as λ-K is restricted on E. coli B and has an efficiency of plating of only 10^{-4} (2).
Table 1. Characteristics and sources of bacterial and phage strains

| Strain designation* | Origin            | Characteristics                        | Source                          |
|---------------------|-------------------|----------------------------------------|---------------------------------|
| B                   | *Escherichia coli* B | Wild type                              | I. Tessman                     |
| 58-161              | *E. coli* K-12    | F<sup>+</sup>, *mer*, *strA*, (λ)<sup>b</sup> | R. H. Symons                   |
| 58-161/58-161       |                  | F<sup>−</sup>, isogenic to 38-161       | Obtained by elimination of sex factor by acridine orange treatment |
| Phage strains       |                   |                                        |                                 |
| AE2                 |                   | Male-specific DNA phage                |                                 |
| M13                 |                   | Male-specific DNA phage                | R. H. Symons (8)                |
| MS2                 |                   | Male-specific RNA phage                | P. H. Hofschneider (9)          |

* Different bacterial and phage strains will be referred to by using the designations given under this column only.

<sup>b</sup> Symbols: F<sup>+</sup>, male, carrying wild type sex factor; F<sup>−</sup>, female, free from sex factor; *mer*, requirement for methionine; *strA*, resistance to streptomycin; (λ), lysogenic for temperate phage lambda.

RESULTS

PFU content of sewage. Data given in Table 3 show that each of the 11 samples tested had sufficient number of PFU values to be detectable by the methods used in this study. However, the range of PFU content was considerable, from 36 (sample VI) to 15,900 (sample VII) PFU per ml, giving a 441-fold difference between the poorest and the richest sample. It is interesting to note that, on the whole, urban samples had a higher PFU content than the rural samples; an average of 1.3 × 10<sup>5</sup> PFU per ml for five rural samples compared with an average of 4.8 × 10<sup>4</sup> PFU per ml for the six urban samples.

Eight samples were assayed both before and after chloroform treatment. The data given in Table 3 show that, in each instance, the PFU contents dropped after chloroform treatment. Such "chloroform sensitive" PFU values constituted from 26.1% of sample II to 60.5% of sample V.

Plaque morphology variation. Plaques produced by each sample were assigned to morphological classes defined by size, clarity, and the periphery. Maximum number of plaque morphology classes observed are listed in Table 4. Samples of rural origin produced a maximum of six different morphological plaque types. On the other hand, all urban samples, with the exception of sample XI, produced seven or more different types of plaques. From this and the observations recorded above, it may be concluded that samples of urban origin in general were richer than the rural samples, both in quantity as well as plaque morphological heterogeneity of the PFU.

Nature of PFU. Sewage samples plated without chloroform treatment on indicator strain 58-161 may have contained four different types of entities potentially capable of forming plaques or giving rise to plaque-like zones of lysis. These are free virions of coliphages, bacteria-harboring colicinogenic factor(s), bacteria lysogenic for one or more coliphages, and, perhaps less likely, phage-infected but unlysed bacteria. If it is assumed that a high level of resistance to streptomycin is uncommon among the bacterial flora of the studied samples and since streptomycin was incorporated in the assay plates, it is likely that the number of plaques given in Table 3 must have been caused by free virions. Study of 77 plaques selected from different samples gives support to such a conclusion. Inoculum from each selected plaque was treated with chloroform and then diluted-plated to obtain isolated plaques, followed by repeating the same procedure once or more on one of the progeny plaques. None of the 77 plaques tested proved to be a colicin-caused zone of lysis (see reference 3, p. 233, for differentiation of phage plaques from colicin-caused lyses). With the possible exception of one plaque, none of the rest was colony-centered, suggesting that the majority of the tested plaques were not of temperate phages. Hence, bacteria lysogenic for coliphages could not have constituted an appreciable fraction, if any, of the PFU content of the sewage samples. Considering the relatively short eclipse period of bacterial viruses and the presence of streptomycin in the assay plates, it is likely that phage-infected bacteria also could not have added appreciably to the PFU contents of sewage samples. Thus, a very high proportion, if not all, of the plaques must have been produced by free virions of coliphages.

Host range. Host range of 77 purified isolates was determined by using three indicator bacterial strains, K-12 F<sup>+</sup>, K-12 F<sup>−</sup>, and B. The data given in Table 5 show that 50% of the tested isolates...
TABLE 2. Description of habitats sampled

| Sample no. | Habitat                                                                 |
|------------|-------------------------------------------------------------------------|
| I (Urban)  | Mouth of the underground sewer pipe discharging its contents into the harbor |
| II (Urban) | Open sewer carrying waste from 20 blocks of multi-story residential buildings |
| III (Rural)| An open puddle made by accumulated discharge of water from the premises of a nearby textile mill |
| IV (Urban) | Concrete lined deep sewer mainly carrying household discharge from densely populated urban area |
| V (Rural)  | A ditch carrying waste water from the village to the fields; likely to be contaminated with animal excrements discharged into the ditch from the cattle sheds |
| VI (Rural) | Open sewer of running water carrying liquid discharge of a public utility; visibly polluted with human fecal matter |
| VII (Urban)| Concrete lined, deep sewer carrying household discharge from densely populated urban area |
| VIII (Rural)| An open ditch carrying sewage from village huts |
| IX (Urban) | Open street gutter in a densely populated area carrying household liquid wastes |
| X (Rural)  | A muddy, unlined ditch carrying waste water from nearby villages; likely to be polluted with animal as well as human excrements |
| XI (Urban) | Open street gutter in a densely populated area carrying household liquid wastes |

were able to grow on all three indicator strains of *E. coli*. As might be expected, such phage strains which show a wider host range than others are somewhat more widely distributed. They are represented in 10 out of 11 samples studied. Isolates of host range restricted to K-12 cells were found less frequently.

**Male-specific phages.** The male-specific local isolates can be subdivided into two distinct classes on the basis of morphology of plaques produced on lawns of 58-161 F* cells. Some produced faint, barely discernible plaques of about 0.25 cm diameter. These, in Table 6, are placed in the “turbid plaque” class. Others, placed in the “clear plaque” class, were characterized by larger and clearer plaques. We have found that, under the same experimental conditions, male-specific phages AE 2 and M13 produce “turbid” plaques. These two phages carry their genetic information in single-stranded deoxyribonucleic acid (DNA) and have cylindrical virion symmetry [AE2, (8); M13, (9)]. Plaques of MS2, another male-specific phage, resemble the plaques of “clear plaque” local isolates. This phage has a spherical virion symmetry and carries its genetic information in a single-stranded ribonucleic acid (RNA) molecule [(4), 5]. Cheng and Dhillon *(unpublished data)* have found that all of the “turbid plaque” male-specific local phage isolates are inactivated by anti-AE2 serum but are unaffected by anti-MS2 serum. Conversely, “clear plaque” male-specific local isolates are inactivated by anti-MS2 serum but not by anti-AE2 serum. From these geological observations, it may be concluded that local isolates of the “turbid plaque” type are single-stranded DNA phages whereas those of “clear plaque” type are RNA phages. Data given in Table 6 show that both types of phages are common in Hong Kong sewage. However, RNA phages appear to be more widely distributed than the single-stranded DNA phages.

**DISCUSSION**

The quantitative data presented in the preceding section demonstrate a rather extensive distribution of coliphages in local sewage. Coliphages unable to form plaques on the K-12 strain used in these assays, namely, 58-161 (λ), either due to their restriction or due to lysogenic immunity of the K-12 host cells, would not be recovered by the assay procedure adopted. Therefore, the actual PFU contents of the sewage samples are likely to be higher than those recorded. Although a variety of phage types have been shown to be present in all the sewage samples analyzed, it is interesting to note that out of 77 isolates studied in some detail only one appears to be of a temperate nature. It may be concluded, therefore, that virulent phages are far more widespread in nature as free virions than the virions of temperate phages. If, under natural conditions, temperate phages are predominantly present as prophage entities in the host cells, then plaing of sewage samples without chloroform treatment on streptomycin-free medium may permit their detection.

The rural and the urban sites sampled differed from one another in many respects. For example, urban samples were probably better aerated from the faster flow of liquid. Also, they were shielded from the direct sunlight and had a much smaller quantity of inorganic matter when compared with
TABLE 3. Total and “chloroform sensitive” plaque-forming units in various sewage samples

| Sample no.     | Plaque-forming units per ml | Per cent loss by chloroform treatment |
|----------------|-----------------------------|---------------------------------------|
|                | A  | B  |                               |
| I (Urban)      | 0.613 $\times 10^3$   | 1.70 $\times 10^3$                     | 26.1 $\times 10^3$                                   |
| II (Urban)     | 2.300 $\times 10^3$   | 1.10 $\times 10^3$                     | 43.3 $\times 10^3$                                   |
| III (Rural)    | 0.0760 $\times 10^3$  | 0.36 $\times 10^3$                     | 60.5 $\times 10^3$                                   |
| IV (Urban)     | 1.940 $\times 10^3$   |                                       |                                                     |
| V (Rural)      | 0.910 $\times 10^3$   |                                       |                                                     |
| VI (Rural)     | 0.036 $\times 10^3$   |                                       |                                                     |
| VII (Urban)    | 15.900 $\times 10^3$  | 9.10 $\times 10^3$                     | 42.8 $\times 10^3$                                   |
| VII (Rural)    | 0.590 $\times 10^3$   | 1.33 $\times 10^3$                     | 33.8 $\times 10^3$                                   |
| IX (Urban)     | 2.010 $\times 10^3$   | 3.33 $\times 10^3$                     | 30.6 $\times 10^3$                                   |
| X (Rural)      | 4.800 $\times 10^3$   |                                       |                                                     |
| XI (Urban)     | 5.800 $\times 10^3$   |                                       |                                                     |

*a Indicator strain: 58-161, F*.
*b PFU/ml, before chloroform treatment.
*c PFU/ml, after chloroform treatment.

TABLE 4. Number of plaque morphology classes represented in sewage samples

| Sample no.     | No. of plaque morphology classes |
|----------------|----------------------------------|
| I (Urban)      | 13                               |
| II (Urban)     | 16                               |
| III (Rural)    | 6                                |
| IV (Urban)     | 7                                |
| V (Rural)      | 3                                |
| VI (Rural)     | 3                                |
| VII (Urban)    | 7                                |
| VIII (Rural)   | 6                                |
| IX (Urban)     | 9                                |
| X (Rural)      | 6                                |
| XI (Urban)     | 3                                |

TABLE 5. Classification of local isolates by host range

| Sample no. | Isolates belonging to various host range classes |
|------------|-----------------------------------------------|
|            | Male-specific a K-12-specific b Nonspecific c |
| I          | 7 1 5                                         |
| II         | 6 2 7                                         |
| III        | 0 1 3                                         |
| IV         | 3 2 2                                         |
| V          | 0 0 3                                         |
| VI         | 1 1 2                                         |
| VII        | 1 0 6                                         |
| VIII       | 1 3 2                                         |
| IX         | 2 2 5                                         |
| X          | 3 0 3                                         |
| XI         | 0 3 0                                         |
| Total      | 24 15 38                                       |

*a Isolates which form plaques only on male strains of E. coli K-12.
*b Isolates which form plaques on male and female strains of K-12 but do not form plaques on E. coli B.
*c Isolates which form plaques on male as well as female strains of K-12 and E. coli B.

rural samples. The PFU contents may have been influenced directly by intervention of one or more of these or other factors or indirectly by their influence on the density of host bacterial cells. From the data available, it is not possible to offer any precise explanation of the differences in the PFU contents observed between rural and urban samples.

Extensive distribution of male specific RNA- and DNA-containing coliphages suggests that host cells permitting multiplication of these virions must be quite widespread in nature. Seven hundred E. coli type of colonies from each of sample I and II were tested, and none was found to be lysed by the RNA phage MS2. This observation, however, does not prove that all cells of the clones tested are insensitive to male-specific phages. Indeed, Meynell and Datta (9) plated MS2 on a random sample of 26 E. coli isolates and observed no plaque formation on any strain. On the other hand, when liquid cultures of the same strains were infected with MS2, six cultures showed an increase in phage titer, indicating the presence of some phage-sensitive cells. The phage response of the six cultures may have a similar or identical genetic basis to certain F- mating-type laboratory strains harboring the plasmid R f*+. Meynell and Datta (8) have
observed that, although such strains do not permit plaque formation by MS2, they do contain a small proportion of phage-sensitive cells that can be demonstrated by phage infection of liquid cultures. MS2 and, possibly, other male-specific phages are also known to be capable of adsorbing to and growing in the sex factor infected cells of other species of the family ENTEROBACTERIA-CEAE; e.g., Proteus mirabilis (6) and Shigella flexneri (7), although neither species will permit plaque formation by MS2. Since we have been unable to isolate E. coli strains from sewage that would permit plaque formation by MS2 and since male-specific phages are widely distributed, it would appear that, in sewage, such phages must normally be propagated on R $f^+$ harboring E. coli cells or cells of other species possessing analogous properties. Recent spread of the infectious drug resistance factor in the natural populations of enteric bacteria and the consequent occurrence of R $f^+$ infected cells in the sewage would tend to support this conclusion.

Wide and frequent occurrence of male-specific phages as observed by us is to be expected in view of the large burst size of such phages, the insusceptibility of RNA genomes to any of the known mechanisms of host restriction (2), and, as mentioned above, host range extending over many genera of the family ENTEROBACTERIA-CEAE.

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