J. gen. Microbiol. (1962), **27**, 143–146
Printed in Great Britain

Hydroxymethylcytosine-containing and Tryptophan-dependent Bacteriophages Isolated from City Effluents

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(*Received* 5 *May* 1961)

SUMMARY

Bacteriophages were isolated from the effluents of the cities of Oxford and Salisbury. They differ in their host range but are alike in that they contain hydroxymethylcytosine and show dependence on tryptophan for adsorption. These properties, which had been described previously only in the T-group of coliphages, are now shown to be widespread.

INTRODUCTION

The much-studied bacteriophages known as the coli T-group can be clearly divided into subgroups on the basis of morphological and biochemical differences. The members of one of these, the T-even subgroup, are indistinguishable in the electron microscope and possess the unusual pyrimidine hydroxymethylcytosine (HMC). The subgroup thus defined is also characterized by different degrees of dependence on tryptophan in adsorption. This dependence appears to be wholly absent only from strain T2.

Fildes & Kay (1957) isolated a phage from Oxford sewage which they named 'phage 3'. It was active on certain strains of *Salmonella typhi* but was later found to lyse a wide range of *Escherichia coli* strains and to have a requirement for tryptophan for adsorption to the host cells. More strains of tryptophan-dependent phages were required for comparative studies and further samples of sewage were examined. This paper describes the range of phages obtained and indicates that the phenomenon of tryptophan-dependence is widespread amongst the bacteriophage population of city effluents. It is shown to be associated with the presence of HMC.

METHODS

Sewage samples. Six (100 ml.) samples of raw inflowing sewage were taken at daily intervals at the sewage works at Oxford and Salisbury. We are greatly indebted to the officials of these works for their assistance. The samples were kept at 4° until delivered to the laboratory. Thymol was added to the Salisbury samples for transmission by post.

Isolation of phages. The sewage samples were centrifuged to remove debris and treated with thymol overnight at room temperature to diminish the bacterial population. Cultures of *Escherichia coli* 518 (Fildes & Kay, 1959) in 200 ml. of a medium containing Difco Nutrient Broth (dehydrated), 20 g.; NaCl, 7·5 g.; DL-tryptophan

(10^{-2}M), 10 ml.; Na Citrate (M), 20 ml.; Na, K phosphate buffer (0·20M-phosphate, pH 7·6), 200 ml. made up to 1 l. with distilled water were set up and infected with 10 ml. purified sewage. After incubation at 37° overnight the lysates were centrifuged and dilutions plated on lawns of *E. coli* 518 on nutrient agar plates containing Difco Bacto-Agar, 15 g.; Difco Nutrient Broth, 20 g.; NaCl, 7·5 g.; Na citrate (M), 10 ml., per litre of water. Plaques of large and small diameters were seen, but only small ones were selected for further propagation in the phosphate + citrate + tryptophan medium. Small plaques were again selected but this time were propagated in cultures of *E. coli* 518 growing on a glucose-ammonia medium augmented with tryptophan (10^{-4}M; Kay & Fildes, 1950). Lysates of these cultures were used to prepare high-titre stocks by the procedure described by Kay (1959).

Host ranges. The twelve specimens of phage were numbered Ox-1 to 6 and Sal-1 to 6. Their host ranges and those of coliphages T2, T4 and T6 were determined by placing drops of serial 10-fold dilutions on nutrient agar plates surface-seeded with bacteria. A large number of strains of *Escherichia coli* were tested and the results recorded as: full titre, equal to that given on *E. coli* 518; low, some fraction of that given on *E. coli* 518; resistant, no plaques seen at the highest concentration tested. From these observations it was clear that all the twelve phages differed from one another and from coliphages T2, T4 and T6 in one or more details of their host specificities.

Morphology. The phages were prepared for examination in the electron microscope by the conventional shadowcasting procedure and by the negative contrast method of Brenner *et al.* (1959). Measurements on phages Ox-1 and Ox-6 (then named 11F and 66F) were given by Bradley & Kay (1960). The other phages have not been so accurately measured but have been judged solely on their overall appearance.

Hydroxymethylcytosine. Preparations of phage containing about 5×10^{12} particles in 5 ml. were centrifuged at 15,000 *g.*, the supernatant fluids discarded and the phage pellet taken up in 98% (w/v) formic acid. After hydrolysis at 175° in sealed glass tubes the free bases were separated by paper chromatography on Whatman No. 1 paper in the *iso*propanol + HCl solvent of Wyatt (1951). Cytosine and HMC are indistinguishable in this solvent but are well separated from the other bases. The areas containing these materials were cut out, eluted in water and again chromatographed in *iso*propanol + ammonia (Hershey, Dixon & Chase, 1953), a solvent which separates cytosine from HMC. Under ultraviolet radiation the identity of the bases could be decided unequivocally.

Polyamines. The presence of the polyamines putrescine and spermidine in coliphages T2 and T4 was first described by Ames, Dubin & Rosenthal (1958). For the purposes of the present paper the polyamines were extracted from about 1×10^{12} phage particles into trichloroacetic acid (3%, w/v) by heating at 45° for 1 hr. After centrifugation the supernatant fluid was evaporated to dryness and applied in a little water to paper chromatograms which were developed in a solvent consisting of the top layer from a mixture of *n*-butanol (225 ml.) + acetic acid (56 ml.) + water (225 ml.). The positions of the spots were revealed by spraying with ninhydrin solution and identified by comparison with authentic specimens of the two polyamines and with material derived from preparations of coliphage T2.

Tryptophan dependence. A phage that shows a requirement of tryptophan for adsorption to its host bacterium depends on it likewise when presented with certain

mineral substances to which it can adsorb (Fildes & Kay, 1959). The adsorption results described in the present paper were obtained by the use of kaolin suspensions (Fildes & Kay, 1959) or by the use of bacterial suspensions treated in the same way.

RESULTS AND DISCUSSION

The properties of the twelve sewage phages are given in Table 1. The relevant details of phage 3, which was also obtained from Oxford sewage at an earlier date and coliphages T2 and T4 are listed for comparison. The similarities between these phages greatly outweigh the differences. Hydroxymethylcytosine, putrescine and spermidine were found in all the specimens examined. The dimensions of the

Table 1. *The properties of sewage bacteriophages*

Phage	Hydroxymethyl-cytosine	Putrescine and spermidine	Dimensions (Å)	Tryptophan requirement
Ox-1	Yes	Not tested	Head, 900 × 650 (*a*) tail, 850 periodicity, 30	Yes
Ox-2	Yes	Not tested	Similar to Ox-1	Yes
Ox-3	Yes	Yes	Similar to Ox-1	Slight
Ox-4	Not tested	Not tested	Similar to Ox-1	Yes
Ox-5	Yes	Yes	Similar to Ox-1	Slight
Ox-6	Yes	Yes	Head, 900 × 700 (*a*) tail, 850 periodicity, 30	Yes
Phage 3 (*b*)	Yes	Yes	Head, 900 × 650 (*a*) tail, 800 periodicity, 30	Yes
Sal-1	Yes	Yes	Similar to Ox-1	No
Sal-2	Yes	Yes	Similar to Ox-1	Yes
Sal-3	Yes	Yes	Similar to Ox-1	Yes
Sal-4	Yes	Yes	Similar to Ox-1	Slight
Sal-5	Yes	Yes	Similar to Ox-1	Slight
Sal-6	Yes	Yes	Similar to Ox-1	Yes
Coli T2	Yes (*d*)	Yes (*e*)	Head, 1000–650 (*c*) tail, 1000 periodicity, 30	No
Coli T4	Yes (*d*)	Yes (*e*)	Similar to T2	Yes (*f*)

(*a*) Bradley & Kay (1960); (*b*) Fildes & Kay (1957); (*c*) Brenner *et al.* (1959); (*d*) Wyatt & Cohen (1953); (*e*) Ames, Dubin & Rosenthal (1958); (*f*) Anderson (1945).

particles differ by slight amounts which might be due to damage suffered during specimen preparation for electron microscopy. The over-all appearance of the virus particles is such that it would be impossible to distinguish between them in the electron microscope. All the sewage phages as isolated, with the exception of Sal-1, showed a definite requirement for tryptophan. Coliphage T2, although of the same morphological and biochemical family, has never been described as showing tryptophan dependence.

The phages isolated during this work are probably highly unrepresentative of the types occurring in the city effluents because the procedure used was a selective one. The phosphate and citrate in the medium would ensure that all the divalent ion-requiring phages would be eliminated. On the other hand, the use of nutrient broth would make certain the retention of those phages that depend on tryptophan for

adsorption to their host bacteria. Consequently the phage population would be enriched in these types and their isolation thereby facilitated. A further selection was made when the first plaques were picked for propagation. Only small plaques were taken from a population of large and small ones. In general, large plaques are given by small phage particles and *vice versa*, the difference being due to their rates of diffusion. The selection procedure therefore favoured the isolation of large particle phages and, as was already known from work on the T-group of coliphages, only the largest of the morphological types, the T-even group, show any tryptophan effect. The present work bears this observation out and amplifies it.

Tryptophan-dependence and the presence of hydroxymethylcytosine as a nucleic acid component are major distinguishing properties of the group of phages under discussion. These properties are not found in any other group that has been examined. The size and shape of the phage particles is both characteristic and complex. They probably represent the most advanced evolutionary form of phage particle. The observation that the same type of phage exists in the effluent from Salisbury as in that from Oxford, when the two cities have quite separate water supplies and effluent systems, suggest that the type of phage in question may be very wide-spread.

The authors would like to thank Miss Muriel Dangerfield and Miss Annelies Pfäffli for technical assistance.

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