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Isolation of a Polyvalent Bacteriophage for *Escherichia coli*, *Klebsiella pneumoniae*, and *Aerobacter aerogenes*[1]

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A lytic bacteriophage isolated from sewage was found to attack strains of *Aerobacter aerogenes*, *Escherichia coli*, and *Klebsiella pneumoniae*, but not members of the genera *Salmonella*, *Proteus*, and *Serratia*. The phage, designated φmp, contained deoxyribonucleic acid with a 50% guanine plus cytosine ratio and a molecular weight of 23.1×10^6 daltons. Single-step growth experiments of φmp plated at 37 C on *A. aerogenes* A2 gave a mean latent period of 20 min, an average burst size of 103 plaque-forming units/infected cell, and an average adsorption rate constant of 3×10^{-10} ml/min. Electron microscopy of φmp revealed a phage with a flexible tail (165 nm long and 6 nm wide). The phage head had a hexagonal outline (62 nm in diameter).

The term polyvalence has been used to describe the lytic capacity of a phage for more than one host (10). Polyvalence, although relatively rare, has been reported for a number of bacterial families among which are *Enterobacteriaceae* (3, 6), *Pseudomonadaceae* (7, 13), and *Actinomycetaceae* (9). Within the *Enterobacteriaceae*, polyvalent phage crosses have been described among *Escherichia*, *Salmonella*, *Shigella*, and *Serratia* species (6). Phages have also been found that attack both *Klebsiella* and *Aerobacter* species (20). Phage interaction between *Escherichia* and *Aerobacter*, or between *Escherichia* and *Klebsiella*, has not been reported. This investigation deals with the isolation, purification, and characterization of φmp, a polyvalent phage that lytically infected strains of *Escherichia coli*, *Aerobacter aerogenes*, and *Klebsiella pneumoniae*.

MATERIALS AND METHODS

Media. Bacterial strains were grown in Penassay Broth (Difco) unless otherwise stated. Solid and semisolid Penassay Agar were prepared by supplementing the broth with 1.5 and 0.7% (w/v) agar (Difco), respectively.

Bacteria. Cultures were obtained from the sources shown in Table 1. All cultures were purified by repeated single-colony isolations and stored at 5 C on Penassay Agar slants. The bacterial strains tested for φmp sensitivity included: *E. coli* (10 strains), *Escherichia freundii* (1), *A. aerogenes* (9), *Aerobacter cloacae* (1), *Enterobacter aerogenes* (4), *Enterobacter cloacae* (1), *K. pneumoniae* (2), *Salmonella typhimurium* (1), *Proteus mirabilis* (2), and *Serratia marcescens* (1). *Enterobacter* and *Aerobacter* genera may be synonymous; however, for identification purposes, we have retained the nomenclature of our sources.

Isolation and purification of φmp. The phage, designated φmp, was isolated from sewage by the procedure of Adams (2). *A. aerogenes* A2 in Penassay Broth at 37 C was used as the host for the isolation. Purification of φmp and other phages used in this study was accomplished by making at least three successive single-plaque isolations. A single plaque from the final plating was stabbed and used to inoculate 25 ml of a log-phase culture in Penassay Broth at 37 C. The resulting lysate was shaken with 1 ml of chloroform and centrifuged twice for 10 min at 12,000 × *g* at 4 C in a Sorvall RC-2 centrifuge. The supernatant fluid was centrifuged at 22,000 × *g* for 3 hr at 4 C, and the phage pellets were suspended in 0.5 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris)-$MgCl_2$ buffer (0.01 M Tris, 0.01 M $MgCl_2$, *p*H 7.3), pooled, and centrifuged at 12,100 × *g* for 10 min at 4 C. The supernatant phage suspensions were further purified and concentrated by the isopycnic centrifugation method of D. S. Ray (thesis, Stanford University, Stanford, Calif., 1965) as follows. To 3.0 ml of the phage suspension was added 2.230 g of CsCl. This suspension was placed in

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an SW39L rotor and centrifuged for 48 hr at 37,000 rev/min and 20 C in a Beckman model L2 or L4 centrifuge. A 23-gauge hypodermic needle was inserted into the tube bottom and 5-drop fractions were collected in tubes containing 0.5 ml of 0.01 M Tris-$MgCl_2$ buffer. Fractions were assayed for phage; several tubes containing a high titer were pooled and dialyzed overnight against 0.01 M Tris-$MgCl_2$ buffer. The dialysate was stored at 5 C over a few drops of chloroform.

Host range and EOP. The host range of ϕmp was established by using the spot test method of Amati (4), and all hosts were subsequently tested for their relative ability to host the phage. Phage used in host range and efficiency of plating (EOP) experiments was grown on *A. aerogenes* A2. The relative EOP was defined as the ϕmp titer on one host relative to the titer on the best host, *A. aerogenes* A2. Penassay Agar (solid) with a Penassay Agar (semisolid) overlay was used for all platings except when strains of *E. coli* K-12 were tested with ϕmp. *E. coli* K-12 formed larger, more easily countable plaques when nutrient agar (Difco) was used. All plates were incubated for 18 to 24 hr at 30 C.

One-step growth experiments. The procedure of Streisinger et al. (23) was generally followed to determine the one-step growth curve of ϕmp. Phage was added at a multiplicity of infection (MOI) of 0.1 to cells of *A. aerogenes* A2 suspended in 0.01 M KCN at 37 C. After 10 min, a 10^{-4} and 10^{-6} dilution was made into Penassay Broth at 37 C, and unadsorbed phage was assayed by the chloroform method (14). The 10^{-4} and 10^{-6} diluents were incubated at 37 C, and portions were removed and plated at intervals through 60 min. Penassay Agar (solid and semisolid) was used with *A. aerogenes* A2 as the indicator host for all growth experiments. Plates were incubated for 24 hr at 37 C.

Electron microscopy. A drop of a purified phage preparation, suspended in 0.01 M Tris-$MgCl_2$ buffer at *p*H 7.3, was placed on a collodion-coated copper grid. The grid was inverted on a drop of 2.0% (w/v) phostungstic acid at *p*H 7.0 for 20 to 30 sec, and the excess liquid was removed with filter paper. Electron micrographs were made with a Phillips 300 electron microscope.

Serology. A slight modification of the technique of Vidaver and Shuster (24) was used to prepare ϕmp antisera. A high-titer ϕmp stock (approximately $5 \times \times 10^{11}$ plaque-forming units (PFU)/ml) suspended in Tris-$MgCl_2$ buffer or SSC buffer (0.15 M NaCl, 0.015 M $Na_3C_6H_5O_7 \cdot 2H_2O$, *p*H 7.0) was mixed with an equal volume of Freund's complete adjuvant. One milliliter of the emulsion was injected subcutaneously in two different dorsal locations on each of the rabbits (male New Zealand Whites). A series of three injections was made at 4-day intervals. Ten days after the final injections, the rabbits were bled by cardiac puncture and the sera were spun free of red blood cells and stored at −15 C until required.

The neutralization procedure was modified slightly from Adams (2). A 1.8-ml quantity of antiserum (1:50 dilution of antiserum was used for all phages except ϕmp, which required 1:500) was mixed with 0.2 ml of phage at 37 C. Samples (0.1 ml) were removed at regular intervals through 30 min, diluted 1:100 in Penassay Broth, and plated on Penassay Agar in triplicate. Plates were incubated 24 hr at 37 C. *A. aerogenes* A2 was used as an indicator host for ϕmp, *E. coli* K-12 was used for virulent λ phage, and *E. coli* B3T-U-RTF was used for the seven T phages.

DNA extraction. Bacterial deoxyribonucleic acid (DNA) was extracted by the Marmur method (18). Phage nucleic acid was extracted by the phenol technique of Mandell and Hershey (17), and identified as DNA by the diphenylamine reaction (12), which is specific for 2-deoxypentoses. DNA concentrations were initially determined by reading the absorbancy at 260 nm (1.0 unit = 50 μg/ml) and also by the diphenylamine method. Both methods yielded similar results, and the former was used routinely.

Per cent GC of phage and bacterial DNA. The guanine plus cytosine (GC) of both ϕmp DNA and the DNA from two hosts (*A. aerogenes* A2 and *E. coli* B3T-U-RTF) was obtained by the method of Schildkraut, Marmur, and Doty (21). About 2 to 3 μg of DNA was mixed with approximately 1 to 2 μg of a reference standard DNA (*Micrococcus lysodeikticus*, buoyant density 1.731 g/ml). CsCl was added to a density of 1.701 g/ml, and the solution was placed in an An-D rotor containing a Kel-F centerpiece. The DNA solutions were centrifuged for 16 hr at 42,040 rev/min and 20 C in a Spinco model E analytical ultracentrifuge. Ultraviolet absorption photographs were taken near the end of the run, and density tracings were made with a Beckman Analytrol densitometer. The per cent GC of ϕmp and bacterial DNA also was determined by the thermal denaturation procedure of Marmur and Doty (19).

Molecular weight of ϕmp DNA. The sucrose gradient method of Burgi and Hershey (11) was followed to determine the molecular weight of ϕmp DNA. A thymine-requiring strain of *A. aerogenes* A2 was grown in 15 ml of 1% tryptone broth (w/v) containing 100 μCi of ^{3}H-thymidine. Log-phase cells were infected with ϕmp at an MOI of 2.0. The phage lysate was purified by differential and isopycnic centrifugation as described above. ^{14}C-labeled coliphage T7 was obtained by growing the phage on cells of *E. coli* B3T-U-RTF, which had been grown in 15 ml of 1% tryptone medium containing 100 μCi of ^{14}C-thymidine. A mixture of the DNA extracted from both phages was layered on a 5 to 20% (w/v) linear sucrose gradient. The gradients were centrifuged in an SW39L rotor at 28,000 rev/min for 4 hr at 4 C. Two-drop fractions were collected from the tube bottom and assayed for radioactivity in a Packard scintillation counter (model 314 EX).

RESULTS

Host range. Of the 32 bacterial strains examined for ϕmp sensitivity by the spot-test method, only strains of *E. coli*, *A. aerogenes*, and *K. pneumoniae* served as ϕmp hosts. The host range included six of the 10 strains of *E. coli*, 1 of 9 strains of *A. aerogenes*, and 1 of 2 strains of *K. pneumoniae* (Table 1). Two strains

TABLE 1. *Host range, efficiency of plating (EOP), and plaque morphology of φmp*

Organism	Source	Spot test[a]	EOP	Plaque morphology (30 C)
Escherichia coli 11775	San Jose State College			
E. coli 0111	San Jose State College			
E. coli B	San Jose State College	+	0.1	Turbid plaque (3.5 mm diameter) with clear center
E. coli K-12	San Jose State College	+	0.2	Turbid plaque (1.0 mm diameter)
E. coli 055	San Jose State College	+	0.3	Turbid plaque (1.0 mm diameter)
E. coli B3T-U-338	Ames collection	+	0.1	Turbid plaque (2.5 mm diameter) with clear center
E. coli Δ+	Ames collection			
E. coli K12F-met-	Ames collection	+	0.3	Turbid plaque (1.0 mm diameter)
E. coli B3T-U-RTF	Ames collection	+	0.1	Turbid plaque (2.5 mm diameter) with clear center
E. coli B3T-original	Ames collection			
E. freundii	San Jose State College			
Aerobacter aerogenes	San Jose State College			
A. aerogenes A3	San Jose State College			
A. aerogenes	Stanford University			
A. aerogenes A2	ATCC 129	+	1.0	Clear plaque (2.0 mm diameter) with small halo
A. aerogenes	Foothill Junior College			
A. aerogenes J6	University of California			
A. aerogenes 1033	University of California			
A. aerogenes 8724	University of California			
A. aerogenes GCOC	Ft. Baker, Calif.			
A. cloacae	ATCC 11367			
Enterobacter aerogenes	ATCC 15038			
E. aerogenes	ATCC 13048			
E. aerogenes	ATCC 23356	+		
E. aerogenes	ATCC 23357	+		
E. cloacae	ATCC 13047			
Klebsiella pneumoniae	San Jose State College			
K. pneumoniae M5A1	University of California	+	3×10^{-4}	Clear plaque (1.0 mm diameter) with small halo
Proteus mirabilis	Ames collection			
P. mirabilis-1	Ames collection			
Salmonella typhimurium	Ames collection			
Serratia marcescens	Ames collection			

[a] Spot tests were made with log-phase cells streaked on Penassay Agar plates and spotted with φmp. Plates were incubated for 24 hr at 37 C. A plus (+) indicates the bacterial streak was cleared at the site of phage application.

of *E. aerogenes* gave positive spot tests, but attempts to obtain plaques on a lawn of either *E. aerogenes* strain failed. Infection of broth cultures of both *E. aerogenes* strains with φmp resulted in a decrease rather than an increase in titer, which suggested that these strains adsorbed but did not reproduce φmp.

The possibility of bacteriocin production by the φmp hosts used to prepare the phage was checked by spotting filtrates of *E. coli* B3T-U-RTF and *A. aerogenes* A2 on a lawn of *E. aerogenes*. Such tests failed to demonstrate bacteriocins.

The EOP and the plaque morphology varied among the different strains of *E. coli* that could host φmp, and in no case did the EOP equal that

obtained on *A. aerogenes* A2 (Table 1). However, when ϕmp was grown and purified on *E. coli* B3T-U-RTF, as opposed to *A. aerogenes* A2, the plating efficiency on the former increased from 0.1 to 0.7. The sevenfold increase was reproducible and did not increase further upon successive passages on *E. coli*. Curiously, a single passage of *E coli*-grown-and-purified ϕmp through *A. aerogenes* A2 was sufficient to return the EOP on *E. coli* to 0.1.

One-step growth curve. Figure 1 illustrates the growth curve of ϕmp in *A. aerogenes* A2 at 37 C in Penassay Broth. Four experiments were performed that yielded an average latent and rise period of 20 and 17 min, respectively. The burst size averaged 103 PFU/infected cell with an average adsorption rate constant of 3.0×10^{-10} ml/min.

Serology. Antiserum, specific for ϕmp, showed no cross-neutralization against coliphages T1-7 or λ virulent. Inactivation of ϕmp by specific antiserum proceeded logarithmically to approximately 90% inactivation, and an identical inactivation rate was obtained with *A. aerogenes* A2 or *E. coli* B3T-U-RTF-grown-and-purified phage (Fig. 2).

DNA analyses. The nucleic acid of ϕmp was found, by the diphenylamine method, to contain deoxyribose. The DNA was assumed to be double-stranded since, like typical double-stranded DNA, it exhibited a 30 to 40% increase in absorbance at 260 nm when heated (Fig. 3). The average T_m obtained for ϕmp DNA was 90.8 C, which corresponded to a 52% GC content (19). The buoyant density of ϕmp DNA was 1.709 g/ml, and was identical for both *E. coli* and *A. aerogenes*-grown-phage (Fig. 4). The per cent GC calculated from the buoyant density by the method of Schildkraut, Marmur, and

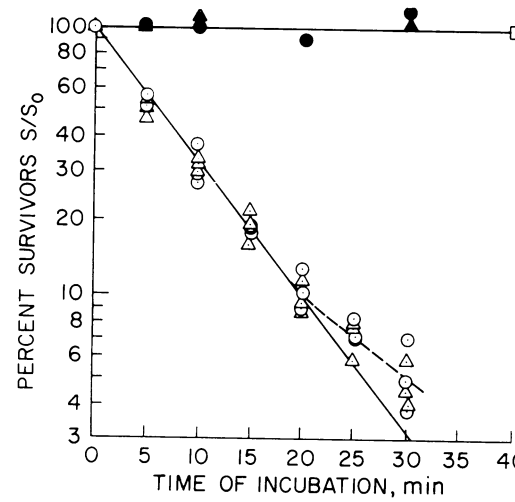

FIG. 2. *Time-survivor curve for ϕmp grown and purified on A. aerogenes A2 (△) or E. coli B3T-U-RTF (⊙). Points on the curve were compiled from the results of three experiments. Phage was mixed with a 1:500 dilution of ϕmp antiserum and incubated at 37 C. Portions were removed at intervals, plated on Penassay plates with A. aerogenes A2, and incubated for 24 hr at 37 C. A control serum (●, ▲) diluted 1:100 and a broth control (⊡) were also included. Dotted line represents the reduced neutralization rate that occurred after about 90% inactivation.*

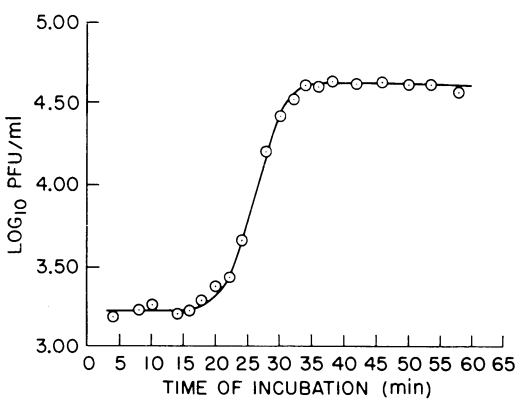

FIG. 1. *One-step growth curve ϕmp. Exponentially growing cells (37 C) were infected for 10 min, in the presence of 0.01 M KCN, with ϕmp (MOI = 0.1). At zero minutes, cells were diluted 10^{-4} and 10^{-6} times into Penassay Broth and incubated at 37 C. Portions were removed from the diluted samples at intervals, plated on Penassay plates with A. aerogenes A2, and incubated for 24 hr at 37 C.*

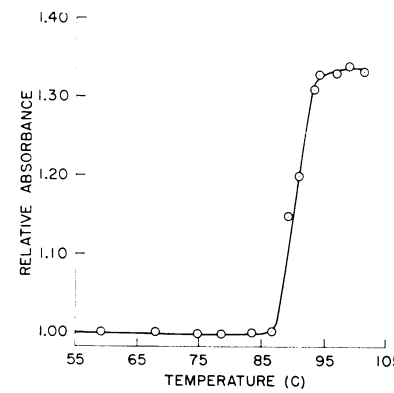

FIG. 3. *Thermal transition of ϕmp DNA in 0.15 to 0.015 M saline-citrate buffer at pH 7.0. DNA solutions were contained in 1-cm pathlength glass-stoppered quartz cuvettes and heated in a Beckman DU spectrophotometer chamber. The absorbance at 260 nm, corrected for thermal expansion, was recorded after each solution was exposed for 15 min to the indicated temperature. The ordinate gives the absorbance reading relative to the absorbance of the unheated sample at 25 C.*

Doty (21) was 50% GC, which is similar to the value obtained from thermal denaturation experiments. The similarity between the two GC determinations suggests that unusual bases are either minor constituents or entirely absent in φmp DNA.

Buoyant densities were also obtained for the DNA of two φmp hosts, *A. aerogenes* A2 (1.715 g/ml) and *E. coli* B3T-U-RTF (1.710 g/ml), and were found to correspond to literature reports for both species (21).

DNA from φmp and coliphage T7 sedimented with an identical rate in a linear 5 to 20% sucrose gradient (Fig. 5). From the similar results of several experiments, the molecular weight of φmp was concluded to be identical to that of phage T7, 23.1 × 10^6 daltons (22).

Morphology. Electron microscopy of φmp preparations, negatively stained with 2% phosphotungstic acid, revealed a phage with a long, flexible tail (165 by 6 nm) and a head that had a regular hexagonal outline 62 nm in diameter, measured apex to apex (Fig. 6). The phage tail apparently lacked a sheath, and no tail fibers have yet been conclusively demonstrated. The size and structure of φmp did not vary when different hosts were used to prepare phage samples for microscopy. According to the bacteriophage classification system of Bradley (8), the morphology of φmp identifies it as a group B bacteriophage.

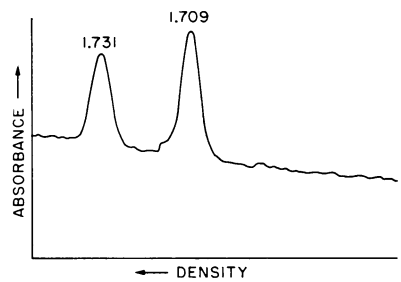

FIG. 4. *Microdensitometer tracing of A. aerogenes A2-grown φmp DNA (1.709 g/ml) and the reference standard M. lysodeikticus DNA (1.731 g/ml), equilibrated in a CsCl density gradient formed by centrifugation in an An-D rotor for 16 hr at 42,040 rev/min and 20 C.*

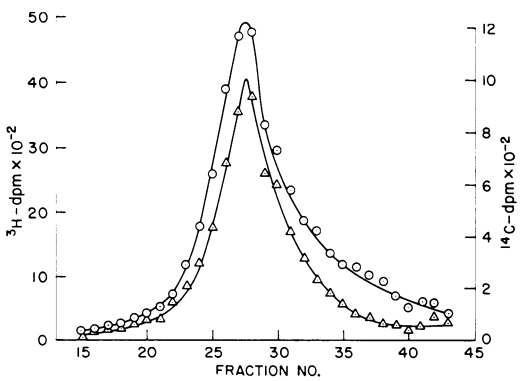

FIG. 5. *Sedimentation pattern of ^{3}H-labeled φmp DNA (⊙) and ^{14}C-labeled coliphage T7 DNA (△). The DNA was sedimented in a 5 to 20% (w/v) linear sucrose gradient at 4 C for 4 hr at 28,000 rev/min in an SW39L rotor. Two-drop fractions were collected from the tube bottom and assayed for radioactivity.*

DISCUSSION

Our results showed that φmp contained double-stranded DNA and possessed a Bradley group B morphology. A review by Ackermann (1) contained the characteristics of more than 400 bacteriophages against which we compared the characteristics of φmp. Although φmp could be

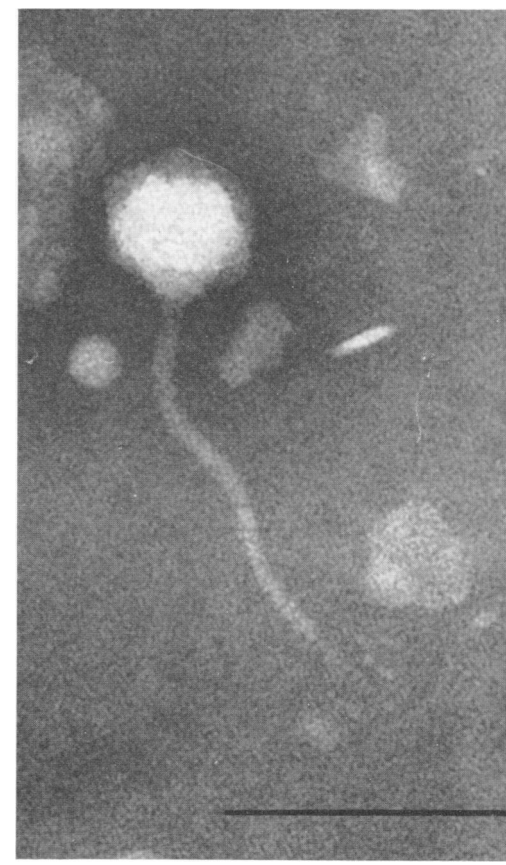

FIG. 6. *Electron micrograph of φmp negatively stained with 2% phosphotungstic acid at pH 7.0 × 380,000. Marker = 0.1 μm.*

distinguished from previously reported phages, the only unique ϕmp characteristic was its host range. Host range mutants are frequently found among phages, and since ϕmp attacked the majority of *E. coli* strains tested, we thought ϕmp might be related to a known coliphage. Luria (16) established that host range mutants do not differ antigenically from their parent strains. Since the eight coliphages we examined were not inactivated by ϕmp antisera, it is unlikely that ϕmp represents a host range mutant of one of these eight common coliphages.

The growth of ϕmp on *E. coli* B3T-U-RTF increased the EOP on this *E. coli* strain, but a single passage through *A. aerogenes* A2 was sufficient to return the EOP on *E. coli* to the original value. Reversion to the initial EOP on *E. coli* was probably not the result of a host range mutation since a much lower reversion rate would be expected if mutation caused the EOP change. Instead, a host-cell modification similar to that described by Arber and Lin (5) was the most likely explanation for the rise in EOP of *E. coli*-grown phage.

The positive spot tests of ϕmp with *E. aerogenes* strains 23356 and 23357 may represent "lysis from without," the disruption of cells caused by the adsorption of large numbers of bacteriophage (2). An alternative explanation can be given based on an observation by Amati (4). He found that *Pseudomonas aeruginosa* and *Serratia marcescens* gave positive spot tests with the P1 phage of *Shigella*, but the phage neither multiplied in nor lysed the cells. P1 phage adsorbed and penetrated cells of both bacteria, but quickly killed them thus aborting the infection. A similar abortive infection may take place when *E. aerogenes* 23356 and 23357 are infected with ϕmp.

The addition of ϕmp to a growing number of polyvalent phages increases the need for a study of this group of bacteriophages, particularly to establish the mechanism of polyvalent action. Polyvalence may reflect more than one receptor site on the phage tail as was found with phage Y of *Pasteurella pestis* and *E. coli* (15). Alternatively, polyvalence may indicate the existence of homologous cell wall receptor sites possessed by the host cells. Further studies with ϕmp are being conducted to establish whether polyvalence is a trait of the phage or the host bacteria.

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