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Isolation and Characterization of a Generalized Transducing Bacteriophage for *Acinetobacter*

NORMA J. HERMAN[1] AND ELLIOT JUNI

Department of Microbiology, The University of Michigan, Ann Arbor, Michigan 48104

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A series of bacteriophages which grow in various strains of *Acinetobacter* have been isolated. One of these, phage P78, which forms turbid plaques on *Acinetobacter* strain 78 is specific for this particular host and fails to attack 389 other independently isolated strains of *Acinetobacter*. Phage P78 appears to be a temperate phage which lysogenizes its host. Various agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, diethyl sulfate, mitomycin C, and ultraviolet light are effective inducers of the lysogen. Phage lysates of wild-type cells are capable of transducing auxotrophs of strain 78 to prototrophy at frequencies ranging from 0.3×10^{-7} to 34×10^{-7} per plaque-forming unit adsorbed. To date, no linkage has been detected between any of the markers studied in two-factor crosses. Donor phage grown in one particular mutant, strain 78 (*arg-1*), has been shown to give rise to significantly higher transduction frequencies than when phage is grown on wild-type or other auxotrophic strains. Phage P78 is rapidly adsorbed to its bacterial host and has a latent period of 25 min, and infection results in a burst size of approximately 50. Some of the physical properties of phage P78 and its DNA are described.

Bacteria comprising the genus *Acinetobacter* are gram-negative coccobacilli; in addition, they are typically nonmotile and oxidase-negative (3). These organisms are commonly found in soil and water (18); some strains are indigenous to man and animals. All members of this group are obligately aerobic and most of them are capable of growing in simple mineral media with acetate as the sole carbon source (3). The finding from this laboratory that one strain of *Acinetobacter* (strain BD413) is competent for genetic transformation (9) has provided the impetus for further work seeking to determine genetic linkages in this group of bacteria. Since transducing phage provides a unique advantage in fine structure mapping, because of the fixed size of donor DNA transduced, a search for such phage was made. Using transformation Sawula and Crawford (16) have shown recently that the genes governing synthesis of tryptophan from chorismic acid map in three chromosomal locations in a manner unlike that found in any other organism to date.

The present paper describes the isolation and characterization of a generalized transducing phage for one strain of *Acinetobacter*. A preliminary report of this work has been presented (N. J. Herman and E. Juni, Bacteriol. Proc., p. 174, 1971).

[1] Present address: Department of Biological Sciences, Fort Hays Kansas State College, Hays, Kan. 67601.

MATERIALS AND METHODS

Growth of bacterial host and phage assay. Strains of *Acinetobacter* were grown in Penassay broth (antibiotic assay medium no. 3, BBL or Difco) at 34 C with shaking. Phage were assayed by using the agar overlay technique (1). Stock cultures were stored on Penassay slants at 4 C and were transferred bimonthly. When a defined medium was required the S-2 minimal medium of Monod and Wollman (14) containing 0.5% L-malic acid was used. For preparation of plates, this minimal medium was mixed with an equal volume of melted 3% agar immediately before pouring.

Chemicals and enzymes. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and diethyl sulfate were obtained from Aldrich Chemical Co. D-Cycloserine, cyclic AMP, and mitomycin C were obtained from Sigma Chemical Co. Deoxyribonuclease I (DNAse) was obtained from Worthington Biochemical Co. Ampicillin was obtained from Bristol Labs. Cesium chloride was obtained from EM Labs, Inc. All other chemicals were reagent grade.

Phage isolation and assay. Samples of activated sludge from the Ann Arbor Sewage Processing Plant were inoculated into flasks containing Penassay broth and incubated with shaking overnight at 30 C. The contents of each flask were centrifuged to remove any sedimentable debris and the supernatant fluid was filtered through a sterile Gelman membrane filter (type GA-8, 0.2 μm mean flow pore size). Filtrates

were plated with *Acinetobacter* strains 25, 43, 46, 53, 58, 78, 83, 99, 108, and BD413 (8) by the agar overlay technique. Plaques were picked and purified by repeated subculture on the respective host strain. Phage stocks of each isolate were prepared by infecting exponential phase bacterial cultures at a multiplicity of infection (MOI) of approximately 1.0 and continuing incubation with shaking until lysis was evident, usually about 2 to 3 h after infection. After removal of cell debris by centrifugation, supernatants were passed through sterile membrane filters, as above, and phage were titered.

Adsorption studies. Phage were added to exponential phase cells at a multiplicity of approximately 0.7 and incubation of the infected cell suspension was continued at 34 C with shaking. Samples were removed at zero time, 1, 2, and 3 min, diluted 100-fold in ice-cold peptone broth, and centrifuged in the cold, and supernatants were diluted and assayed for free phage.

One-step growth experiments. One-step growth and single burst experiments were performed by the methods outlined by Adams (1).

Isolation and induction of lysogens. Lysogens of strain 78 were obtained by picking viable cells from the center of a turbid plaque of phage P78 and purifying away from any possible free phage by repeated streaking on Penassay plates. No plaques were visible when phage P78 was plated with an indicator lawn of bacteria isolated in this manner.

Induction of lysogenic strains was observed using the method of Mayer et al. (13).

Isolation of bacterial mutants. Auxotrophs of strain 78 were obtained following mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine using the methods of Adelberg et al. (2). In order to enrich for auxotrophs, exponential phase cultures of mutagenized cells grown in malate-minimal medium were treated with a combination of cycloserine (400 μg/ml) and ampicillin (2,000 μg/ml) for 2 h, at which time the viable count was reduced to approximately 0.1%. Cells were then washed free of antibiotics, diluted appropriately, and plated on Penassay agar. Colonies appearing on these plates were replicated to malate-minimal agar plates and those which failed to grow on this medium were tested for specific growth-factor requirements (4).

Transduction experiments. Auxotrophs of strain 78 were grown overnight in Penassay broth, centrifuged, resuspended to a concentration of 5×10^9 to 10×10^9/ml, infected with phage P78 at an MOI from about 0.2 to 5.0, and incubated for 5 min at 34 C with aeration. Bacteria were then sedimented by centrifugation and the pellet was resuspended in malate-minimal medium to the same volume as the original suspension, and 0.2-ml samples were spread on malate-minimal agar plates. Colonies of prototrophic transductants were scored after incubation at 34 C for 48 h. For each transduction experiment phage lysates were plated on Penassay agar to test for sterility. Equal samples of the auxotrophs, to which no phage were added, were also plated on malate-minimal agar to determine the rate of spontaneous reversion to prototrophy.

Antiserum. Antiserum against phage P78 was prepared in rabbits by a series of five intravenous injections of 2 ml of phage suspension (10^{10} PFU/ml).

Purification of phage P78. Lysates for phage purification were prepared by infecting about 600 ml of early log-phase cells with phage at an MOI of about 1.0 and incubating with aeration until lysis occurred. In some experiments cells were grown to early log phase (about 1.5 h) in 1-liter quantities, were centrifuged, and were concentrated by resuspending in 0.1 the original volume of Penassay broth. They were infected at a final MOI of about 1 and allowed to incubate with aeration until lysis occurred. Crude phage lysates were centrifuged at 8,000 rpm in a Sorvall RC2-B centrifuge for 20 min to remove cell debris; phage were pelleted by centrifugation for 2 h at 25,000 rpm in a Beckman L-2 centrifuge. Pellets were resuspended in 10 ml of TMK buffer (5×10^{-3} $MgCl_2$ plus 0.3 M KCl, buffered with 0.01 M Tris-hydrochloride, pH 7.2; reference 15) and treated with DNase (8 μg/ml) for 1 h at room temperature. Solid CsCl was added to a final concentration of 47.5% (wt/wt) and the mixture was divided equally among three 5-ml cellulose nitrate tubes. Mineral oil was layered over the CsCl-phage suspension which was then centrifuged at 35,000 for 48 h at 20 C. Fractions were collected and the pooled phage bands (about 1.5 ml) were dialyzed overnight against 1 liter of 1X SSC (0.15 M NaCl plus 0.015 M Na_3 citrate).

Isolation of phage DNA. Phage P78 DNA was prepared essentially as described by Yelton and Thorne (20) and stored in SSC at 4 C. DNA concentration was estimated in a Zeiss spectrophotometer assuming that an absorbancy of 1.0 at 260 nm (1 cm light path) corresponds to a concentration of 50 μg of DNA/ml.

Isolation of bacterial DNA. DNA from host strain 78 was isolated by the method of Marmur (12).

Equilibrium centrifugation of phage and host DNA in CsCl. A 1.37-g amount of CsCl was dissolved in 0.79 ml of SSC; 0.20 ml of phage DNA (5.4 μg DNA) was added along with 5 μg of *Micrococcus lysodeikticus* DNA (ρ = 1.731) and *Bacillus subtilis* SPO-1 phage DNA (ρ = 1.742 ± 0.001) as standards. The refractive index of the sample was adjusted to 1.4002 as determined in a Bausch & Lombe refractometer. Centrifugation was continued for 22.5 h at 20 C in a Beckman E analytical ultracentrifuge with a Kel F centerpiece. Ultraviolet absorption photographs were taken using Kodak commercial film and scanned with a Heath microdensitometer. The same technique was used for determining the density of bacterial DNA. Density of DNA was calculated by using the equation of Mandel et al. (10). The GC percentage of DNA was determined from the density of each sample (10).

Sedimentation velocity of phage DNA. Sedimentation was carried out at 20 C in a Beckman E analytical ultracentrifuge with ultraviolet absorption optics. An aluminum centerpiece was employed in these experiments. The four DNA concentrations used in this study ranged from 6 to 51 μg/ml; dilutions of DNA were made to the desired concentration in 1X SSC. Phage DNA was centrifuged at 44,770 rpm and photographs were taken at 8-min intervals. Absorption photographs were scanned with a Heath mi-

crodensitometer. Molecular weight of the phage DNA was calculated by using the equation of Eigner (5).

Electron microscopy. Phage, purified in CsCl and dialyzed against buffer, was used. Preparations were negatively stained with 2% phosphotungstic acid (pH 7.2) by coating droplets of the phage-stain mixture on Formvar-carbon-coated grids (400 mesh). Electron micrographs were taken on AEI electron microscopes, models EM6B and Corinth-275, at 60 kV with liquid nitrogen cooled decontaminator.

Transformation experiments. Competency of strain 78 was tested by the method of Juni (8) in which an auxotroph is incubated continuously with wild-type prototrophic DNA during growth on a complex medium and the resulting cell-DNA mixture is streaked on minimal medium where any prototrophic transformants will give rise to colonies. To determine whether genetic recombination observed in the present study resulted from transformation rather than from transduction, phage suspensions were treated with DNase (10 μg/ml) for 30 min, to destroy any transforming DNA possibly present, prior to mixing with recipient bacterial cells.

RESULTS

Temperature nature of phage P78. When plated on *Acinetobacter*, strain 78, phage P78 produces plaques which have turbid centers surrounded by small clear rings. Bacteria isolated from the turbid centers are not lysed by P78 and are lysogenic. Induction of the lysogen is demonstrated in Fig. 1B where an inducing agent (diethyl sulfate) was applied to a mixture of sensitive and lysogenic cells. The massive induction evident on this plate contrasts markedly with the pattern observed on the control plate to which no inducing agent was added (Fig. 1A). In similar tests the lysogenic strain was induced with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and mitomycin C. It was also shown that maximum induction occurred after exposure to ultraviolet light for 10 s at a dose of 810 μW/cm^2. Longer exposure to ultraviolet light resulted in a considerable reduction in the number of viable phage.

Adsorption and one-step growth experiments. As indicated in Fig. 2, phage P78 adsorbs quite readily and fairly completely to its host strain. Within 3 min after mixing, greater than 90% of the added phage particles are adsorbed to strain 78. Phage P78 seems to be quite specific for its host strain regarding adsorption since it failed to adsorb to several other strains of *Acinetobacter* with which it was mixed. Furthermore, P78 failed to lyse 389 independently isolated *Acinetobacter* strains.

A typical one-step growth curve is shown in Fig. 3. The latent period is 25 min with a burst size of approximately 50. Single burst experiments gave an average burst size of approximately 40, based upon the results of six independent determinations. Cell lysis was evident at the termination of the one-step growth experiment.

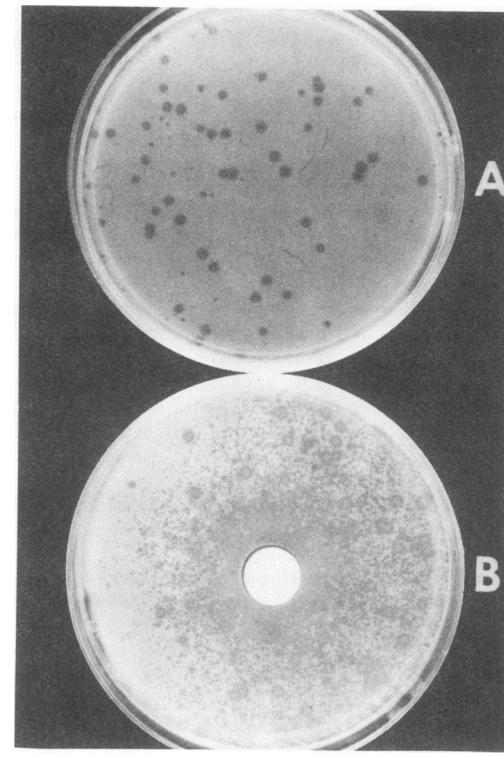

FIG. 1. *Induction of lysogenic cells. Late log-phase lysogenic cells were mixed with phage-sensitive cells and plated by the agar overlay technique. An antibiotic assay disk placed in the center of the plate was soaked in a 10% solution of DES in ethyl alcohol and incubated at 34 C overnight; induction of the lysogen is shown in Fig. 1B. Fig. 1A is the control plate containing a mixture of the lysogen and phage-sensitive cells. No induction was observed in a control plate in which the central disk was soaked with ethyl alcohol.*

A spontaneous clear-plaque mutant, isolated from wild-type phage stock, was shown to adsorb readily to sensitive or lysogenic bacteria although it was unable to form plaques when plated on the lysogen.

Transduction experiments. All alleles tested could be transduced by phage P78. Representative results for transduction of a group of auxotrophs to prototrophy are shown in Table 1 where frequencies of transduction range from 0.3×10^{-7} to 34×10^{-7} per PFU adsorbed. Transduction frequencies at different MOI are included for several mutants; these frequencies decreased slightly with increase in MOI, probably due to simultaneous infection and killing by phage particles. The degree of bacterial killing varied somewhat with the particular marker in the recipient strain being transduced.

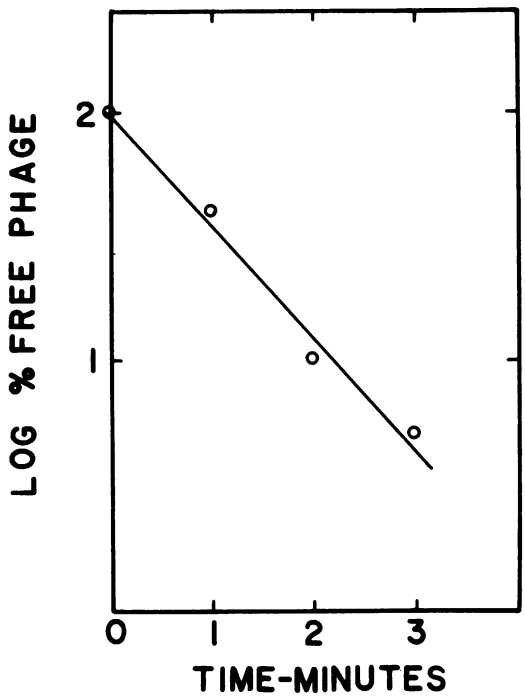

FIG. 2. *Adsorption of phage P78 to phage-sensitive host cells. The details of the experiment are given in Materials and Methods.*

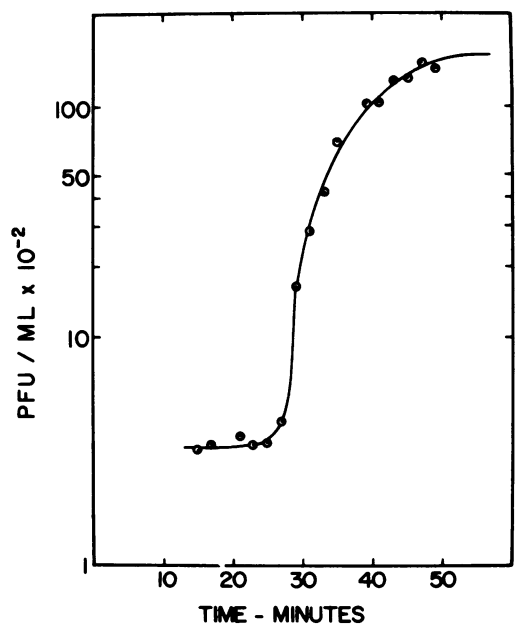

FIG. 3. *One-step growth curve for phage P78. Phage was added to log-phase cells at an MOI of about 5. Adsorption was allowed to continue for 3 min at 34 C in a shaking water bath. The culture was diluted immediately into Penassay broth at 34 C and 0.1-ml samples were removed at the appropriate time intervals for plating with the host strain.*

TABLE 1. *Transduction of auxotrophs to prototrophy with the wild-type strain 78 as donor*

Marker	MOI	Transductants per 10^7 PFU
his-1	0.9	14
his-2	1.0	5
ser-1	0.65	2
ser-1	1.3	1.8
ser-1	6.5	1.1
met-1	0.7	1
met-1	3.4	2.3
met-1	6.9	0.8
cys-1	0.2	4.2
cys-1	0.8	1.2
cys-1	1.6	0.9
cys-1	8.1	0.3
arg-1	0.3	28
arg-2	0.1	2
ilv-1	0.17	1
ilv-1	1.7	0.8
ade-1	0.4	16
ade-1	2.4	4.6
ade-1	10.0	0.06
leu-1	0.9	34
trp-1	0.4	2.6
trp-1	2.4	1.2
trp-1	17.0	0.9

When phage is plated on strain 78 (*ade-1*), only clear plaques are seen. Since lysogenization of these cells is unlikely to occur under such conditions, it is to be expected that there will be a decrease in frequency of transduction of this mutant as the MOI is increased (Table 1). In view of the observations of Hong et al. (7) and Grodzicker et al. (6) that *Salmonella* phage P22 and lambdoid phages form clear plaques on mutant hosts with defective adenylate cyclase, we attempted to determine whether adding cyclic AMP to the growth medium would restore turbid plaque morphology when phage P78 was plated on strain 78 (*ade-1*). These experiments gave negative results suggesting that the defect in strain 78 (*ade-1*) may not involve alteration of intracellular cyclic AMP levels. It is also possible that, unlike the enteric bacteria, *Acinetobacter* may not be able to take up cyclic AMP from the growth medium.

No linkage has been observed so far with any pair of mutants tested in two-factor cross studies. When donor phage was grown in strain 78 (*arg-1*) it was noted that the frequencies of transduction of other auxotrophs to prototrophy were significantly higher than when donor phage was grown in wild-type cells (Table 2). For each case shown in Table 2 the transduction frequency was higher for phage grown in strain 78 (*arg-1*), although the relative increase varied from 6- to 110-fold for the various auxotrophs

TABLE 2. *Comparison of transduction frequencies using wild-type phage and 78 (arg-1) phage*

Recipient marker	MOI	Transduction frequency × 10^7		Fold increase
		Wild-type donor	78 (*arg-1*) donor	
arg-2	0.07	16	96.4	6
ser-1	0.17		33.2	
ser-1	0.13	10.4		
trp-1	0.6	3.9	149	38
ilv-1	0.7		72	
ilv-1	1.7	0.8		
cys-1	0.9	1.2	11	9
met-1	0.65	1	110	110

transduced. The particular defect in arginine biosynthesis in strain 78 (*arg-1*) does not appear to be concerned with the increased frequencies of transduction observed for phage grown on this mutant strain since phage grown on a prototrophic transductant of strain 78 (*arg-1*) was also found to display higher frequencies of transduction compared with phage grown on wild-type cells. Because strain 78 (*arg-1*) was obtained by mutagenesis using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine it is reasonable to assume that this potent mutagen also altered this strain, in an as yet unknown manner, in its ability to produce transducing particles.

Since at least one strain of *Acinetobacter* is known to be competent for genetic transformation (9) the possibility was considered that DNA, released from phage-infected cells, could be transforming the recipient auxotroph to prototrophy and that transduction was not involved in formation of the recombinants observed. It was shown, however, that strain 78 is not competent for transformation. Auxotrophs of this strain were not transformed to prototrophy after treatment with DNA from wild-type cells. When DNase was included in transduction mixtures no decreases in recombination frequencies were observed. To further verify that gene transfer in this system is phage mediated, it was demonstrated that the presence of antiserum prepared against wild-type phage completely inhibits the appearance of prototrophic recombinants.

Physical properties of phage P78. Table 3 lists some of the properties of phage P78 and its DNA. An electron micrograph of this phage (Fig. 4) reveals that it has a polyhedral head and a relatively short tail. The properties of phage P78 resemble those of a phage (BP1) which infects another strain of *Acinetobacter* (18) but fails to infect strain 78. Furthermore, phage P78 is unable to infect the host strain for phage BP1.

TABLE 3. *Properties of phage P78 and its DNA*

Determination	Measurement
Head size	50 nm
Tail length	13.3 nm
Plaques	1–2 mm diam.
Latent period	25 min
Burst size	44
Density in CsCl	1.524 g/cc
DNA	
Buoyant density	1.699 g/cc
GC %	39.8
Molecular wt	34.2×10^6

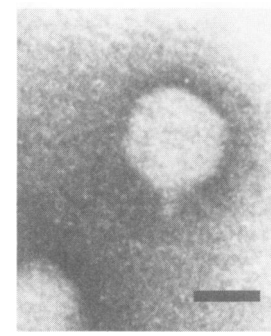

FIG. 4. *Electron micrograph of phage P78 negatively stained with phosphotungstic acid. Scale marker: 25 nm.*

Isolation of phages for other strains of Acinetobacter. It is not difficult to isolate phages for a variety of acinetobacters from sewage. In the present study 10 phages were isolated on different host strains. In general, each phage was specific for the host on which it was isolated and failed to infect other strains of *Acinetobacter*. This result is not surprising in view of the finding that there are a large number of different surface antigens on various acinetobacters (11). Bacterial strain 78 is not typable with any of the type sera already studied (11) (W. B. Cherry, personal communication). Of the 10 phages studied only P78 appears to be temperate, the others giving clear plaques when plated on their respective hosts.

DISCUSSION

Of the 10 *Acinetobacter* phages isolated in this study only one (phage P78) was investigated in some detail, primarily because it appeared to be a temperate phage. Although phage P78 does not attack the strain of *Acinetobacter* competent for transformation (strain BD 413), it is possible that treatment with mutagens may permit isolation of variants having a host range that includes this particular strain. Phage P78 is the first transducing phage

reported for any strain of *Acinetobacter* and should prove useful in mapping studies of at least one member of this genus. The rapid and almost complete adsorption of phage P78 to its bacterial host is also an attractive feature in working with this system. Phage P78 is the only known *Acinetobacter* phage which can transduce, since phage BP1 has been shown to be nontransducing (R. Twarog, personal communication).

As indicated above, phage P78 mediates generalized transduction, all auxotrophic mutants of strain 78 being transduced to prototrophy by phage preparations grown on the wild-type host. As is the case in other transducing systems, we have demonstrated variation in the frequencies of transduction of different auxotrophs to prototrophy, and also a dependency on the MOI. Of particular interest in this connection are the results obtained with strain 78 (*ade-1*), where clear plaques were observed when phage was plated on this mutant.

During our search for possible genetic linkage of various auxotrophic markers the observation was made that phage grown on strain 78 (*arg-1*), and used as donor in transduction experiments, consistently gave rise to increased frequencies of transduction of other auxotrophs to prototrophy, compared with the frequencies observed when donor phage was grown in wild-type cells (Table 2). Since phage grown on a prototrophic transductant of strain 78 (*arg-1*) also results in increased frequencies of transduction of several auxotrophic markers, the bacterial mutation responsible for this phenomenon appears to be unrelated to the genes for arginine biosynthesis. Although a report has appeared (17) describing mutants of *Salmonella* phage P22 with higher or lower frequencies of transduction, compared with transduction frequencies observed using wild-type phage, our studies indicate that there must also be bacterial determinants which control the relative numbers of transducing particles produced.

The size of phage P78 DNA (molecular weight = 34.2×10^6) suggests that this generalized transducing phage will be useful in mapping relatively small regions of the host genome. With the accumulation of a greater variety of host mutants it should be possible to begin preliminary mapping with this transduction system.

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