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MB78, a Virulent Bacteriophage of *Salmonella typhimurium*

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The isolation and some properties of a virulent bacteriophage of *Salmonella typhimurium*, MB78, which is morphologically, serologically, and physiologically unrelated to P22, are reported. The phage has a noncontractile long tail with partite ends. It cannot multiply in minimal medium in the presence of citrate. MB78-infected cells are, however, killed in such medium. This phage cannot grow in rifampin-resistant mutants of the host. The latent period of growth of this phage is much shorter than that of P22. Both *sieA* and *sieB* genes of the resident P22 prophage are required to exclude the superinfecting MB78 phage, whereas all temperate phages related to P22 are excluded by either one or both of the genes individually. Restriction endonuclease cleavage patterns of P22 and MB78 are distinctly different. The absence of homology between the two phages P22 and MB78 suggests that MB78 is not related to phage P22.

In the course of studies with the temperate phages of *Salmonella typhimurium*, a virulent bacteriophage of the microorganism has been isolated. This phage is morphologically, serologically, and physiologically different from the temperate phage as well as the other virulent phage Felix O1 (13) of *S. typhimurium* reported so far. The present report deals with the purification and characterization of this new phage, which has been named MB78.

MATERIALS AND METHODS

[^{3}H]thymidine (6,700 Ci/mol) was purchased from New England Nuclear Corp., Chicago, Ill. ^{32}P in the form of orthophosphoric acid was the product of Bhabha Atomic Research Centre, Bombay, India. 2,5-Diphenyloxazole (PPO) and dimethyl 1,4-bis-(5-phenyloxazolyl)benzene (POPOP) were products of the Amersham/Searle Corp., Arlington Heights, Ill. Cesium chloride was obtained from Schwarz/Mann, Orangeburg, N.Y. All other chemicals were commercial preparations of analytical grade.

Bacterial strains. *S. typhimurium* (LT2, strain 18) and the *sieA*$^+$ *sieB*$^+$ and *sieA sieB* lysogens were kindly supplied by M. Levine of the Department of Human Genetics, University of Michigan, Ann Arbor. *sieA sieB*$^+$ and *sieA*$^+$ *sieB* lysogens were isolated in our laboratory by lysogenizing LT2 with the corresponding phage strains obtained from D. Botstein, Department of Biology, Massachusetts Institute of Technology, Cambridge. The lysogens thus produced were checked for the presence of the respective prophages. It should be mentioned here that the presence of the immunity repressor prevents the multiplication of superinfecting homoimmune phage P22 in a P22 lysogen. However, upon induction of prophage in *sieA*$^+$ *sieB*$^+$ and *sieA*$^+$ *sieB* lysogens, only the prophage multiplies and the superinfecting phage is excluded, whereas in *sieA sieB* and *sieA sieB*$^+$ lysogens both the superinfecting phage and the prophage can multiply (18, 19). All of the homoimmune and heteroimmune superinfecting phages related to P22 are excluded by *sieA*$^+$ lysogens, but only heteroimmune phages are excluded by *sieB*$^+$ lysogens.

The strain referred to as 18/MC$_4$ is a thiolutin-resistant mutant of *S. typhimurium* derived from strain 18. It is a conditionally nonpermissive host for phage P22 that is unable to support phage development at 40°C (11). At 30°C 18/MC$_4$ can support phage development.

Phage strains. Wild-type phage P22 C^+ and its clear-plaque-forming mutant C_1 were obtained from M. Levine (12). Phages MG40 (7), MG178 (M. Grabner, Ph.D. thesis, Johns Hopkins University, Baltimore, Md., 1967), and L (2) were supplied by P. Amati, International Laboratory of Genetics and Biophysics, Naples, Italy. The wild-type phage, P22 C^+, lysogenizes the host. Mutation in the C_1 region of the phage genome results in the failure to establish lysogeny and thus leads to phage development and lysis of the cells.

Growth media. The cells were grown in minimal medium, Luria broth, or Casamino Acids-supplemented minimal medium (M9CAA). The compositions of the media are described elsewhere (5, 12, 16). Growth was followed by measuring absorbancy of the cell suspension in a Hilger type colorimeter at 610 nm.

Preparation of phage lysate. *S. typhimurium* 18 was grown in M9CAA medium (16) to 2.6×10^8 cells per ml at 37°C. Phage was added at a multiplicity of infection of 0.10 and incubated with aeration till lysis was over. Chloroform was added to lyse the unlysed cells, if any.

Purification of the phage. Bacterial debris was removed from phage lysate by centrifugation at 10,000 rpm for 20 min in a GS3 rotor of a RC5B Sorvall centrifuge. The lysate was concentrated by a two-phase system using 6.5% polyethylene glycol, 0.2% dextran sulfate, and 0.3 M NaCl (14).

The phage was finally purified by high-speed centrif-

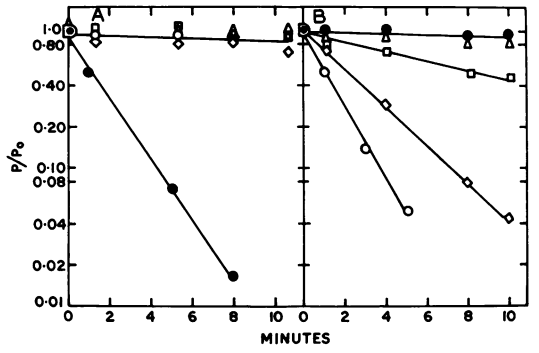

FIG. 1. Kinetics of neutralization of P22, MB78, and other related phages by anti-MB78 (A) and anti-P22 (B) antisera. Different phages (10^6 PFU/ml) were separately treated with either anti-P22 or anti-MB78 sera (final K = 0.5) at 37°C. At different times the number of infected particles was determined by plating samples from the incubation mixture after suitable dilution. Different phages used were MB78 (●), P22 (○), L (◇), MG178 (□), and MG40 (△).

ugation in a stepwise gradient of CsCl (ρ = 1.4 to 1.6 g/ml) as described by Botstein (3).

Preparation of ^{32}P-labeled phage. LT2 cells were grown in a low-phosphate medium (3) to which carrier-free $H_3{}^{32}PO_4$ (10 μCi/ml) was added 5 min after infection. The resulting phage lysate was purified as described earlier.

Preparation of ^{3}H-labeled phage. LT2 cells (2.6×10^8 per ml) growing in M9CAA at 37°C were infected with phage at a multiplicity of infection of 10. Deoxythymidine at a concentration of 3.3 μmol/ml was added along with [^{3}H]thymidine (16 nmol containing 2.2×10^8 cpm). After lysis, phage particles were concentrated and purified as described before.

Isolation of phage DNA. An equal volume of 0.1× SSV (1× SSV is 0.015 M NaCl–0.03 M EDTA, pH 8.0) was added to the purified phage (10^{14} PFU/ml), which was extracted twice with SSV-saturated phenol. The aqueous layer was collected, and the last trace of phenol was removed by washing with chloroform. DNA was precipitated by adding double the volume of ice-cold 95% ethanol. Ethanol was removed by drying the tube in a vacuum desiccator. DNA was dissolved in 0.01× SSV. Specific activities of ^{32}P-labeled P22 and MB78 DNAs were 0.8×10^5 and 1.3×10^5 cpm/μg of DNA, respectively.

Equilibrium density gradient centrifugation. To avoid long-term centrifugation, a short-range gradient (ρ = 1.45 to 1.6 g/ml) of cesium chloride was prepared in a 5-ml nitrocellulose tube. A mixture of purified ^{3}H-labeled phage was layered on the top of the freshly prepared gradient and centrifuged at 170,000 × *g* for 3 h in a swing-out rotor of a VAC 60Z centrifuge at 3 to 4°C. Since the cesium chloride gradient was prepared in the tube before centrifugation and the centrifugation was only for 3 h, we tested to show that the gradient is at equilibrium under this condition.

***Eco*RI and *Hpa*I endonuclease cleavage.** Endo R·*Eco*RI was purified according to the method of Thomas and Davis (20). The preparation was free of contaminating endonuclease activity as checked by assaying for a longer period and with double the volume of enzyme. *Hpa*I was a commercial preparation from Bethesda Research Laboratory. *Eco*RI digestion of 0.5 μg of P22 or MB78 DNA was carried out at 37°C for 2 h in a total volume of 20 μl containing 100 mM Tris-hydrochloride (pH 7.5), 50 mM NaCl, and 5 mM $MgCl_2$ and a sufficient amount of enzyme to complete the digestion. The reaction was terminated by the addition of 10 μl of 0.1 M EDTA. Similarly, *Hpa*I digestion was carried out in a reaction mixture (20 μl) containing 10 mM Tris-hydrochloride (pH 7.5), 20 mM KCl, 7 mM $MgCl_2$, and 10 mM mercaptoethanol for 2 h at 37°C. The reaction was terminated in the same way as for *Eco*RI.

Agarose gel electrophoresis. Horizontal slab gel electrophoresis was carried out in 1% (wt/vol) agarose prepared in electrophoresis buffer containing 40 mM Tris-hydrochloride, 1 mM EDTA, 5 mM sodium acetate (pH 8.2), and 0.5 μg of ethidium bromide per ml (21).

DNA samples were mixed with glycerol (10%) and bromophenol blue (0.005%) and then layered under buffer into the slots of the gel. Just before layering, the samples were heated for 5 min at 70°C to disrupt hydrogen bonding between short cohesive ends. Electrophoresis was performed at 100 V (120 mA) for 4 h at room temperature (30°C). The bands were visualized by placing the gels over a UV light source (1, 15). The gels were photographed with a Polaroid Land camera using an orange filter and Polaroid type 667 Coaterless black and white Land films.

The convention suggested by Smith and Nathans (17) was used to name the DNA fragments generated by the action of *Eco*RI and *Hpa*I restriction endonucleases. The bands that resulted from electrophoresis of endonuclease digests of P22 or MB78 DNA in 1% agarose gels were assigned letters in order of increasing electrophoretic mobility.

Hybridization. Hybridizations of ^{32}P-labeled MB78 DNA to P22 DNA immobilized on nitrocellulose filters and vice versa were performed by the method of Denhardt (6).

Electron microscopy. The samples were negatively stained with uranyl acetate. Micrographs were taken in a Philips EM200 electron microscope with an operating voltage of 60 kV.

RESULTS

Isolation of phage MB78. In an attempt to isolate a mutant of P22 which would grow on the conditional nonpermissive host at 40°C, approximately 3×10^7 PFU from a stock of nitrosoguanidine-treated clear-plaque mutant (C_1) of phage P22 were plated on the conditionally nonpermissive host, 18/MC_4 (11), and incubated at 40°C. After a large number of plates had been scored, a large, clear plaque, quite distinct from that of P22, was observed. The plaque might have originated from an airborne phage. The phage was isolated from this plaque and purified by repeated plating on 18/MC_4. Not only was the plaque size of the phage much bigger than that of P22 C_1, but its morphology was also quite distinct; it

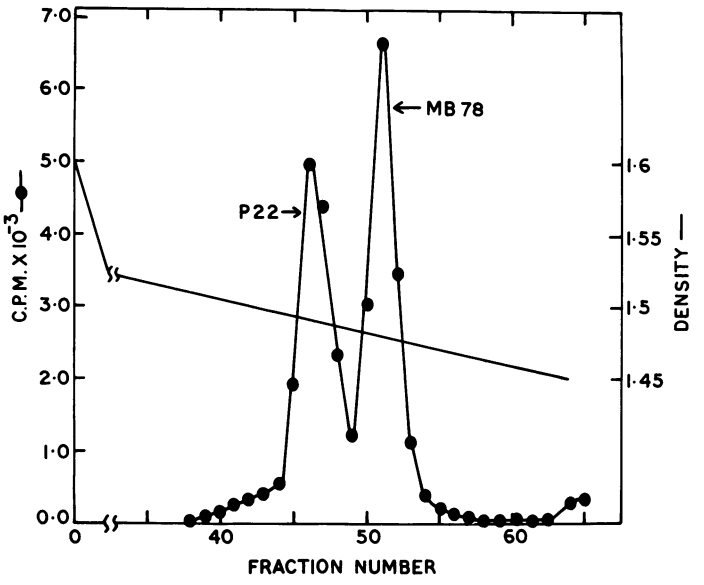

FIG. 2. Relative buoyant densities of P22 and MB78. Purified ^{3}H-labeled phages, P22 (C_1) and MB78 (1 μl of each containing 1 × 10^4 to 2 × 10^4 cpm), were layered on the top of the gradient. The remaining procedure is described in the text.

showed a very clear center surrounded by a kind of halo resulting from a turbid zone surrounded by a narrow clear ring.

Growth of MB78. The phage grows on the smooth and not the rough strains of *S. typhimurium*. It cannot multiply in cells growing in minimal medium. Citrate present in minimal medium inhibits the growth of MB78 and not that of P22. MB78 multiplies in minimal medium M9. In M9CAA, infected cells lyse earlier than those in Luria broth and M9. In minimal medium the phage adsorbs to the host and injects its DNA, which cannot replicate. Infected cells are killed, however, and the extent of killing is dependent on the multiplicity of infection. Phage MB78 has a shorter latent period and the rate of phage development is faster than that of P22.

Antigenic property. Antisera raised against MB78 do not inactivate P22 or its related phages L (2), MG40 (7), and MG178 (Grabner, thesis) (Fig. 1A). Antisera against phage P22 also do not cross-react with MB78 (Fig. 1B). The inactivation of MG40, MG178, and L phages by P22 antisera was observed as reported in the literature (7; Grabner, thesis).

Buoyant density. The relative buoyant densities of the two phages, MB78 and P22, were measured by cesium chloride density gradient centrifugation. The phages were identified from their plaque morphology. Their relative positions in the gradient were also confirmed by labeling one phage and leaving the other unlabeled. It is clear from the results presented in Fig. 2 that the new phage is lighter than P22.

Morphology. In contrast to P22, phage MB78 has a long, flexible tail which ends in a prominent and partite base (Fig. 3). The diameter of the head is comparable to that of P22 (~60 nm [~600 Å]). The normal length of the tail is approximately 90 to 100 nm. About 3 to 4% of the phage in the population have longer tails of variable length up to 0.5 μm (98, 105.7, and 500 nm). It is tempting to speculate that ~100 nm is the unit length of the tail.

Superinfection immunity. Phage P22 possesses a superinfection exclusion system besides the immunity repressor (18, 19). P22 is excluded by the *sieA* exclusion system of the resident prophage, whereas the phages related to P22 (L, MG40, and MG178), possessing different immunity systems, can be excluded by either of the two systems, *sieA* or *sieB*. It is clear from the results presented in Table 1 that both *sieA* and *sieB* exclusion systems are necessary for the exclusion of phage MB78. Except for the *sieA*$^+$ *sieB*$^+$ lysogen, the other three types of *sie* lysogens are killed as a result of superinfection by MB78. It may be emphasized that under this condition there is neither any phage production nor lysis of the cell. Thus the decrease in the colony-forming ability represents the killing effect of the phage.

***Eco*RI and *Hpa*I cleavage fragments of P22 and MB78 DNA.** DNAs isolated from phages P22 and MB78 were digested with restriction endonucleases *Eco*RI and *Hpa*I, and the digestion patterns were compared. The patterns are entirely different. As reported in the literature (9, 10, 21),

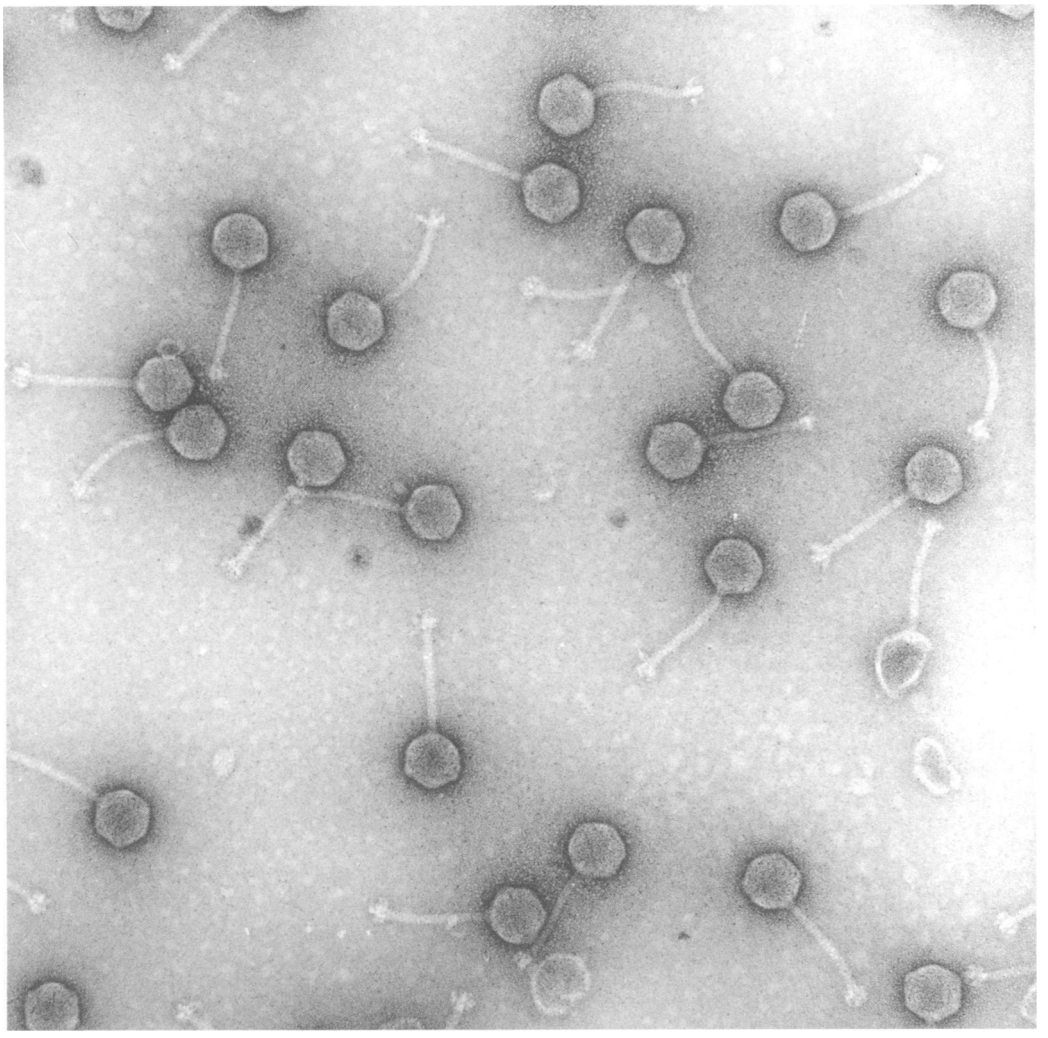

FIG. 3. Electron micrograph of phage MB78. Total magnification, ×200,000.

TABLE 1. Viability of P22 lysogens after superinfection with phage MB78[a]

Lysogens used	% Survival	
	P22 infected	MB78 infected
sieA$^+$ *sieB*$^+$	100	100
sieA *sieB*	100	2–5
sieA$^+$ *sieB*	100	2–5
sieA *sie B*$^+$	100	2–5

[a] Four P22 lysogens (*sieA*$^+$ *sieB*$^+$, *sieA sieB*, *sieA*$^+$ *sieB*, and *sieA sieB*$^+$) growing exponentially in M9CAA at 37°C were infected with phages MB78 and P22 (C_1) at a multiplicity of infection of 10. Ten minutes after infection, superinfected lysogens were diluted and plated on tryptone agar plates. The plates were incubated at 37°C to allow the viable cells to form visible colonies.

*Eco*RI digestion of P22 DNA produces 8 fragments (Fig. 4a), whereas 10 fragments are obtained from MB78 DNA (Fig. 4b). The *Hpa*I restriction endonuclease produces 13 fragments from P22 DNA (Fig. 4c), whereas 23 fragments are obtained from MB78 (Fig. 4d). It was suggested that *Eco*RI bands A and D from P22 are unusual due to limited circular permutation of the mature DNA (9, 10). Likewise, the band G after *Eco*RI digestion of MB78 seems to be present in less than a molar ratio. Bands A, B, C, and E of the *Hpa*I digest of MB78 DNA and bands A, C, and D of the *Hpa*I digest of P22 DNA are also present in less than a molar ratio. This suggests that, like P22, MB78 DNA is also circularly permuted.

DNA-DNA hybridization. To study homology between the two phages P22 and MB78, the

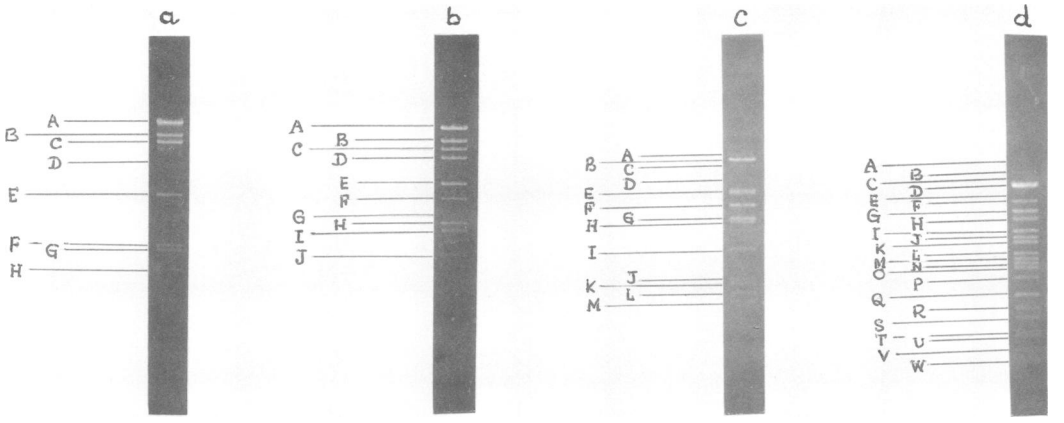

FIG. 4. Agarose gel electrophoresis of *Eco*RI and *Hpa*I endonuclease digestion fragments of P22 and MB78 DNAs. (a) P22, *Eco*RI; (b) MB78, *Eco*RI; (c) P22, *Hpa*I; and (d) MB78, *Hpa*I. A 0.5-μg sample of P22 or MB78 DNA was digested with *Eco*RI or *Hpa*I endonuclease for 2 h at 37°C and analyzed in 1% agarose gel. Electrophoresis was performed in a 0.6-by-10-cm horizontal slab gel at 100 V for 4 h. The samples were heated to 65°C for 5 min before layering on the gel. All of the bands are labeled by capital letters in order of increasing electrophoretic mobility.

DNAs isolated from these phages were allowed to hybridize with each other. Denatured DNA loaded on a filter was allowed to hybridize with ^{32}P-labeled denatured DNA present in the hybridization liquid. P22 DNA loaded on a filter hybridized with P22 DNA in the liquid but not with MB78 DNA (Table 2). Similarly, MB78 DNA hybridized with MB78 DNA and not with P22. Filters loaded with salmon sperm DNA were used for a control experiment. As expected, the salmon sperm DNA did not hybridize with either P22 or MB78 DNA. These results confirm that there is no detectable homology between the two phages.

DISCUSSION

Phage P22 is the most widely studied phage of *S. typhimurium*. Other temperate bacteriophages of *S. typhimurium*, like MG40, MG178, L, etc., share many properties in common with P22. No temperate strain of MB78 was obtained even after screening a large number of particles from a mutagenized stock of the phage. Therefore it is probably a virulent phage. MB78 is morphologically distinct from the temperate phage P22. It also does not resemble Felix-O1, the virulent phage of *S. typhimurium* (13). Felix-O1 has a contractile tail sheath with delicate tail fibers, whereas MB78 possesses a noncontractile, flexible tail with prominent partite ends. Felix-O1 adsorbs poorly to the smooth strain of *S. typhimurium*, whereas MB78 grows only in the smooth strain of the host. MB78 resembles morphologically some of the phages isolated by Young et al. (22) but differs from those also in host specificity. The phages isolated by Young et al. (22) are temperate and grow on rough strains of *S. typhimurium*. MB78 is serologically distinct from P22 and its other related phages, MG40, MG178, and L. One of the peculiar features of this new phage is its inability to multiply in minimal medium in the presence of citrate. In citrate-containing minimal medium

TABLE 2. Hybridization between P22 and MB78 DNA[a]

DNA on filter	^{32}P-labeled DNA in solution	Input cpm	Hybridized cpm	% Hybridization
Salmon sperm	P22	2,600	70	2.7
Salmon sperm	MB78	4,200	70	1.66
P22	P22	2,600	2,000	76.6
P22	MB78	4,200	120	2.9
MB78	P22	2,600	53	2.0
MB78	MB78	4,200	3,900	92.2

[a] A 2-μg sample of cold DNA as indicated above was loaded on each filter and incubated with 0.02 μg of ^{32}P-labeled P22 or MB78 DNA. Blank filters included in each hybridization vial bound 1% of the total ^{32}P-labeled DNA. The values given are the average of three annealing reactions performed in parallel. All values are corrected for blank.

the phage is absorbed and the DNA is injected. The phage exhibits a strong killing effect. Both the exclusion genes *sieA* and *sieB* are necessary to save a P22 lysogen from the killing effect of the superinfecting MB78 phage. The response of the phage to the superinfection exclusion gene might suggest a relationship between the two phages, P22 and MB78. It should, however, be remembered that even λimm^{21} phage encodes for a repressor which is able to repress the early functions of a quite unrelated phage, i.e., P22 (4). Furthermore, restriction endonucleases *Eco*RI and *Hpa*I fragments of P22 and MB78 DNA suggest that the base sequences of the two phages are quite dissimilar. This is also confirmed by DNA-DNA hybridization experiments. The nonhomology between the two phages suggests that the phage MB78 did not arise from recombination between P22 and a cryptic phage, if any, present in the strain 18/MC_4. At 40°C the phage MB78 can grow on the thiolutin-resistant strain 18/MC_4 (11), whereas P22 cannot. Defective phage particles are produced when P22 infects strain 18/MC_4 and infection is carried out at 40°C (11). This suggests that at least some of the host functions required for the development of P22 are not required for MB78.

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