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Lysogenic Pneumococci and Their Bacteriophages

HARRIET P. BERNHEIMER

Department of Medicine, State University of New York, Downstate Medical Center, Brooklyn, New York 11203

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About half of pneumococci recovered from pediatric patients and one-third of isolates from adult patients yielded bacteriophages active against one or more of four noncapsulated indicator strains of pneumococcus. Strains of capsular types most frequently causing pediatric infections were associated with lysogeny. Classical restriction-modification phenomena have been demonstrated in vivo with some of the temperate phages, and correlation of restriction with the presence of one or the other of the two known pneumococcal restriction endonucleases has been established. The temperate phages differ serologically and in several other characteristics from virulent pneumococcal phages previously described. All pneumococcal phages so far studied can be classified into a minimum of three serological groups.

Pneumococcal phages were discovered relatively recently (17, 21). The first phages were recovered by inoculating cultures of noncapsulated pneumococci with samples collected from pharyngeal swabbings of humans with upper respiratory infections but similar phages could be recovered from healthy subjects. The original bacterial host of these phages has not been identified. In experiments carried out several years ago using fresh clinical isolates of pneumococci from patients with pneumococcal disease, I reported that four of twelve strains examined could be induced with mitomycin C to yield phage lytic for one noncapsulated pneumococcal strain (4). A larger study was undertaken to determine more accurately the frequency of lysogeny in pneumococcus, to explore any possible relationship between lysogeny and pneumococcal virulence, and to compare phages recovered from pathogenic pneumococci with the virulent phages isolated directly from the human throat.

MATERIALS AND METHODS

Pneumococcal strains tested for lysogeny. Strains recovered from the ear or nasopharynx of children with otitis media were received from the same source and were handled in the same manner as described previously (4). Strains from adult patients were received as soon as possible after isolation from diagnostic laboratories at either State University Hospital or Kings County Hospital, Brooklyn, N.Y. Cultures received on plates or in broth were transferred to Neopeptone (Difco Laboratories) beef heart infusion broth containing 2% citrated horse blood and were incubated overnight before testing. Strains were tested without prior knowledge of capsular type except where noted or, for cultures from pediatric patients, without knowledge of the name of the patient. After the survey was completed, results from replicate isolates of the same pneumococci from the same patient were deleted from the study. The South African, multiply antibiotic-resistant strains were received from Robert Austrian, University of Pennsylvania. The capsular types of these strains were known at the time of testing.

Indicator strains. Four noncapsulated pneumococcal strains were used routinely: #649, SVIR3 (3); #213, R36A streptomycin resistant (6); #7, A66R2 (S-III4 in 3); and #264, 8R1 (16). These strains were derived from type 1, type 2, type 3, and type 8 encapsulated pneumococci, respectively. Noncapsulated mutants from a type 6 and a type 7 strain were tested regularly for a period of a few weeks, but their use was discontinued because they yielded no new information. Indicator strains were grown each day in Trypticase soy broth (Difco) to a cell density of approximately 2×10^7 colony-forming units (CFU) per ml.

Growth and treatment of test cultures. Organisms from 16- to 18-h cultures were transferred into CH medium (6) at a dilution of 1:15 (vol/vol) and were incubated at 37°C to a density corresponding to 2×10^7 to 4×10^7 CFU/ml. A portion was removed, and to it was added mitomycin C (Sigma Chemical Co.) to a final concentràtion of 0.1 μg/ml. Incubation of control and mitomycin treated cultures was continued; beginning at 90 min after the addition of mitomycin, treated cultures were examined every 30 min. If lysis was evident, both the treated and control cultures were removed to an ice bath. Three hours after the addition of mitomycin, all remaining cultures were cooled, and all tubes were centrifuged at 5,000 rpm for 15 min. At the beginning of the study, the supernatant solutions were filtered through GA-6 (Gelman) filter disks before testing for the presence of phage, but later this was discontinued.

Test for bacteriophage. In the early part of the study, tests were carried out as previously described

(4). Subsequently, a spotting technique was used; M1 (21) soft blood agar (1.5 ml) was added to 0.1 to 0.2 ml of each indicator strain, and the mixtures were overlaid on hardened M1 agar. To obtain good lawns with indicator strain A66R2, the concentration of Neopeptone in the bottom agar layer was increased to 1.0%, and 2.5 ml of M1 soft blood agar containing 0.4 ml of culture was used for the overlay. All lawns were allowed to dry at room temperature for at least 1 h before spotting with 0.1 ml of a 1:1 mixture of culture supernatant and M1 soft blood agar. The supernatants of all control and mitomycin-treated cultures were tested whether or not visible lysis had occurred. A maximum of five spots were placed on a plate and after drying, the plates were incubated at 37°C overnight and then examined. All putative plaques were replated on the same indicator strain on which they were observed originally. No pneumococcal culture was considered to be lysogenic unless putative plaques could be replicated.

Nomenclature of phages. Until such time as the recovered phages have been fully characterized and it is established how they differ, the phages recovered from lysogenic pneumococci should be designated *Streptococcus pneumoniae* phage HB-1, HB-2, etc. For brevity, designations in this paper will be ϕHB-1, ϕHB-2, etc.

Preparation of low-titer phage stocks. CH medium (1 ml) was inoculated with about 10^7 CFU of the appropriate pneumococcal host strain and after 15 min of incubation at 37°C, two plaques of the desired phage were transferred with Pasteur pipettes from overnight cultures on solid medium into the bacterial culture. After 1 h of incubation at 37°C, 9 ml of CH medium was added to each tube (for lysates made on host strain A66R2, 5 ml of CH medium was added) and incubation was continued for 5 to 6 h. The cultures became turbid and then either lysed or the bacteria became visibly clumped and settled to the bottom of the tube. The tubes were chilled and centrifuged at 5,000 rpm for 10 min, and the supernatants were removed and assayed for activity. Glycerol (15%, vol/vol) was added and the lysates were frozen and stored at −70°C. The titers of the lysates varied from 10^6 to 10^8 plaque-forming units (PFU) per ml. The unconcentrated lysates were used directly in some experiments and were also used as inocula for large-scale phage preparations.

Concentrated preparations of ϕHB-2 and ϕHB-3. One-liter lysates of each phage grown in CH medium on pneumococcal strains R36A and 8R1, respectively, were concentrated ×100, using polyethylene glycol (PEG) by the method of Yamamoto et al. (22). The PEG pellets were washed and the phage were collected in 50 mM Tris (pH 7.8; Sigma Chemical Co.)-10 mM Mg^{2+} (TM buffer). The phage preparations were stored in 15% glycerol at −70°C.

Antisera to ϕHB-2 and ϕHB-3. A 1.5-ml amount of each phage preparation containing, respectively, 1.2×10^{10} and 2.5×10^9 PFU of phage was mixed with 1.5 ml of complete Freund adjuvant. Two rabbits were immunized with each of the phage suspensions by injecting 1 ml of the mixtures into the footpads and thigh muscles. Each rabbit received booster inoculations at 3 and 5 weeks of 1 ml of a 1:1 suspension of phage in incomplete Freund adjuvant; the booster injections were given 0.5 ml intramuscularly and 0.5 ml subcutaneously. The rabbits were bled 6 days after the last injection, then rested, reinoculated, and bled again.

Adsorption of phage and neutralization by antisera. Standard techniques were employed (1) for adsorption of phage and neutralization by antisera.

Electron microscopy. Phage HB-3, concentrated with PEG as above, was centrifuged on step gradients of CsCl (7) in TM, and the phage band was collected and dialyzed against several changes of TM. Drops of purified phage were spread on carbon films and stained with 2% phosphotungstate at pH 7.5 by the method of Haschemeyer and Meyers (10). All grids were examined at 80 kV with a Siemens Elmiskop 1A electron microscope.

RESULTS

Frequency of phage recovery. Of 110 pneumococcal strains recovered from pediatric patients, 49 (44.5%) yielded phage lytic for one or more indicator strains. The rate of lysogeny among strains recovered from adult patients was lower; 31% of 29 strains yielded phage. Phages were recovered from some of the strains only upon addition of mitomycin, whereas other strains yielded phage in both treated and untreated controls (data not presented). The incidence of lysogeny in relation to pneumococcal capsular type is recorded in Table 1. For the most part, lysogenic organisms recovered from children and from adults are of the same capsular types and represent the types that most frequently cause infections in pediatric populations; in this study, 75% of all pediatric strains were of the types yielding phage.

Lysogeny in multiply antibiotic-resistant strains. The isolation in South Africa of strains of *Streptococcus pneumoniae* resistant to many antibiotics was recently reported (2). Of 23 of the pneumococcal isolates tested, 19 were lysogenic (Table 2). Two of the negative cultures represent the only type 19A strains examined which did not yield phage.

Activities of phages on indicator strains. Each control and mitomycin-treated lysate was tested on four indicator strains. The phages recovered differed in patterns of activity toward the indicator strains; some cultures yielded phages which gave plaques on only one strain, some gave plaques on two or more strains (Table 3). Most of the phages grow only on strain A66R2 or strains which differ from A66R2 solely in the site of the point mutation in the type 3 capsular genome. There was no correlation between the capsular type of a pneumococcus and the indicator strain on which the phage recovered from it would grow, e.g., one type 6A pneumococcus yielded a phage that grew on

TABLE 1. *Lysogeny in relation to pneumococcal capsular type*

Type[a]	No. positive/total[b]
Pediatric patients	
1	0/1
3	4/5[c]
4	2/8
5	0/2
6A	8/11
6B	2/9
7C	0/1
7F	0/3
9N	0/2
10A	0/3
11A	0/5
14	7/12
15B	0/3
15C	0/3
18B	0/1
18C	1/4
19A	7/7
19C	0/1
19F	4/7
20	0/1
21	0/1
22F	0/1
23F	11/18
24B	1/1
24F	2/2
35F	0/1
Adult patients	
3	0/1[d]
4	0/1
6A	1/2
6B	1/2
8	1/6
9A	0/1
11A	0/1
14	1/1
17	0/2
19A	1/1
19F	2/5[e]
23F	1/2
29	1/1
31	0/1
33B	0/1
Untyped	0/2

[a] Types according to the Danish system of nomenclature (11).
[b] Values indicate number of strains yielding phage per total number of strains tested.
[c] Three strains were selected as type 3.
[d] Phage heads seen by electron microscopy as reported previously (4).
[e] Lysates of the three negative strains were tested on only two indicator strains.

strain SVIR3, a second 6A strain yielded a phage that grew on strain R36A, and a third yielded a phage that grew only on strain A66R2. In subsequent testing, all phages isolated on strain R36A would grow and give plaques on A66R2 but gave no plaques on strain 8R1 or SVIR3. All phages recovered originally on strain 8R1 grew on strain SVIR3, but none grew on strains A66R2 and R36A. Phages originally isolated on strain SVIR3, with one exception, grew on strain 8R1 but, again, none gave plaques on strains A66R2 and R36A. Insofar as has been tested, all phages which on isolation grew on strain A66R2 but not on R36A continue to grow only on A66R2. The phages could be divided into four groups on the basis of host specificity (Table 4).

Further experiments carried out with several

TABLE 2. *Lysogeny in antibiotic-resistant pneumococci from South Africa*

Locale[a]	No. of strains	Capsular type	Lysogenic	Susceptible indicator strain
1	5	19A	+	A66R2, R36A
	1	19A	−	
2	1	19A	+	A66R2, R36A
	6	19A	+	A66R2
	1	19A	+	SVIR3[b]
	1	19A	−	
	1	6A	+	A66R2
	1	4	+	A66R2
3	4	19A	+	A66R2
	1	13	−	[b]
	1	43	−	[b]

[a] Strains recovered in one hospital in Durban (locale 1) and two hospitals in Johannesburg (locales 2 and 3).
[b] Strains not antibiotic resistant.

TABLE 3. *Growth of phages on indicator strains at time of isolation from American strains of pneumococcus*

No. of phages	Susceptible indicator strains
33	A66R2
11	A66R2 and R36A
2[a]	A66R2, R36A, and SVIR3
2[a]	A66R2, R36A, and 8R1
3[a]	A66R2 and 8R1
1[a]	A66R2 and SVIR3
4	8R1 and SVIR3
1	8R1
1	SVIR3

[a] Phage grew initially on bacterial strains of two specificity groups. See text.

TABLE 4. *Growth of recovered, tested phages on indicator strains*

Group	Susceptible indicator strain(s)
1	A66R2
2	A66R2 and R36A
3	SVIR3 and 8R1
4	SVIR3

phages revealed that the phages would adsorb at least as well to nonhost as to host pneumococcal strains and that the magnitude of restriction was high (Table 5).

Restriction-modification in pneumococcus. The presence of pneumococcal restriction endonucleases and/or the pattern of DNA methylation of the 4 indicator strains were investigated (S. Lacks, personal communication). Pneumococcus strain R6, in which the restriction endonuclease *Dpn*I was first described (12) was cloned from strain R36A, and it is assumed that R36A, like R6, has unmethylated DNA and makes *Dpn*I. The DNA of strain A66R2 also is unmethylated, whereas the DNAs of strains 8R1 and SVIR3 are methylated. In addition, strain 8R1 was found to contain the second pneumococcal endonuclease, *Dpn*II (13). In view of these

TABLE 5. *Activities of several phages on four indicator strains*

Phage[a]	Indicator strain	Adsorption (%)[b]	Titer (PFU/ml)[c]
HB-1 (R36A)	R36A	74	7.3×10^6
	8R1	83	$<10^3$
	R36A	—[d]	2.2×10^6
	A66R2	—	1.3×10^6
HB-1 (8R1)	R36A	95	$<10^3$
	8R1	93	2.7×10^8
HB-2 (R36A)	R36A	—	4.1×10^9
	A66R2	—	1.4×10^9
	R36A	—	1.5×10^7
	8R1	98.5	$<10^2$
HB-3 (8R1)	R36A	97.6	<10
	8R1	94.5	1.5×10^7
	SVIR3	—	4.8×10^6
HB-3 (R36A)	R36A	—	3.4×10^6
	8R1	—	$<10^2$
HB-5 (SVIR3)	SVIR3	40	1×10^7
	8R1	25	$<10^2$
	R36A	30	$<10^2$
	8R1	30	$<10^2$
	A66R2	20	$<10^2$
HB-6 (SVIR3)	8R1	84	2.3×10^6
	SVIR3	75	1.6×10^6

[a] Pneumococcal strain name indicates strain on which phage was grown.

[b] Adsorption tests carried out by the method of Adams (1) for 20 min at 37°C.

[c] Samples (0.1 ml each) of varied dilutions of phage lysates were plated in triplicate.

[d] —, Not done.

findings, the pattern of growth of the group 2 and the group 3 phages (Table 4) can be explained by classical restriction-modification mechanisms, but an additional explanation must be sought for the behavior of phages of groups 1 and 4.

A host-modified preparation of phage HB-3 (originally of host specificity group 3) was obtained by growing cells of strain R36A with the phage in liquid medium. After 205 min of incubation (but not before this time) two plaques were recovered on strain R36A from a portion of the infected culture. Taking dilutions into account, these plaques represent a recovery of 10^4 PFU from an input of 2.7×10^7 PFU. The behavior of the host-modified phage HB-3 (R36A) is recorded in Table 5. Experiments designed to obtain a host-modified preparation of phage HB-2 that would grow on strain 8R1 have thus far been unsuccessful.

Although eight of the pneumococcal lysates yielded phages which, on primary isolation, apparently crossed the restriction barrier (Table 3), in subsequent tests, the phages would replicate and give plaques only on the bacterial strain from which they were recovered or on a strain of the same specificity group (Table 5, phage HB-1). The type 19A pneumococci from which phage HB-1 (8R1) and phage HB-1 (R36A) were isolated were recovered from the same patient on three occasions at monthly intervals, and each time the mitomycin-treated lysate gave plaques on strains R36A, A66R2 and 8R1, but on replication the recovered phages conformed to the restriction patterns noted above.

Activity of pneumococcal phages toward streptococci. In a series of experiments, the adsorption of the virulent phages ω2 and ω3 (21) to 11 *Streptococcus sanguis* strains was examined. The phages did not adsorb to four strains found by Cole et al. (8) to fall into Rosan's (20) antigenic group I nor to four strains belonging to group II but did adsorb (80 to 90%) to two strains of the heterogeneous group and to strain K208, of "no type." The phages replicated in liquid medium only on the last strain, yielding progeny phage that gave plaques on strain R36A. No plaques were formed on strain K208. None of four temperate phages tested adsorbed to any *S. sanguis* strain (data not presented).

Serological reactions. Antisera to phage HB-2 and HB-3 were raised in rabbits. These phages were isolated, respectively, from a type 19F pneumococcus recovered in Alabama from the ear of a child with otitis media and a type 6B pneumococcus from the blood culture of an adult in Brooklyn, N.Y., with pneumococcal bacteremia. Phage HB-2 belongs to host specificity group 2 and HB-3 to host specificity group 3.

Antisera to both phages neutralized all phages tested of groups 2, 3, and 4 (Table 6). The sera did not neutralize the ω phages nor any of 3 phages of host specificity group 1 (Table 7). The latter group of phages was serologically different from the ω phages.

Morphology of phage HB-3. Negative-stained particles of purified phage HB-3 were examined in the electron microscope (Fig. 1).

TABLE 6. *Neutralization tests*

Phage	Host specificity group	Antiserum[a]	K value (min^{-1})[b]
HB-3	3	Anti-HB-3 no. 1	81-170
HB-1 (R36A)	2		45
HB-1 (8R1)	3		29
HB-2	2		29
HB-4	2		178
HB-3	3	Anti-HB-3 no. 2	42
HB-2	2		37
HB-5	4		21
HB-6	3		31
HB-2	2	Anti-HB-2 no. 1	59-147
HB-3	3		65
HB-2	2	Anti-HB-2 no. 2	45
HB-1 (R36A)	2		27
HB-1 (8R1)	3		20

[a] Two antisera for each phage were employed.

[b] K value determined by the method of Adams (1). Phage and antisera were incubated for 15 min at 37°C and immediately diluted 1:100, and 0.1-ml samples were assayed for phage. In controls, preimmunization serum was used in place of antiserum.

TABLE 7. *Neutralization tests*

Phage[a]	Host specificity group	Antiserum	Serum dilution	Remaining PFU (%)[b]
ω3	—[c]	Anti-ω2	1:100	0.01-0.034
HB-1 (R36A)	2		1:10	89.2
HB-1 (8R1)	3		1:10	71
HB-3	3		1:100	84
			1:10	39.4
HB-7	1		1:100	83
HB-8	1		1:100	100
ω3	—[c]	Anti-HB-3 no. 1	1:100	91
HB-7	1		1:100	78
HB-8	1		1:100	100
HB-9	1		1:100	60
			1:10	35

[a] Phages HB-7 and HB-8 were recovered from multiply antibiotic-resistant type 19A South African pneumococcal strains. Phage HB-9 was recovered from a fully antibiotic-sensitive type 19A strain from Alabama.

[b] Phage and antisera are incubated and assayed as described in footnotes to Table 6.

[c] The ω phages grow on all indicator strains.

Morphologically, HB-3 resembles Dp-1 (17) more closely than it does the ω phages (14, 18); the head is 65 nm in diameter, the tail is 156 ± 1 nm in length, and no tail fibers are evident. The tails of many of the phage appeared to be cross-striated and to have bifurcate ends. The latter phenomenon may be due to attachment of debris to the ends of the tails.

Lysogeny versus pseudolysogeny. The type 19F (496) and type 6B (3961) pneumococcal strains from which phages HB-2 and HB-3 were recovered were passed three times in broth containing 5% antiserum raised to phage HB-3. Strain #496, on induction with mitomycin, yielded as much phage as a control culture passed three times in blood broth without antiserum; strain #3961 passed in antiserum yielded less phage than did its control culture but was still lysogenic.

DISCUSSION

The frequency of lysogeny in pneumococcus reported here is probably lower than the true frequency. Many cultures that lysed when grown with mitomycin yielded no phage; it is possible that some of these strains carried defective phage or phage that might be lytic for indicator strains other than those used in this study. During the course of the investigation, it was found that the titer of some lysates was significantly lowered by filtration through the GA-6 filters that were used routinely for culture supernatants. Filtration was discontinued, but some cultures yielding low titers of phage may have been falsely recorded as negative because of removal or inactivation of phage. The presence of bacteriocins was suspected in some lysates, but the possibility of their presence was not pursued.

Lysogeny was associated only with pneumococci of certain capsular types. However, the numbers of strains tested of other capsular types were too small to preclude the possibility that additional strains of the same type might yield phage. It is striking, though, that strains most frequently associated with pediatric infections are of those types in which lysogeny is found regardless of whether the pneumococcus is recovered from a pediatric or adult patient. A much larger study would be needed for a definitive relationship to be established between lysogeny and capsular type in pneumococcus.

Studies of the multiply antibiotic-resistant strains were undertaken to determine whether there was a relation between lysogeny and the presence of putative plasmids carrying drug resistance genes. It has been reported that no plasmids can be detected in these strains (L. W. Mayer and V. B. Ploscowe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, D26, p. 36) nor has a

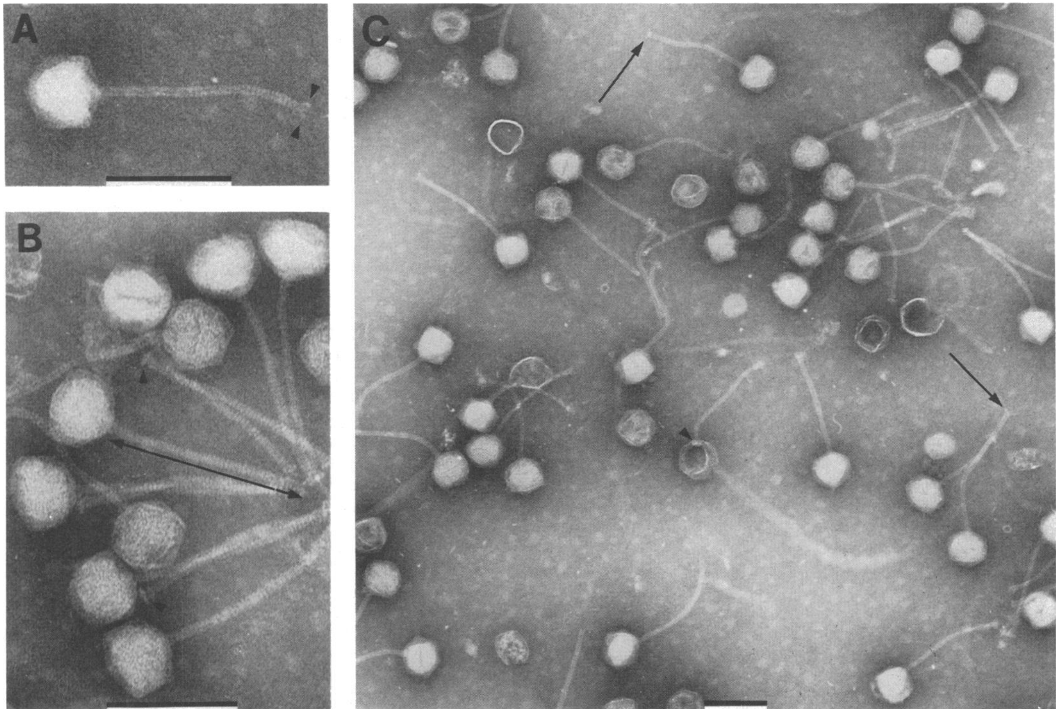

FIG. 1. *Electron micrographs of phage HB-3. (A) Single phage particle showing striated tail with bifurcate end (arrow heads). (B) Several phage particles, some showing striated tails. Double-headed arrow measures tail length: 155.2 nm. Arrow heads indicate plates on head ends of disjointed tails. (C) Long arrows point to bifurcate tails, and arrow head points to plate in empty head. Bars indicate 100 nm.*

plasmid been found in a lysogenic, chloramphenicol and tetracycline-resistant type 19 pneumococcus isolated in Paris (9). It seems not unlikely, though, that the introduction into the pneumococcus of resistance genes occurred via a plasmid and in this connection, it is interesting that the only South African strain yielding a phage that grew on SVIR3 was a fully sensitive type 19A strain. Two resistant type 19A strains did not yield any lytic phage, but both strains lysed completely when grown with mitomycin, and lysates should be examined for the presence of defective phage.

Eight pneumococcal strains yielded on primary isolation phages which grew on indicator strains of both restriction groups. It remains to be determined whether the lysogenic strains are comprised of a mixture of cells, some of which methylate their DNA and others not, or whether the phages were modified by one of the indicator strains in the first cycle of growth.

When classified by host specificity, the temperate phages fall into four groups (Table 4), and it has been shown that classical restriction-modification mechanisms alone can account for the growth patterns of phages in only two groups. Because so many of the phage grow on both strains R36A and A66R2, it seems unlikely that modification by strain A66R2 or a second endonuclease in the strain R36A is responsible for the behavior of phage in host specificity group 1. Unless it can be shown that the phage themselves code for a modifying enzyme, the action of which limits growth to strain A66R2, the simplest explanation for the results is that R36A lacks a receptor for these phages. The finding that the few strains of group 1 phages that have been examined are serologically different from the phage in the three other groups lends support to this hypothesis. To date, the temperate phage fall into at least two serological groups based on reactions with antisera to phage HB-2 and HB-3. However, group one is comprised of the largest number of phage, and serological differences may be found among them.

In contrast to the data presented here demonstrating restriction-modification phenomena in vivo for two groups of temperate phages and selectivity of growth of others, the ω phages gave plaques on all four indicator strains used in the study (5); they are resistant to both pneumococcal restriction endonucleases, *Dpn*I and *Dpn*II (13), presumably because of a substituted base in their DNA (19). The ω phages also differ

serologically from any of the temperate phages studied in this report. It was previously reported that phage Dp-1 was neutralized by antiserum to ω2 as was a phage recovered from a type 8 pneumococcus (4). Preparations of the latter phage are no longer available and no phages have been recovered from five additional type 8 strains. Morphologically, phage Dp-1 and HB-3 resemble each other more closely than they do the ω phages. It is not known whether any of the temperate phages, like Dp-1, contain lipid (15).

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