

# Isolation of Bacteriophages Active Against *Neisseria meningitidis*

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Five distinct bacteriophages have been isolated from strains of *Neisseria meningitidis*. Filtrates with titers of  $10^{-4}$  to  $10^{-6}$  were produced with a modified Swanstrom and Adams semisolid agar procedure, employing Eugonbroth with added agar and an incubation temperature of 30 C. Of 49 strains of *N. meningitidis* (groups B and C), 25 were lysed by one or more of the phages, but there was no lysis of other *Neisseria* and *Mima polymorpha* strains.

In 1953, Stone et al. (Bacteriol. Proc., p. 39, 1953) described the isolation of a bacteriophage active against a chromogenic *Neisseria*, later identified as *N. perflava* (9). Phelps and Kellogg (Bacteriol. Proc., p. 115, 1965) reported similar isolations from a stock culture of *N. perflava* ATCC 1055 and a strain subcultured from the nasopharynx of a normal individual. Bacteriophages for *N. meningitidis* have not been described.

Since studies with military personnel in recent years have shown group B *N. meningitidis* to be the predominate serotype in carriers and cases of meningitis, we attempted to demonstrate bacteriophages in this species. This report will describe methodology and properties of the first five bacteriophages isolated.

## MATERIALS AND METHODS

**Organisms.** All strains of *N. meningitidis* were recent isolates from body fluids of meningitis cases and from the nasopharynges of carriers. Nonpathogenic *Neisseria* strains were isolated on chocolate-agar from the nasopharynx of normal individuals. A modified Thayer-Martin medium (11) was used for the initial isolation of *N. meningitidis*. These "drug plates" were made as follows. Chocolate-agar was prepared with Mueller Hinton Medium (Difco) and 4% sheep blood. After cooling, lincomycin (The Upjohn Co., Kalamazoo, Mich.) was added to a final concentration of 0.006 mg/ml. Previously measured frozen polymyxin B (Chas. Pfizer & Co., Inc., New York, N.Y.) was thawed and added to yield a final concentration of 25 units/ml. Sugar fermentations were performed by the procedure of Evans et al. (Bacteriol. Proc., p. 56, 1964), employing a modified Mueller Hinton Medium with 1% carbohydrate, phenol red, and 1% rabbit serum. For serological classification, slide agglutination tests were used with live suspensions made from overnight

drug plates and Difco immune sera. All *Neisseria* species were identified according to *Bergey's Manual* (7th ed.). The *N. gonorrhoeae* cultures were fresh isolates submitted to this laboratory. All *Mima polymorpha* cultures, including one *oxidans* variety, were from our stock culture collection.

Primary isolates were lyophilized by standard procedures or were frozen in a modified Greaves medium (8) consisting of 5% bovine albumin and 5% sodium glutamate.

**Isolation of bacteriophages.** Twelve strains of *N. meningitidis* (group B) from nasopharyngeal carriers were used in the preliminary studies. Subcultures on chocolate-drug-agar were incubated overnight in a candle jar at 37 C and used to inoculate 100 ml of Eugonbroth (BBL; lot 406642). To obtain satisfactory growth in a liquid medium, the pH was adjusted to 7.7 before sterilization. After overnight incubation aerobically at 37 C, the broths were centrifuged and the supernatant fluids were filtered (0.45- $\mu$  membrane filters; Millipore Corp., Bedford, Mass.). The sterile filtrates were checked for lytic activity (see below) against the homologous strain, the other 11 test strains, and 6 more group B carrier strains, making a total of 18 test strains for each filtrate. Four filtrates (M1-M4) showed marked lytic activity against the homologous strain only and were used for further studies. Early phage lysates showed minor cross-reactions which were eliminated when high-titer preparations were used (see below).

M-5 was isolated at a later time and demonstrated similar minor reactions.

**Propagation of phage isolates.** A modified Swanstrom and Adams semisolid agar technique (10) and a broth method (1) were compared. The semisolid technique consistently produced lysates of higher titer. Eugonbroth (200 ml; pH 7.7) with 2% added agar was sterilized and poured into a sterile, dry Roux bottle to form a base layer. To 290 ml of Eugonbroth with 0.5% added agar, 4 ml of a 2% Trypticase (BBL) suspension of host cells, from an overnight chocolate-drug plate grown at 37 C in a candle jar, equivalent

to the density of the no. 7 MacFarland tube, was added. Then, 6 ml of bacteriophage, diluted with 2% Trypticase so that the final dilution was equivalent to the previously determined titer, was added to the cell-soft agar mixture. The entire mixture was poured over the base layer in the Roux bottle, and the culture was incubated at 30 C for 15 hr. (Preliminary experiments indicated the superiority of incubation at this temperature compared to 37 C.) After incubation, maximal yield was obtained from the soft agar by squeezing through sterile gauze, coarse filtration, and centrifugation. Membrane filters were used to obtain a bacteria-free lysate, which was stored at 5 C.

**Titration and typing procedures.** Test strains, including the host organism, were grown overnight on freshly prepared chocolate-drug plates in a candle jar at 37 C. Freezing all fresh *N. meningitidis* isolates in modified Greaves medium at -60 C prevented any major changes in the propagating or test strains used in this study. Freshly prepared chocolate-drug plates proved superior to any other medium for growth of propagating or test organisms. A suspension in 2% Trypticase was made equivalent to a no. 2 MacFarland tube (600 million cells per ml). Eugonagar plates (pH 7.7, 1.5% agar), after overnight incubation, were flooded with 1.0 ml of the cell suspension, and the excess was drained off. (A 6- to 8-hr Eugonbroth culture, heavily inoculated to obtain a final density equivalent to a no. 2 MacFarland tube and grown on a 37 C shaker, was tried for flooding plates; however, since there was more variation in the organism and its susceptibility to phage with a broth culture than with a suspension made from an overnight drug plate, the agar method was used routinely.) After drying at room temperature for 15 min, 0.01 ml of each phage, diluted 10-fold in 2% Trypticase (pH 7.0), was dropped on the surface. Because of the moisture content, no more than one phage was added to a single plate. The plates were allowed to remain an additional 15 min at room temperature before incubation and were incubated in an upright position in metal petri-dish cans used as candle jars to prevent spreading of the phage on the surface of the agar. All plates were incubated for 15 hr at 30 C before being read for lytic activity. The titer of each lysate was determined as the highest dilution exhibiting more than 50 plaques or confluent lysis in 0.01 ml (3). Figure 1 demonstrates the susceptibility of one strain to the five phages. This plate is one from preliminary experiments incorporating all phages on one plate. Individual plaques from all five phages measured 0.8 to 0.9 mm in diameter. Phage-typing studies of *N. meningitidis* strains were performed in a similar manner, by use of 0.01 ml of the previously determined titer.

**Bacteriophage counting.** A modification of the standard two-layer agar method for determining plaque-forming units was used (1). Diluted bacteriophage (0.5 ml) was mixed with 0.5 ml of a suspension of an indicator strain. After standing at room temperature for a short time, the mixture was added to 5 ml of 0.7% Eugonagar and poured over a base layer. Plates in triplicate were incubated at 30 C for 15 hr.

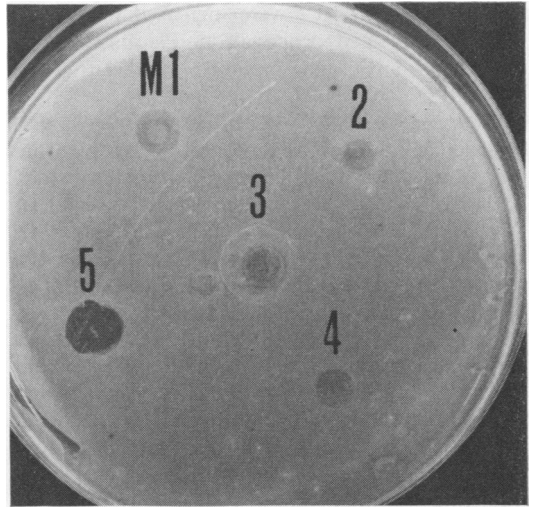


FIG. 1. Reactions of one strain of *Neisseria meningitidis* to the five phages. M1, less than 20 plaques; M2, 20 to 50 plaques; M3, more than 50 plaques; M4, more than 50 plaques; M5, semiconfluent lysis. Interpretation: phage pattern M3/4/5.

## RESULTS AND DISCUSSION

**Size of bacteriophages.** Filtration through 100-m $\mu$  membrane filters showed a fall in titer from  $10^{-3}$  to less than  $10^{-1}$ . No lytic activity passed the 50-m $\mu$  and 10-m $\mu$  filters. Electron micrographs showed particles consisting of head-like structures. No tail-like structures were seen. These observations on phages of *N. meningitidis* show sizes similar to those described by Stone et al. (9) for the heads of *N. perflava* phages and by Phelps and Kellogg (Bacteriol. Proc., p. 115, 1965) for the heads of phages of *N. perflava*. Both of these reports described tail structures, however. Further electron microscopic studies of the meningococcal phages are underway.

**Biological characteristics.** In early attempts to isolate bacteriophages from *N. meningitidis*, a number of different media were used, including Brain Heart Infusion (BHI; Difco), Trypticase Soy Broth (BBL), and Eugonbroth. The addition of calcium chloride (400  $\mu$ g/ml) to one lot of BHI increased the production of bacteriophage. The addition of calcium chloride to Trypticase Soy Broth, agar, or the diluent, 2% Trypticase, was unnecessary. There is apparently enough residual calcium to meet the requirement. We have had a similar experience with the propagation of staphylococcal phages.

Of the above-mentioned media, the highest phage titers were obtained with semisolid Eugonagar. The first lysates with titers of 50 plaques or

more at  $10^{-1}$  to  $10^{-3}$  were increased to  $10^{-4}$  to  $10^{-6}$  by the third propagation. Plaque-counting by the two-layer agar method revealed titers of  $10^7$  to  $10^9$ .

**Temperature stability.** Temperature sensitivity was determined for two phages. A Temperature Gradient Bar (Lab-Line Instruments, Inc., Melrose Park, Ill.) was set between 25 and 44.8 C. Additional water baths were used at 51 and 60 C. Phage M1 was reduced from a titer of 50 to 100 plaques to less than 50 plaques after 30 min at 38.6 C and was completely inactivated at 51 C in 30 min. M5 was slightly more resistant to higher temperatures, maintaining its titer after 30 min at 42.3 C; however, it was completely inactivated at 51 C.

Concentrated phage filtrates stored at 5 C maintained a constant titer for as long as 1 year, following an initial drop in 13 days. For example, the titer of M1 (lot 3) 48 hr after harvesting was  $10^{-6}$ . After 13 days of storage, the titer was  $10^{-5}$ , thereafter remaining the same. Other concen-

TABLE 1. *Stability of diluted bacteriophages at 5 C*

| Storage time  | Bacteriophage  |           |           |           |           |
|---------------|----------------|-----------|-----------|-----------|-----------|
|               | M1             | M2        | M3        | M4        | M5        |
| <i>days</i>   |                |           |           |           |           |
| 14            | $10^{-3a}$     | $10^{-2}$ | $10^{-3}$ | $10^{-3}$ | $10^{-3}$ |
| 22            | $10^{-2}$      | $10^{-1}$ | $10^{-3}$ | $10^{-3}$ | $10^{-3}$ |
| 30            | — <sup>b</sup> | —         | $10^{-3}$ | $10^{-2}$ | $10^{-3}$ |
| 37            | —              | —         | —         | $10^{-2}$ | $10^{-2}$ |
| Stock control | $10^{-4}$      | $10^{-3}$ | $10^{-3}$ | $10^{-3}$ | $10^{-3}$ |

<sup>a</sup> Highest dilution with 2+ to confluent lysis.

<sup>b</sup> No reaction.

trated phages decreased 2 logs in titer after several months of storage but maintained this titer for more than 1 year. Freezing at  $-20$  C resulted in 2-log decreases in phage titer.

*Neisseria* phages (lot 2) diluted in 2% Trypticase (pH 7.0) and stored at 5 C were tested at various intervals up to 37 days (Table 1). M4 and

TABLE 2. *Bacteriophage typing of Neisseria and Mima polymorpha strains<sup>a</sup>*

| Strains                | Serotype | Bacteriophage |    |    |    |    | Phage pattern |
|------------------------|----------|---------------|----|----|----|----|---------------|
|                        |          | M1            | M2 | M3 | M4 | M5 |               |
| <i>N. meningitidis</i> |          |               |    |    |    |    |               |
| 20 strains             | B        | —             | —  | —  | —  | —  | —             |
| 84A                    | B        | 2+            | 3+ | 4+ | 3+ | 3+ | M1/2/3/4/5    |
| 96A                    | B        | ±             | 3+ | 2+ | 3+ | ±  | M2/3/4        |
| 41                     | B        | —             | 2+ | 2+ | —  | —  | M2/3          |
| 52                     | B        | 2+            | +  | 2+ | —  | —  | M1/3          |
| 58                     | B        | —             | —  | 2+ | —  | ±  | M3            |
| 60                     | B        | +             | —  | +  | 2+ | +  | M4            |
| 64                     | B        | 2+            | 2+ | —  | 2+ | +  | M1/2/4        |
| 4 strains              | C        | —             | —  | —  | —  | —  | —             |
| I-3-20                 | C        | 2+            | +  | +  | ±  | —  | M1            |
| C6                     | C        | —             | 2+ | —  | —  | —  | M2            |
| C9                     | C        | 2+            | ±  | —  | —  | —  | M1            |
| C10                    | C        | ±             | 2+ | ±  | —  | —  | M2            |
| <i>N. catarrhalis</i>  |          |               |    |    |    |    |               |
| 4 strains              |          | —             | —  | —  | —  | —  | —             |
| <i>N. gonorrhoeae</i>  |          |               |    |    |    |    |               |
| 4 strains              |          | —             | —  | —  | —  | —  | —             |
| <i>N. perflava</i>     |          |               |    |    |    |    |               |
| 2 strains              |          | —             | —  | —  | —  | —  | —             |
| <i>N. sicca</i>        |          |               |    |    |    |    |               |
| 1 strain               |          | —             | —  | —  | —  | —  | —             |
| <i>M. polymorpha</i>   |          |               |    |    |    |    |               |
| 6 strains              |          | —             | —  | —  | —  | —  | —             |

<sup>a</sup> Symbols: —, no lysis; ±, less than 20 plaques; +, 20 to 50 plaques; 2+, more than 50 plaques; 3+, semiconfluent lysis, cell debris present; 4+, confluent lysis.

TABLE 3. Bacteriophage-typing of case strains of *Neisseria meningitidis*<sup>a</sup>

| Case    | Serotype | Bacteriophage |    |    |    |    | Phage pattern |
|---------|----------|---------------|----|----|----|----|---------------|
|         |          | M1            | M2 | M3 | M4 | M5 |               |
| 1       | B        | 2+            | —  | 3+ | ±  | 4+ | M1/3/5        |
| 2       | B        | 3+            | 2+ | 3+ | +  | 3+ | M1/2/3/5      |
| 3       | B        | 3+            | 2+ | 4+ | 3+ | 3+ | M1/2/3/4/5    |
| 4       | B        | +             | 2+ | 3+ | 3+ | 2+ | M2/3/4/5      |
| CDC 537 | B        | 3+            | 2+ | 2+ | +  | 2+ | M1/2/3/5      |
| 39I     | B        | 2+            | 3+ | 3+ | —  | —  | M1/2/3        |
| 341V    | B        | 4+            | 2+ | +  | ±  | +  | M1/2          |
| 17I     | B        | ±             | ±  | 2+ | ±  | 3+ | M3/5          |
| 14B-8   | B        | 3+            | 2+ | 4+ | 2+ | 2+ | M1/2/3/4/5    |
| 74-VI   | C        | 2+            | —  | 2+ | 2+ | 2+ | M1/3/4/5      |
| 51-V    | C        | 2+            | ±  | —  | —  | 2+ | M1/5          |
| 28aI    | C        | 3+            | ±  | 2+ | 2+ | 2+ | M1/3/4/5      |
| XXXVII  | C        | 2+            | 2+ | 2+ | 2+ | 3+ | M1/2/3/4/5    |
| 47-VI   | C        | ±             | +  | ±  | 2+ | 2+ | M4/5          |

<sup>a</sup> See Table 2 for explanation of symbols.

M5 were the most stable upon dilution, the titer falling only 1 log after 37 days. The control stock listed was the concentrated phage diluted and titered for comparison when the diluted phages were checked at 37 days.

Tests with one phage compared two diluents, phosphate buffer (pH 7.0) and 2% Trypticase (pH 7.0). The dilutions and titrations were done on the same day. The titer of the phage diluted with phosphate buffer was  $10^{-1}$ , whereas that portion diluted in Trypticase remained at  $10^{-4}$ , the previously determined titer. Thus, Trypticase is preferable as diluent.

Lyophilization of these phages, applying a gradual decrease in temperature as recommended by Clark (4), was attempted but a constant low temperature could not be maintained during the drying process with the available equipment, resulting in a considerable loss in titer. Other methods are under study.

Treatment of the concentrated filtrate with 0.2% trypsin for 2 hr at 37 C, as used by Kingsbury (8) to inactivate bacteriocins of *N. meningitidis*, had no effect on the bacteriophage titer.

The specificity of these phages and their use in typing *N. meningitidis* strains were evaluated (Table 2). Group B and C meningococcal strains from carriers were compared with a number of other *Neisseria* species, and with *M. polymorpha*. *M. polymorpha*, on smears, may resemble *Neisseria*, and it has been isolated from throat, genitourinary tract, and spinal fluid (2, 5, 6). Of 27 group B strains from carriers, 7 showed lytic reactions with one or more of the five phages used. Twenty strains were nonreactive. Four of eight group C carrier strains showed reactivity; in general, a significant (2+) reaction occurred

with only one phage. Eleven strains of other *Neisseria* were totally unreactive, as were six strains of *M. polymorpha*.

Phage-typing of strains of *N. meningitidis* isolated from spinal fluid of cases of meningitis is shown in Table 3. Nine group B and five group C strains all reacted with two or more phages.

These results suggest that phage reactivity is specific for *N. meningitidis*. Group C as well as group B meningococci may be lysed by these five phages, but virulent meningococci (case strains) show much greater phage susceptibility than do strains isolated from normal carriers. Further study of this aspect of phage-typing is currently underway.

The above studies were performed on original isolates or with strains which had been transferred not more than twice in vitro. We observed that serial agar passage may result in loss of phage susceptibility, although the serological reaction is retained.

The results of phage-typing suggest that we are dealing with five distinct phages. Additional phages have been observed, and separation and classification by the use of immune sera is planned.

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