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Two Bacteriophages of *Clostridium difficile*

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Two temperate bacteriophages of differing morphology and host range were isolated by screening 94 isolates of *Clostridium difficile*. Phage 41 had a 300-nm flexible tail, whereas phage 56 had a shorter tail with a contractile sheath. Electron microscopy of phage 56 lysates exposed to elevated magnesium concentrations showed small virus-like particles which were 21 nm in diameter. The addition of $MgCl_2$ to semisolid agar overlays enhanced both the titer and plaque size of phage 56. Phage 56 was more temperature labile than phage 41 and demonstrated unusual lability in buffer at pH 7.0. One-step growth and adsorption experiments revealed that both phages had latent periods of about 60 min, but phage 56 adsorbed to its indicator strain more efficiently. Phage 56, which was obtained from a toxigenic strain of *C. difficile*, was used to lysogenize its nontoxigenic indicator strain, but no conversion to toxigenicity was observed in this strain.

Clostridium difficile has been implicated as the major cause of antibiotic-induced pseudomembranous colitis, a disease of the large bowel in which diarrhea and lesions of the mucosa are clinical features (5). To date, two toxins associated with the disease have been identified: a cytotoxin that is toxic to various tissue culture cell lines and an enterotoxin that can be assayed by the rabbit ileal loop method (4, 9; H. S. Taylor, G. M. Thorne, and J. G. Bartlett, Clin. Res. **28**:285A). Sell et al. (7) recently reported on the first bacteriophages and bacteriocins of *C. difficile* and described a preliminary typing scheme for the species (2, 7). In this paper we describe two bacteriophages of *C. difficile* and examine the possibility of toxin conversion in this species.

Ninety-four clinical isolates of *C. difficile* were obtained from the Department of Microbiology, Victoria General Hospital, Halifax, courtesy of T. Marrie. The cultures were grown in cooked meat medium (Difco Laboratories, Detroit, Mich.) at 37°C and stored in this medium at room temperature. Brain heart infusion broth (Difco) to which was added glucose (1%, wt/vol) and sodium thioglycolate (0.1%, wt/vol) (BHIGT medium) enabled good growth of *C. difficile*. This medium was boiled for 10 min and cooled before inoculation. Inoculated cultures were incubated at 37°C. Semisolid agar used for overlays in the plaque assay was composed of BHIGT medium, 0.6% agar (Difco), and 0.4 M $MgCl_2$. All cultures grown on solid media were incubated anaerobically at 37°C either in a Gas-Pak system (BBL Microbiology Systems, Cockeysville, Md.) or in an anaerobic glove box (Forma Scientific, Marietta, Ohio).

Lysogenic strains were identified by one of two methods. Five-hour cultures of the 94 isolates of *C. difficile* were spotted onto blood agar plates and incubated for 24 h. Each plate was then overlaid with 2.5 ml of semisolid agar containing a few drops of one of the 94 strains. After 24 h of incubation, the plates were observed for plaques or growth inhibition at the sites of inoculation. An alternative method was that of Riley and Mee (6) used for bacteriocin detection in *Bacteroides* species, in which sterilized Millipore membranes (0.22-μm pore size; Millipore, Ltd., Mississauga, Ontario, Canada) were placed on the surface of blood agar plates, the potential producing strains were spotted onto the membranes, and after 24 h of incubation, the membranes were removed and the plates were lightly swabbed with a 1:5 dilution of a 5-h culture of all the respective test strains. After 24 h of incubation, the plates were observed for plaques or growth inhibition.

Plaque assays were performed on plates containing BHI agar (Difco) plus 0.6% human erythrocytes. This medium improved the visibility of plaques and assured good growth of *C. difficile*. Phages were assayed by depositing 100 μl of appropriate phage dilutions onto the surface of the agar and overlaying with 2.5 ml of semisolid agar containing 1 drop of a 5-h broth culture of the indicator strain. All plaque assays were done in duplicate or triplicate. Isolated plaques were first picked to broth and propagated with the indicator strain. Subsequent phage stocks were prepared by serially passing phage lysates in young broth cultures of the indicator strains of *C. difficile*.

Of 94 isolates of *C. difficile*, 2 spontaneously released bacteriophages active against other strains in the collection. Phage 41 produced plaques on four different isolates with various degrees of efficiency, and phage 56 produced plaques on five different isolates. There were no common hosts for the two phages. Upon initial isolation, plaques of phage 41 were 1 mm in diameter and plaques of phage 56 were 0.4 mm in diameter. The producing strains were inducible with UV light, and 10-fold or greater increments in PFU were obtained in cultures that were irradiated for 15 s at a distance of 50 cm from two General Electric germicidal lamps (G15T8). The membrane technique of Riley and Mee (6) was best for detecting phage-producing strains; a number of other strains produced bacteriocin-like zones of inhibition without plaques on the indicator lawns.

We noted that phage 56 showed a marked response to divalent cations (Fig. 1). Salts ($MgCl_2$, $MgSO_4$, $CaCl_2$, and NaCl) were added to the semisolid agar overlays to obtain final concentrations of up to 0.5 M. Whereas NaCl had little influence on plaque size and number, a substantial increment in the size and number of plaques was observed when $MgCl_2$ was present. The optimal concentration of 0.4 M $MgCl_2$ showed a 6.4-fold increase in the number of plaques. The plaque count was likewise enhanced by $MgSO_4$ and $CaCl_2$, but the lesser solubility of these salts discouraged their use at higher concentrations. In contrast, phage 41 showed little response to any of the above ions.

High-titer phage lysates (10^{10} PFU/ml) were negatively stained with uranyl acetate (1% aqueous solution containing

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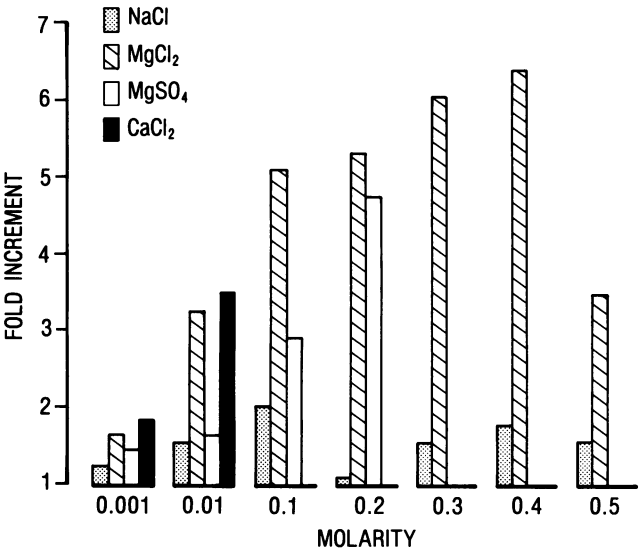

FIG. 1. Effect of salts on plaquing efficiency of phage 56. $MgCl_2$, $MgSO_4$, $CaCl_2$, and NaCl were added to semisolid agar overlays to obtain final concentrations up to 0.5 M. Phage 56 titer was assayed with these various overlays.

50 μg of bacitracin per ml) either directly or after centifugation through a 35% sucrose solution for 5 h at 80,000 × *g*. Preparations were examined in a Philips 300 electron microscope operating at 80 kV and calibrated with bacterial spinae (1). Phage 41 had a head which was hexagonal in profile and measured 62 by 56 nm (Fig. 2a). A flexuous tail measured 300 by 12.5 nm and demonstrated striations of 4.5-nm spacing indicative of a stacked disk construction. Some type of base plate was apparent, suggestive of coiled fibrils. Phage 56 had a somewhat smaller hexagonal head (60 by 55 nm), a tail core 100 by 9 nm, and a contractile tail sheath 17.5 nm in diameter. Figures 2b and c, respectively, show the uncontracted and contracted tail sheaths.

When phage 56 lysates or centrifuged pellets were treated with 2 M $MgCl_2$, a 21-nm virus-like particle was also observed (Fig. 2d). These particles were usually observed to be adsorbed to some characteristic material in the preparation which was lipid-like in appearance and were only rarely found in its absence (Fig. 2e). Without $MgCl_2$ added, the particles were undetectable. Some of these particles appeared hollow or empty whereas others demonstrated three-dimensional features with clearly visible subunit structures on their surface (Fig. 2f). Centrifugation of the phage 56 lysate in a cesium chloride density gradient (1.35 g/cm^3, 30,000 × *g*, 50 h) revealed two bands, one containing the 21-nm particle at 1.3-g/cm^3 density, the other containing the tailed phage at 1.44-g/cm^3 density. Again, magnesium was required for detection of the 21-nm particle by electron microscopy. Treatment of the purified tailed phage with $MgCl_2$ did not yield any of the small particles, indicating that the salt was not responsible for dissociating the tail structures into small subunit structures. No peak in plaque titer was associated with the 1.3-g/cm^3 density band when fractions of the gradient were assayed, suggesting that the 21-nm particle did not produce plaques on the indicator strain, whereas the titer of the tailed phage correlated with the 1.44-g/cm^3 density band.

Phage 41 was stable in broth up to and including 40°C. At 45°C there was a 53% survival of PFU, whereas at 50°C only 2% survived a 10-min exposure. Phage 56 was more sensitive to elevated temperatures and demonstrated a 1-log drop in titer when exposed to 40°C for 10 min. The response of phage 41 to various pH values over 30 min was unremarkable. The phage was acid labile but was stable throughout pH 5 to 9. Phage 56 was stable at pH 4, 5, and 8 but unstable at pH 6, 7, and 8.8. The drop in PFU at pH 6 to 7 was reproducible with new buffers and in either citrate phosphate or Tris-hydrochloride buffer adjusted to these pH values. This loss in activity did not occur when 0.1 M $MgCl_2$ was added to the buffer. The addition of NaCl to the buffer also improved the survival of PFU at pH 7.0 but at 100-fold-higher molarity than required for $MgCl_2$, and the addition of 1% bovine serum albumin was without protective effect. The phage showed no loss of viability in BHIGT medium at pH 7.0.

Adsorption of these phages was determined by adding appropriately diluted phage stocks to 5-h cultures of the indicator strains, incubating them at 37°C, and removing samples for centrifugation at 4°C at 30-s intervals. About 40% of phage 41 PFU were adsorbed to strain 36 in 30 s after which adsorption was slower, reaching 65 to 70% over the 9.5-min period studied. Adsorption of phage 56 to strain 79 was virtually complete by 30 s. The results of several experiments suggested that 0.1 M $MgCl_2$ did not influence adsorption of either phage.

One-step growth experiments for phages 41 and 56 were performed in an anaerobic glove box. The propagating strain was grown overnight in cooked meat medium, after which 1 ml was added to 10 ml of boiled BHIGT medium in a small Erlenmeyer flask. The culture was incubated for 2 h at 37°C in a glove box, after which 1 ml of phage was added. Both phages had latent periods of 60 min. The release period for phage 41 was 15 min, and the relative burst size represented a 15-fold increase in phage titer. The release period for phage 56 was 40 min, and the burst size was about 122.

To determine whether phage 56 might control cytotoxin synthesis, nontoxigenic strain 79 was lysogenized with phage 56 (from toxigenic strain 56) by infecting strain 79 with a high multiplicity of infection. The lysogenized strain 79 was then grown on sporulation agar (Difco plus 5% sheep erythrocytes) for 48 h, the growth was washed off the agar, and the resulting spore suspension was heated at 90°C for 10 min to kill any contaminating phage and vegetative cells. The spores were plated for growth, and the resulting bacteria were shown to release phage spontaneously and were inducible with UV light. Broth cultures (24 h) of strain 79, lysogenized strain 79, and strain 56 were assayed for cytotoxin as described by Silva (8) with MRC-5 cells. Neutralization with *Clostridium sordellii* antitoxin (Wellcome Research Laboratories, Beckenham-Kent, United Kingdom) was used to confirm *C. difficile* toxin. No cytotoxic activity was observed in the supernatant fluid of strain 79 or lysogenized strain 79, whereas toxigenic strain 56 had toxic activity at a dilution of 1:1,000.

We have thus described two (and perhaps three) new bacteriophages of *C. difficile* and have presented data which clearly differentiate them. Sell et al. (7) described the first isolation of bacteriophages and bacteriocins in this species, and although no detailed description of their phages was made, phage 56 resembles Cld 1. We are not aware of any previous description of 21-nm virus-like particles among the clostridia although Hendry et al. (3) have described somewhat similar particles referred to as θ med-1 in *Bacillus medusa*. They indicated that their 25-nm particles contained single-stranded RNA but could not find a host for the particles. These particles, associated with sporulation in *B.*

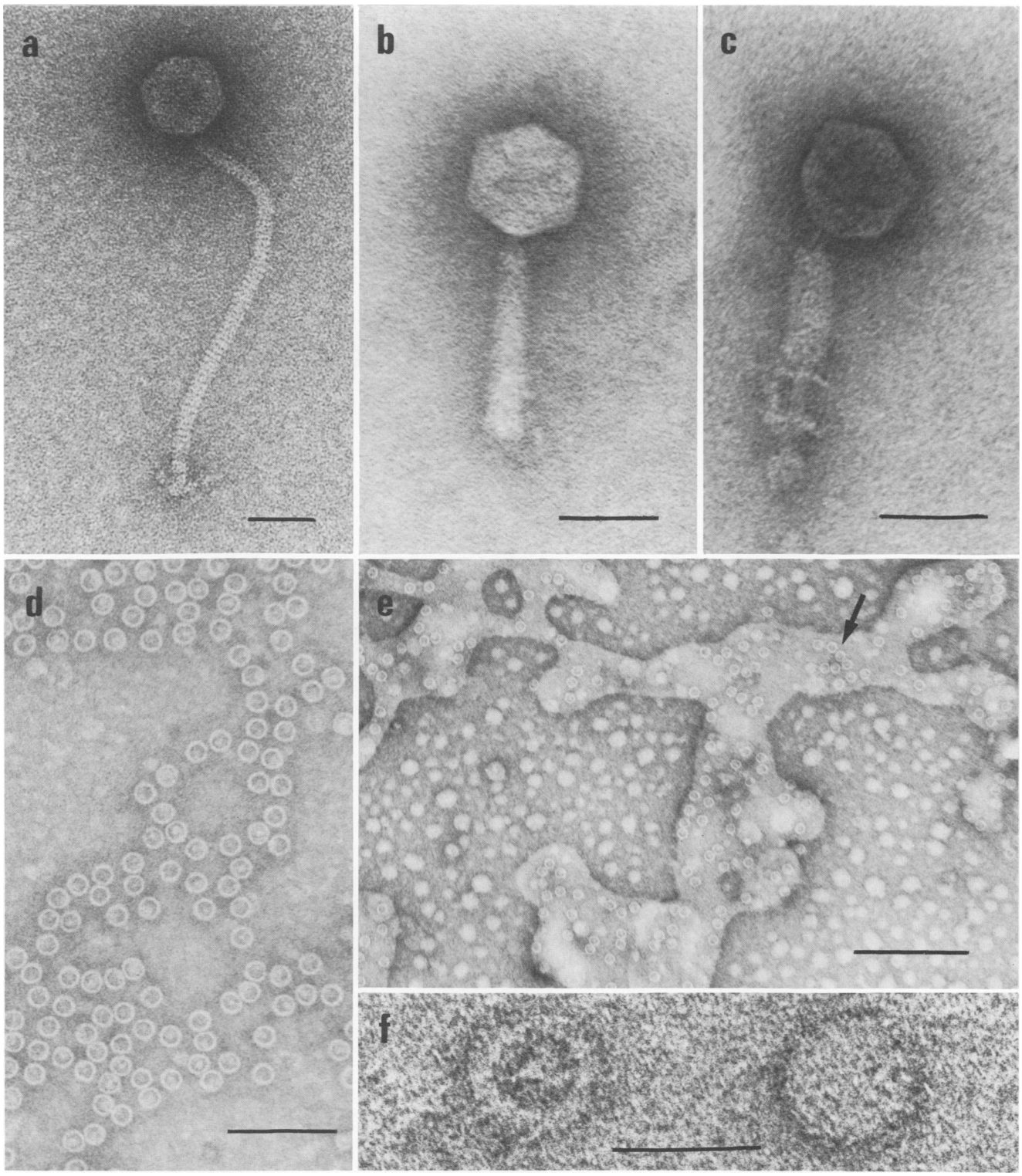

FIG. 2. (a) Bacteriophage 41 stained with uranyl acetate. Bar, 50 nm. (b) Bacteriophage 56 stained with uranyl acetate showing uncontracted tail sheath. Bar, 50 nm. (c) Bacteriophage 56 stained with uranyl acetate showing contracted tail sheath and base plate. Bar, 50 nm. (d) Small "viral" particles in the presence of 2 M $MgCl_2$ (stained with uranyl acetate). Bar, 100 nm. (e) Small "viral" particles (arrow) adsorbed to lipid-like material in the lysate (stained with uranyl acetate). Bar, 200 nm. (f) Small particle showing empty or ring-form on the left and full or complete form on the right (stained with uranyl acetate). Bar, 25 nm.

medusa, were also found by electron microscopy to be attached to "fragments of thin sheets of unknown origin" (3). Certainly, further studies are required to characterize these particles found in *C. difficile* to determine whether they are truly viruses or, if not, the role they may play in the physiology of the organism. The relationship of bacteriophages to toxin synthesis, although negative in our preliminary experiments, requires further attention.

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LITERATURE CITED

1. **Easterbrook, K. B., J. H. M. Willison, and R. W. Coombs.** 1976. Arrangement of morphological subunits in bacterial spinae. Can. J. Microbiol. **22**:619–629.
2. **Hawkins, C. C., B. P. Buggy, R. Fekety, and D. R. Schaberg.** 1984. Epidemiology of colitis induced by *Clostridium difficile* in hamsters: application of a bacteriophage and bacteriocin typing system. J. Infect. Dis. **149**:775–780.
3. **Hendry, G. S., J. B. Gillespie, and P. C. Fitz-James.** 1976. Bacteriophage and bacteriophage-like structures carried by *Bacillus medusa* and their effect on sporulation. J. Virol. **18**: 1051–1062.
4. **Lyerly, D. M., D. E. Lockwood, S. H. Richardson, and T. D. Wilkins.** 1982. Biological activities of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**:1147–1150.
5. **Moar, J. J., and J. Silva.** 1981. Pseudomembranous enterocolitis and the aetiological role of *Clostridium difficile*. S. Afr. Med. J. **60**:623–625.
6. **Riley, T. V., and B. J. Mee.** 1981. Simple method for detecting *Bacteroides* spp. bacteriocin production. J. Clin. Microbiol. **13**:594–595.
7. **Sell, T. L., D. R. Schaberg, and F. R. Fekety.** 1983. Bacteriophage and bacteriocin typing scheme for *Clostridium difficile*. J. Clin. Microbiol. **17**:1148–1152.
8. **Silva, J.** 1981. Tissue culture assay for *Clostridium difficile* cytotoxin. The Upjohn Co., Kalamazoo, Mich.
9. **Sullivan, N. M., S. Pellett, and T. D. Wilkins.** 1982. Purification and characterization of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**:1032–1040.