Bacteriophage and Bacteriocin Typing Scheme for *Clostridium difficile*

THOMAS L. SELL,† DENNIS R. SCHABERG,* AND F. ROBERT FEKETY

Division of Infectious Diseases, Department of Internal Medicine, University of Michigan Medical Center, Ann Arbor, Michigan 48109

The study of the epidemiology of infection with *Clostridium difficile* would be aided by a way to type individual bacterial isolates. We therefore sought bacteriophages for use in typing. With mitomycin C exposure (3 μg/ml), filtrates from 10 strains of *C. difficile* had plaque-forming lytic activity on other *C. difficile* strains. Individual phage were passaged and made into high-titer stock preparations for typing. Electron microscopy revealed tailed phage particles from one such preparation. In addition to phage, inhibitory activity without distinct plaque formation consistent with bacteriocins was observed for 20 strains. *C. difficile* isolates from 16 patients taken 1 to 14 days apart were similar in their phage sensitivity pattern, whereas isolates from separate geographic locations showed a great variety of patterns. We conclude that bacteriophage should be useful for typing strains of *C. difficile*.

Clostridium difficile is the primary etiological agent for pseudomembranous colitis and is often associated with so-called nonspecific cólitis after antibiotic usage. Because major predisposing factors for the development of this disease, such as surgery and antimicrobial or cancer chemotherapy, are common among hospitalized patients, many cases of pseudomembranous colitis are hospital acquired. Although the organism is a strict anaerobe, it forms aerotolerant spores which can be demonstrated in the environment about culture-positive patients with diarrhea. The spores can persist in the hospital environment for as long as five months (7). Clusters of pseudomembranous colitis have been described, both in pediatric and adult hospitalized populations, but study of the epidemiology of these outbreaks has been hindered by the lack of a suitable system for typing the organisms isolated (5, 12; R. J. Sherertz, R. L. Marshall, and F. A. Sarubbi, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 21st, Chicago, Ill., abstr. no. 707, 1981). Serotyping has not been useful for *C. difficile*. Bacteriophage are potentially useful for typing and have been described with many clostridial species (1, 10). Therefore, we sought phage active upon *C. difficile* for use in a typing scheme.

MATERIALS AND METHODS

Bacterial isolates. *C. difficile* isolates from stool specimens of different patients from different geo-

---

† Present address: Veterans Administration Medical Center, Division of Infectious Diseases, Allen Park, MI 48101.

graphic areas submitted to our laboratory were selected for study. They were each identified by growth on cycloserine-cefoxitin-fructose agar, biochemical characteristics, and fermentation product analysis by gas liquid chromatography (6). Other *Clostridia* species were obtained from the clinical microbiology laboratory of the University of Michigan Hospital. All cultures were grown at 37°C in an anaerobic glove box (Coy Laboratory Products, Ann Arbor, Mich.).

Lysate production. Isolates were grown in 10 ml of brain heart infusion (BHI) broth for 24 h. After incubation, mitomycin C was added to a final concentration of 3 μg/ml. After an additional incubation for 24 h, broth cultures were centrifuged at 5,000 × g for 10 min, and the supernatant was collected after passage through a 0.45-μm membrane filter (Gelman Sciences, Inc., Ann Arbor, Mich.).

Filtrate assay. Filtrate drops were assayed for lytic or inhibitory activity on soft agar lawns prepared as described previously by Adams (3). BHI agar (1.5%) was used as the base with 0.75% BHI agar as an overlay. A total of 50 μl of a 24-h BHI broth culture of *C. difficile* adjusted to approximately 5 McFarland units was used to seed the 3-ml molten overlay. Filtrate drops were applied to the cooled soft agar overlay, using a Steers replicator. Each filtrate was tested on 25 to 35 lawns seeded with different *C. difficile* isolates.

Phage propagation. When plaques were observed, they were picked with a Pasteur pipette and passaged three times on the original sensitive lawn. Stock preparations for further routine testing were prepared by growing plaques to near confluence with the addition of phage to the overlay in the molten state and then, after incubation, flooding the plate with 5 ml of BHI broth and allowing the flooded plate to sit for 2 to 4 h. Decanted broth was sterilized by passage through a 0.45-μm membrane filter and stored at 4°C.

Bacterial typing. A bacterial isolate seeded as a soft agar lawn was said to be sensitive to a phage if more than five plaques were observed with a standard Steers replicator drop. Isolates with between one and five plaques per drop were rarely observed. Growth inhibition was said to occur when we observed uniform decreased growth under a Steers replicator drop. For an isolate to be termed resistant to a specific phage or filtrate, the absence of plaques or inhibition in the presence of a positive control was necessary. Stock phage preparations were used at a concentration that would produce confluent lysis on the indicator lawn; for phage stock in which confluent lysis could not be obtained, the highest available concentration was used.

Electron microscopy. After concentration by centrifugation, phage preparations were placed on a carbon-coated Formvar grid, negatively stained with 2% phosphotungstic acid at neutral pH, and examined with a Philips 400 electron microscope.

RESULTS

Ten of 254 filtrates (3.9%) assayed formed plaques which could be passaged. Individual plaques were very small, measuring 0.1 to 0.9 mm in diameter on the soft agar lawns, and were best viewed with back lighting (Fig. 1). Plaques were visible when lawns were grown on a 0.75% agar overlay, but not on a 1.5% agar overlay. Preparations with greater than 10^5 PFU/ml could usually be easily obtained for most phage and produced confluent lysis on sensitive lawns. Plaques were generally clear at 24 h, becoming turbid after incubation for 48 h. A standard nomenclature was applied to the stock preparations, with the letters Cld and a number denoting the order of phage isolation (2). Cld 1, the first phage isolated, was prepared for electron microscopy, and a virion with sheathed-tail morphology similar to other previously described clostridial phage was apparent (Fig. 2) (1, 9, 10).

In general, the phage were sensitive to heat, resistant to chloroform, and inactivated by 1 h of incubation at 56°C. They were stable for at least 4 weeks under refrigeration at 4°C.

Another 20 of the filtrates exhibited inhibition of lawn growth without discrete plaque formation. Upon serial dilution of these filtrates, no discrete plaques were apparent, and any inhibition of lawn growth disappeared after a 64-fold dilution (Fig. 3). This inhibitory property could not be serially passaged on sensitive lawns, was labile, and disappeared after 2 weeks of storage under refrigeration or after 60 min of exposure to heat. It can best be described as a bacteriocin. Bacteriocin or phage production was not limited to isolates from any single geographic area. (Isolates were obtained from the following areas [number of isolates]: Ann Arbor, Mich. [11]; Houston, Tex. [6]; Southfield, Mich. [4]; Traverse City, Mich. [1]; Gastonia, N.C. [1]; and Dayton, Ohio [1].)
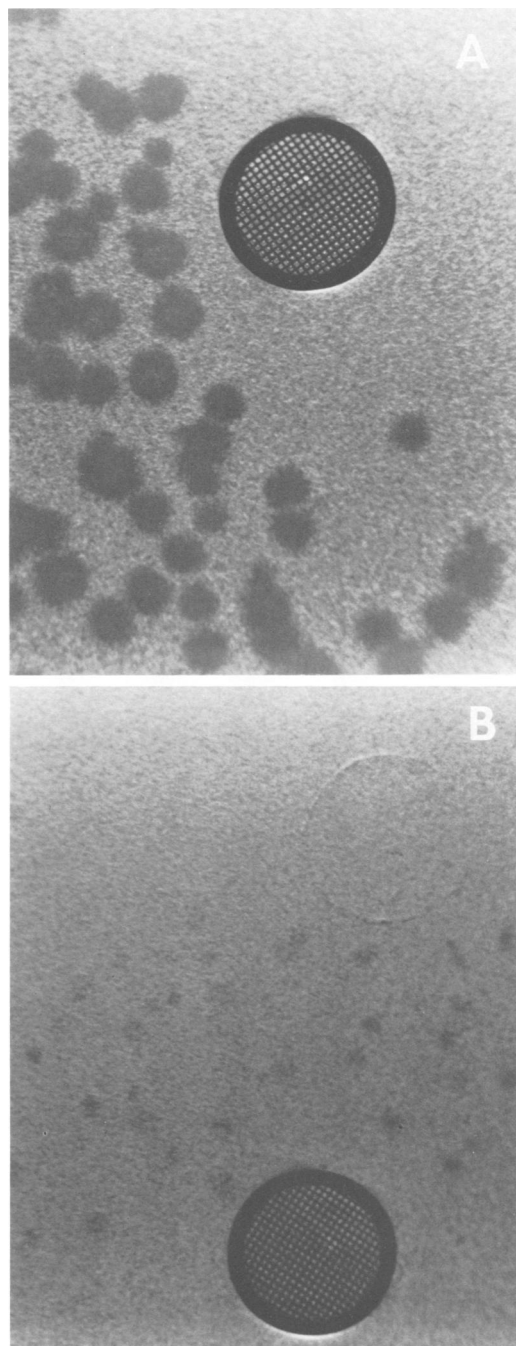

FIG. 1. Two different phage plaque morphologies on *C. difficile* soft agar lawns. The electron microscope grid included for scale is 3 mm in diameter. (A) Turbid center is apparent. (B) Plaques are pinpoint in size and are visible with black lighting only.

When all 10 available stock preparations of phage and freshly prepared bacteriocins were applied to isolates from 29 randomly selected
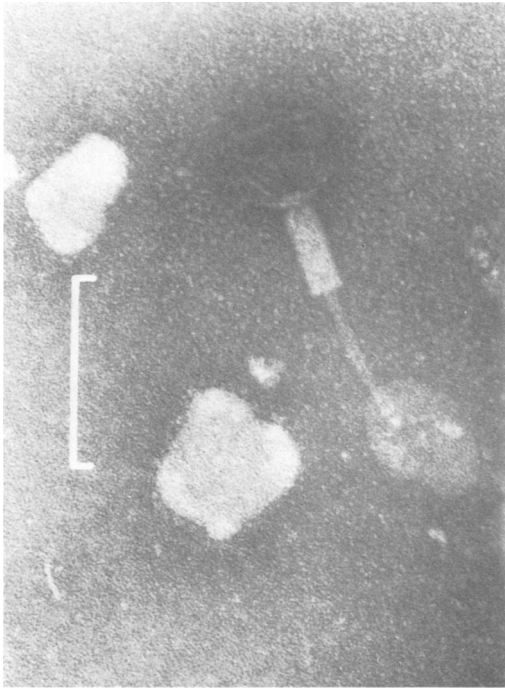
FIG. 2. Electron micrograph of Cld 1 particle with negative staining. Bar, 100 nm. The particle consists of a head, sheath, and tail.

three representative bacteriocins. On those 51 isolates sensitive to both phage and bacteriocin, an association between phage groups of activity and bacteriocin groups of activity was apparent.

Isolates of *C. difficile* from 16 patients were compared with isolates taken from the same patient 1 to 14 days later. In nine (56%) of the instances, the typing pattern of the first isolate was identical to that of the second isolate. In three (19%), there was a difference in a reaction to a single bacteriocin or phage. In two (12%), there was an identical reaction to at least one phage or bacteriocin with different reactivity to more than one. In two (12%), there was a major difference or dissimilarity between the members of the pair. When isolates were retested without knowledge of prior test results, the identical pattern of phage or bacteriocin sensitivity (or both) was always seen.

No other clostridial species tested, including four isolates of *C. sordellii*, one of *C. bifermentans*, one of *C. sphenoides*, and one of *C. perfringens*, showed lysis by phage or inhibition by bacteriocin from *C. difficile*. One of eight bacteriocin producers tested was observed to produce bacteriocin in the absence of mitomycin C.

DISCUSSION

Many phage and bacteriocins active on various clostridial species have been isolated previously, but none for *C. difficile*. In our laboratory, stool specimens are often tested for the presence of *C. difficile*. Because our goal was the development of a typing scheme to differentiate isolates from our large collection, the method we have developed has been aimed at obtaining as many active phage as possible. The use of the Steers replicator has been essential to our approach of screening isolates for phage production, as well as for their sensitivity to phage preparations. We were especially interested in host range and, on the basis of overlapping ranges, can group the phage based upon activity. Within a group, there is only partial overlapping of activity range so that for our purposes relating to typing, no two phage isolated are identical. Further studies to define morphology and serological relatedness should clarify the relatedness of each phage. For typing purposes, phage preparations should prove useful and, upon control strains tested at least 10 times, have given identical results. To our knowledge, phage have not been used for typing any other clostridial species to date.

Bacteriocins have been applied for the purpose of typing clostridial species (8, 11, 12). Because the inhibitory activity is more labile and present in lower titer compared with phage, frequent fresh mitomycin C filtrate preparations

patients, 20 isolates (69%) were susceptible to either phage or bacteriocin. Isolates from diverse geographic locations showed a variety of sensitivity patterns (Table 1). Several more recently acquired bacteriocins and phage were not available for the earliest susceptibility studies. However, when all 254 isolates were reviewed for sensitivity to the initial 10 preparations, 132 (52%) were sensitive to either phage or bacteriocins. Forty of the 132 isolates (30%) were lysed by phage but were not sensitive to bacteriocins. Fifty-one of the 132 (39%) were sensitive to both a phage and a bacteriocin. Forty-one of the 132 (31%) were not sensitive to phage but were inhibited by bacteriocin. Twenty isolates were sensitive to more than one phage. Cld 1 and Cld 8 were active on the same lawn 13 times, and Cld 2, 6, 9, and 10 were active on the same lawn in various combinations on seven occasions. No phage preparation appeared conclusively identical to another based upon host range. With the phage-sensitive bacterial isolates, at least nine distinct phage groups could be discerned.

Forty-nine isolates were sensitive to more than one bacteriocin. Many of the bacteriocin preparations had an identical spectrum of inhibition, but at least three distinct ranges of activity were apparent. For typing purposes, we selected
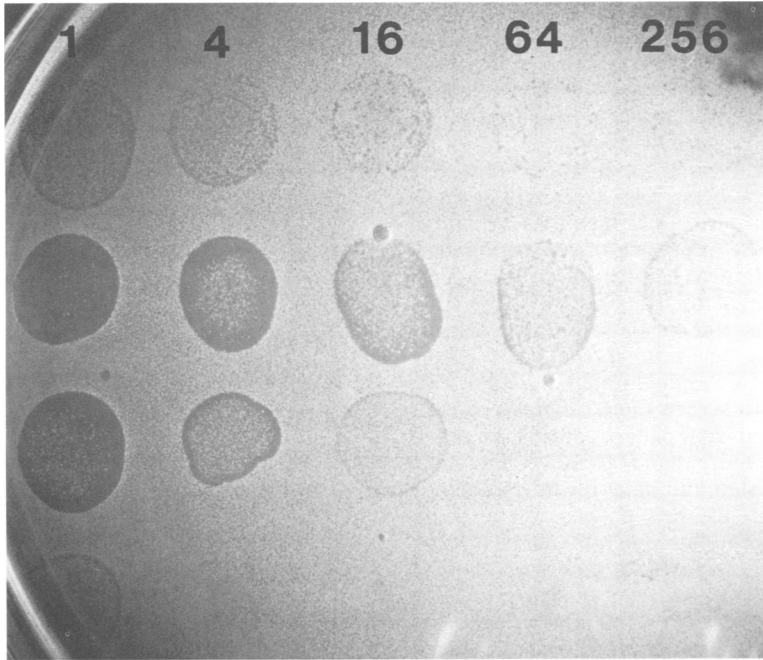
FIG. 3. Soft agar lawn seeded with *C. difficile* onto which drops of serial fourfold dilutions of two bacteriophage (Cld 1 in the top row and Cld 8 in the second row) and two bacteriocins (83 in the third row and 2329 in the bottom row) have been applied with a Steers replicator apparatus. The bacterial strain is sensitive to all four preparations, which produce similar clearing in the undiluted drops. Upon dilution, bacteriophage have endpoints with individual plaques. Endpoints of 2329 (1:16) and 83 (undiluted) show no discrete plaques. Numbers on top show dilution.

are required. Because there is no way to selectively isolate and propagate the bacteriocins, it is possible that there is more than one bacteriocin within any given stock preparation. Furthermore, a given isolate when exposed to mitomycin C may fail to produce active bacteriocin about 20% of the time, which emphasizes the requirement for stringent controls. Reproducibility of bacteriocin typing is not as great as phage typing because of the greater variation in the preparations. Although the nature of this inhibitory activity remains poorly characterized at this time, the association of phage activity groups and bacteriocin activity groups suggests

TABLE 1. Bacteriophage-bacteriocin susceptibility of 16 *C. difficile* isolates from human stool[a]

Isolate no.	Phage no.							Bacteriocin no.				Geographic source of tested isolates
	1	2	6	7	8	9	10	83	2329	1320	1537	
900		+	+			+	+			+	+	Huntsville, Ala.
362	+							+				Winchester, Va.
458											+	Southfield, Mich.
578	+							+	+			St. Paul, Minn.
358											+	Columbus, Ohio
403	+				+			+				Freeport, Ill.
361				+								Washington, D.C.
594					+			+				St. Louis, Mo.
464				+								Columbus, Ohio
739	+					+	+				+	Gastonia, N.C.
1,029								+	+			Ann Arbor, Mich.
955				+								Ann Arbor, Mich.
100	+				+			+	+			Indianapolis, Ind.
409							+			+		Winchester, Va.
1,710			+			+	+				+	Westland, Mich.
1,704		+								+		Ann Arbor, Mich.

[a] All testing was done on the same day with replicate tests.

that they may share receptor sites. The addition of bacteriocin preparations to the typing scheme that uses bacteriophage increases the percentage of typable strains by 50% and is probably useful, but better standardization and stabilization of bacteriocin preparations is desirable.

Both production and sensitivity to phage or bacteriocin have been observed with isolates from widespread geographic locations. Although it is likely that the frequency of susceptibility of clinical isolates to a battery of typing agents could be increased by addition of phage from other continents, the phages that are already available should be useful in the study of the epidemiology of *C. difficile*-related disease. We are continuing to screen the *C. difficile* isolates we have obtained from patients during the past 5 years. We have more than 800 isolates that have not yet been tested and have recently obtained additional apparently new phages. We are continuing this work to improve our typing system.

Typing of isolates from clusters of cases of *C. difficile* disease should resolve whether these outbreaks actually represent cross-infection or coincidence. Such information would directly relate to the need for isolation of culture-positive symptomatic patients and environmental decontamination. In addition, since many patients with colitis who are treated will have a relapse (2), typing of isolates from such patients could differentiate between relapse and reinfection and assist in management of this difficult group of colitis patients.

ACKNOWLEDGMENTS

LITERATURE CITED

1. **Ackermann, H.** 1974. Classification of bacteriophages of Bacillus and Clostridium. Pathol. Biol. **22:**909–917.
2. **Ackermann, H., A. Auduviev, L. Berthiaume, L. A. Jones, J. A. Mayo, and A. K. Vidaver.** 1978. Guidelines fr bacteriophage characterization. Adv. Virus Res. **23:**1–24.
3. **Adams, M. A.** 1959. Bacteriophage, p. 443. Interscience Publishers, New York.
4. **George, W. L., N. A. Volpicell, D. B. Stiner, D. D. Richman, E. J. Liechty, H. Y. I. Mok, R. D. Rolfe, S. M. Finegold.** 1979. Relapse of pseudomembranous colitis after vancomycin therapy. N. Engl. J. Med. **401:**414–415.
5. **Greenfield, C., A. Burroughs, M. Szawathawski, N. Bass, P. Noone, and R. Pounder.** 1981. Is pseudo-membranous colitis infectious? Lancet **i:**371–372.
6. **Holdeman, L. V., E. F. Cato, W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed., p. 79–106.
7. **Kim, K. H., R. Fekety, D. Batts, D. Brown, M. Cudmore, J. Silva, and D. Waters.** 1981. Isolation of *Clostridium difficile* from the environment and contacts of patients with antibiotic-associated colitis. J. Infect. Dis. **143:**42–50.
8. **Mahoney, D. E., and A. Li.** 1978. Comparative study of ten bacteriocins of *Clostridium perfringens*. Antimicrob. Agents Chemother. **14:**886–892.
9. **Nieves, B. M., F. Gil, and F. J. Castillo.** 1981. Growth inhibition activity and bacteriophage and bacteriocinlike particles associated with different species of *Clostridium*. Can. J. Microbiol. **27:**216–225.
10. **Ogata, S., and M. Hongo.** 1979. Bacteriophages of the genus *Clostridium*. Adv. Appl. Microbiol. **25:**241–273.
11. **Satija, K. C., and K. G. Navayan.** 1980. Passive bacteriocin typing of strains of *Clostridium perfringens* type A causing food poisoning for epidemiologic studies. J. Infect. Dis. **142:**899–902.
12. **Tagg, J. R., A. S. Dajani, and L. W. Wannamaker.** 1976. Bacteriocins of gram-positive bacteria. Bacteriol. Rev. **40:**722–756.
13. **Walters, B., R. Stafford, R. Roberts, and E. Seneviratne.** 1982. Contamination and crossinfection with *C. difficile* in an intensive care unit. Aust. N.Z. J. Med. **12:**255–258.