JOURNAL OF BACTERIOLOGY
Vol. 87, No. 2, p. 408–415 February, 1964

ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGES ACTIVE ON *CLOSTRIDIUM SPOROGENES*[1]

JOHN V. BETZ[2] AND KENNETH E. ANDERSON

Department of Biology, St. Bonaventure University, St. Bonaventure, New York

Received for publication 25 July 1963

ABSTRACT

BETZ, JOHN V. (St. Bonaventure University, St. Bonaventure, N.Y.), AND KENNETH E. ANDERSON. Isolation and characterization of bacteriophages active on *Clostridium sporogenes*. J. Bacteriol. **87**:408–415. 1964.—Twelve bacteriophages active on the anaerobic species *Clostridium sporogenes* were studied. Four of these were isolated by the authors, and eight were obtained from L. S. McClung of Indiana University. The 12 phages studied could be distinguished into three groups on the basis of their plaque morphology, host range, receptor sites, and serological relationships. One group contained ten of the phages which were serologically related. These were separated into three subgroups on the basis of plaque morphology, host range, and receptor sites. The heat sensitivities of four phages were correlated with their classification in this scheme. Of 25 strains of *C. sporogenes* tested for lysogenicity, none was found to be lysogenic, but 9 produced bacteriocin-like substances and 20 were sensitive to one or more of these.

The existence of bacteriophages for a member of the genus *Clostridium* was first established by Cowles (1934), who isolated from sewage a phage which was active on two strains of *C. tetani*. Since then, several groups of workers have isolated phages for other members of this genus. McClung (1956) summarized and reviewed most of the available literature on phages for the genus *Clostridium*. Spencer (1953) listed reports by several Russian investigators on phages for *C. perfringens* and other toxigenic *Clostridium* species. Frenkel (1940) reported isolation from river water of phages active on *C. sporogenes*. However, no further work on phages for this species has appeared.

The general purpose of this study was to investigate phage-host interactions in an obligately anaerobic species. We chose *C. sporogenes* as our host organism for this purpose, because it is common in nature and ordinarily is not pathogenic. It is easier to grow than many other strict anaerobes, and is thus more amenable to the manipulations routinely involved in phage research. This paper deals with the first phase of these studies, the isolation and characterization of bacteriophages active on this species.

MATERIALS AND METHODS

Bacterial strains. The 25 strains of *C. sporogenes* used in this study were kindly made available to the authors by L. S. McClung of Indiana University; their numbers, as given in Table 1, refer to their designations in his collection.

Media. The media used in this study were variations of a medium designated S broth, which had the following composition: Proteose Peptone (Difco), 5.0 g; Polypeptone (BBL), 5.0 g; NaCl, 5.0 g; yeast extract (Difco), 1.0 g; glucose, 3.0 g; sodium thioglycolate, 0.5 g; and distilled water, 1 liter. The pH was adjusted to 7.6 to 7.8 with NaOH before autoclaving for 10 min at 121 C. Sterilization lowered the pH to 7.0 to 7.2. The oxidation-reduction potential of freshly autoclaved S broth which had been cooled quickly to 37 C averaged −60 mv.

The medium used for maintenance of stock cultures and to produce cells for seed layers was S/H broth. This was made by tubing 10 to 15 ml of S broth over a 1- to 3-mm column of Difco Beef Heart for Infusion, and autoclaving as described.

Hard and soft agars for double-layer phage plating contained, respectively, 2.0 and 0.6% Difco agar in S broth.

Incubation. Temperature of incubation for

[1] This work is part of a Ph.D. thesis submitted by one of the authors (J.V.B.) to the Graduate School of St. Bonaventure University. A preliminary report of this work was made (Betz and Anderson, 1963).

[2] Present address: The Botany Program, The University of South Florida, Tampa.

phage and bacterial growth was 37 C in all experiments.

Assay of phage. Assay of phage by plaque count was carried out by a double agar layer method similar to that described by Adams (1959), with modifications to protect the bacteria from atmospheric oxygen. Base layers of 60 to 75 ml of hard S agar were prepared in petri dishes (20 by 100 mm) with porous porcelain tops to allow surface moisture to evaporate overnight. Freshly sterilized tubes (13 by 100 mm) containing 2.5 ml of soft S agar were kept molten in a 45-C water bath. To these were added 0.2-ml samples of a late log phase S/H culture of the host bacteria, containing 0.8×10^9 to 1.0×10^9 viable cells per ml. Serial dilutions of the phage-containing material were made through blanks of S broth, and 0.1- or 1.0-ml portions of appropriate dilutions were added to seeded soft agar tubes, mixed, and plated. Plaques were counted after 18 to 20 hr of anaerobic incubation.

Anaerobic incubation methods. When a large number of plates were ready for incubation at the same time, an anaerobic oven (1.58 ft^3 capacity) from National Appliance Co., Portland, Ore., was used. After loading and sealing, the oven was flushed three times by evacuating to 26-in. vacuum and filling with nitrogen. After a final evacuation, the oven was filled with 10% CO_2 in H_2 as the final anaerobic gas phase. Small numbers of plates were incubated in Brewer jars under an atmosphere of illuminating gas.

Phage isolation procedures. Two methods were used in attempts to isolate phages for *C. sporogenes* from natural sources.

(i) About 3 liters of a fluid sample such as sewage or river water were saturated with ammonium sulfate (technical grade, 800 g per liter) at room temperature and set overnight in a refrigerator at 8 C. The floc which formed was skimmed off and resuspended in 50 to 100 ml of S broth. Portions (5 ml) of this suspension were added to actively growing S/H cultures of various strains of *C. sporogenes* and incubated overnight at 37 C. After incubation, the mixtures were centrifuged, and drops of the supernatant fluids were spotted on seed layers of the presumptive host strains.

(ii) About 1 liter of a fluid or solid sample, such as soil, manure, or septic tank material, was mixed with 2 liters of freshly steamed S broth and inoculated with 10-ml samples of actively growing S/H cultures of several *C. sporogenes* strains. After incubation for 48 hr, a sample was taken and centrifuged at low speed; the supernatant fluid was spotted on seed layers of the bacterial strains originally used as presumptive hosts.

Purification of phage stocks. Lysed areas on seed layers were scraped from the agar surface with a sterile Nichrome wire, transferred to S broth, diluted, and plated to yield isolated plaques. These were stabbed, diluted, and replated twice in series. If the plaques were uniform on the third plating, one was scraped from the agar surface, inoculated into a young culture of the host bacteria, and a stock was prepared as described below.

Preparation of phage stocks. A young S broth culture of the host bacteria, containing ca. 5×10^8 viable cells per ml, was inoculated with enough phage to give an input multiplicity of one phage per ten cells, and incubated at 37 C. Under these conditions, lysis began after 3 hr and was virtually complete by 6 hr. The lysates were centrifuged at low speed and passed through a Millipore filter (0.45-mμ pore diameter). Phage stocks with titers of 10^9 to 10^{11} plaque-forming units (PFU) per ml were routinely obtained. The sterile filtrates lost only about 1 log unit of activity when stored at 8 C for up to 6 months.

Screening of bacterial strains for lysogenicity. To determine whether any of the 25 bacterial strains actively produced phage during growth, the supernatant fluids from mature S/H cultures of each strain were spotted on seed layers of every other strain. After anaerobic incubation, the plates were examined for signs of lysis in the spotted areas.

Production of antisera. Young rabbits were injected subcutaneously twice a week for 3 weeks with 5 ml of phage stocks containing at least 10^{10} PFU per ml. At 2 weeks after the last injection, the rabbits were bled by ventricular puncture. Blood samples were allowed to clot in sterile tubes and centrifuged to sediment clot and cells. The sera were decanted into sterile bottles and stored at 8 C.

For use in determining antigenic similarity among the phages, 0.2 ml of antisera, or of antisera diluted in S broth, were mixed with an equal volume of phage stock containing about 2×10^8 PFU per ml. The mixtures were incubated at 37 C for 15 min, then spotted on seed layers of the host strains to determine whether most or all of the phage particles had been neutralized.

As a control, 0.2 ml of sera from a rabbit which had not been inoculated with any of the phages was mixed with each antigen, incubated, and spotted to determine whether "normal" rabbit serum carried substances capable of inactivating these phages.

Phage-resistant mutants. C. sporogenes 213 was found to be sensitive to all of the phages in our possession. Phage-resistant mutants of this strain were selected by mixing ca. 10^8 log-phase cells and 10^9 phage particles in a seed layer and incubating anaerobically. Colonies of survivors were isolated, subcultured, and plated as for a viable count. Individual colonies were reisolated and challenged with the selecting phage in broth culture and by spotting on seed layers. Those isolates which were not perceptibly lysed were considered resistant. They were shown not to be lysogenic by their failure to produce a zone of lysis when spotted on seed layers of the phage-sensitive parent strain. They failed to adsorb more than 1% of the challenging phage in 5 min under conditions in which the parent strain adsorbed 99% of the challenging phage in the same time. We concluded that these phage-resistant strains were mutants of *C. sporogenes* 213 that had suffered an alteration in their surface layers, destroying the phage-receptor sites.

Host range. The host ranges of the phages were determined by spotting ca. 10^7 particles of each phage on seed layers of each of the 25 bacterial strains and the phage-resistant mutants derived from strain 213. Plates were incubated as described and examined for lysed areas.

Heat resistance. The rates of thermal inactivation of several of the phages at 60 C in S broth were determined by incubating a dilution of each phage containing initially 10^7 to 10^8 PFU per ml at 60 C, and sampling at intervals for assay of survivors by plaque count.

RESULTS

Isolation of phages. Four phages active on various strains of *C. sporogenes* were isolated.

Phage S2 was isolated from a septic tank by the mixed-culture technique, and gave only very turbid and incomplete lysis on its original host, strain 2835. It produced clear lysis on seed layers of strain 1009, however, and, after purification and propagation on this strain, gave a strongly lytic reaction with the original host, strain 2835. Like most of the other phages studied, it gave "bull's-eye" plaques, 3 to 4 mm in diameter (Fig. 1).

Phage K was isolated by the mixed-culture technique for strain 1009 from the same septic tank which yielded phage S2, but at a different time. Like S2, it gave bull's-eye plaques, but

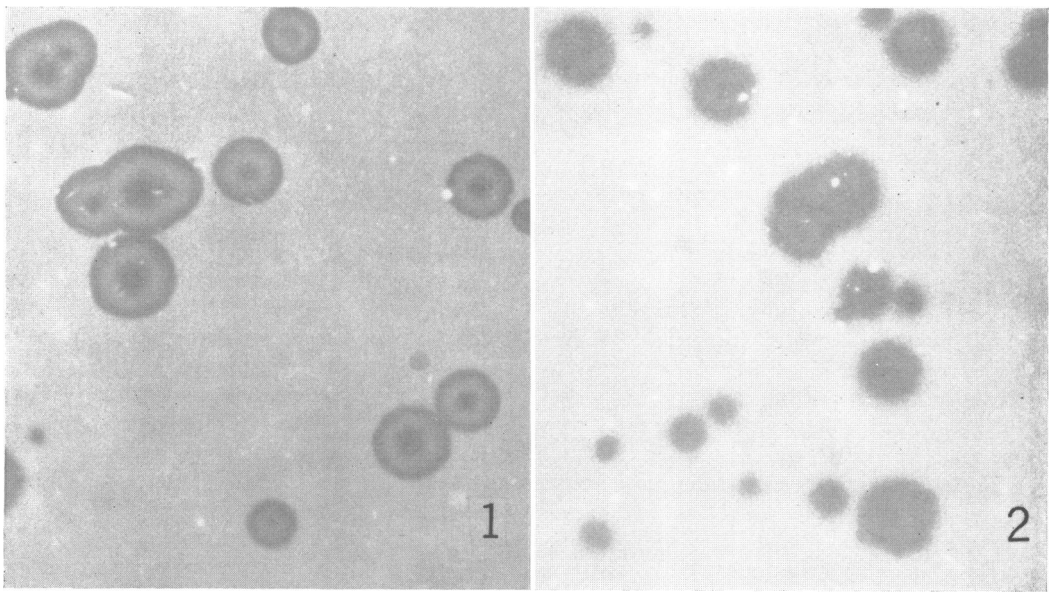

FIG. 1. *Plaques of phage S2 on Clostridium sporogenes 1009. Magnification: 3×.*
FIG. 2. *Plaques of phage 1 on Clostridium sporogenes 175. Magnification: 3×.*

gave clear lysis of all host strains from the time of its isolation.

Phage M was isolated by the mixed-culture technique for strain 1009 from a mixture of about equal parts of soil and manure taken from a barnyard. Like phage S2, it was originally quite temperate, but propagation on a variety of host strains failed to increase its virulence. It formed very turbid plaques, 3 to 4 mm in diameter.

Phage F1 was isolated from sewage by the ammonium sulfate flocculation technique. It was originally quite lytic for its original host, strain 213, and gave bull's-eye plaques 3 to 4 mm in diameter.

Phages 1, 14, 15, 16, 17, 18, 21, and 22 had been isolated from chicken feces by the ammonium sulfate flocculation technique in the laboratory of L. S. McClung of Indiana University (*unpublished data*), and stocks of these were kindly made available for our study. Phages 1 and 14 were routinely propagated on strain 175, and gave clear or very slightly turbid plaques 3 to 4 mm in diameter (Fig. 2). Phages 15, 16, 17, 18, 21, and 22 were routinely propagated on strain 213, and gave bull's-eye plaques similar to those of phages S2, F1, and K.

TABLE 1. *Sensitivity of some strains of Clostridium sporogenes to inhibitory substances produced by other strains of C. sporogenes**

Strain tested	Strains producing inhibitory substances								
	416	1071	1050	175	386	397	420	423	371
174	+	+	−	+	−	+	−	−	+
175	+	−	−	−	−	−	−	+	+
212	−	−	+	−	−	−	−	+	+
213	−	+	+	−	+	+	+	+	+
225	+	+	−	−	−	−	−	−	−
361	−	−	−	−	−	−	−	−	−
371	+	−	−	−	−	−	−	−	−
379	+	−	−	−	−	−	−	−	−
386	−	−	−	−	−	−	−	−	−
396	+	−	−	−	−	−	−	−	−
397	−	−	−	+	−	−	−	−	−
416	−	−	−	−	−	−	−	−	−
420	−	+	−	−	−	−	−	−	−
423	−	+	−	−	−	−	−	−	−
613	−	−	−	−	−	−	−	−	−
815	+	+	+	−	+	−	+	+	−
845	+	+	−	−	−	−	+	−	−
935	+	+	−	−	−	−	−	−	−
985	+	−	−	−	−	−	−	−	−
1009	+	−	−	+	−	−	−	−	+
1010	+	+	−	+	−	−	+	+	−
1012	−	−	−	−	−	−	−	−	−
1050	+	+	−	−	−	−	−	−	−
1071	−	−	+	−	+	−	−	−	−
2835	+	+	+	−	−	−	−	−	−

* Symbols: + indicates inhibition; − indicates no inhibition.

TABLE 2. *Sensitivity of three phage-resistant mutants of Clostridium sporogenes 213 to challenge by the 12 phages**

Phage	Mutant bacterial strain		
	213/18A	213/18B	213/21B
1	+	+	+
14	+	+	+
15	−	−	−
16	−	−	−
17	−	−	−
18	−	−	−
21	−	−	−
22	−	−	−
S2	−	−	−
F1	−	−	−
M	+	+	+
K	+	+	+

* Symbols: + indicates lysis; − indicates no lysis.

Plaque morphology. As is obvious from Fig. 1 and 2, these phages did not give plaques which were uniform in size. Since repeated serial isolations of single plaques failed to yield phage stocks which gave plaques of uniform size, we do not believe that the heterogeneity of plaque size exhibited by these phages is an indication of more than one phage type present in our stocks. Rather, we feel that the different sizes of plaques are a reflection of the different rates at which infected bacteria become able to support phage multiplication as anaerobic conditions are gradually achieved after plating. We are studying this phenomenon further. The largest (most mature?) plaques formed by each phage had a characteristic size and morphology, and it was these which were scrutinized in an attempt to distinguish or relate the different phages on the basis of their plaque morphology.

Occurrence of lysogenic and bacteriocinogenic strains. None of the 25 strains of *C. sporogenes* was found to be lysogenic as tested. However, nine strains produced substances which inhibited other strains in the seed layer spot test. The clear areas did not resemble those produced by phage,

and a reproducing lytic principle was not recovered from any of them by transferring them into broth cultures of the sensitive bacterial strains. If about 50 cells of an inhibitor-producing organism were plated on a seed layer of a sensitive strain, each cell produced a small plaque-like area, 3 to 5 mm in diameter, with a colony of the inhibitor producer at its center. No clear areas without central colonies were found, and no clear areas were produced by plating serial dilutions of sterile filtrates of the inhibitor-producing cultures on sensitive strains. Thus, these toxic substances resemble the bacteriocins produced by other genera (Ivanovics, 1962). Table 1 lists the strains producing toxic substances and the sensitivity of the other strains to these.

The most active inhibitor-producing strains were 416, which inhibited 14 other strains, and 1071, which inhibited 11 other strains. These two strains and their toxic substances differed in at least two respects. Strain 416 was sensitive to none of the phages in our collection, whereas strain 1071 was at least partially lysed by ten of the phages. Heating the supernatant fluid of a broth culture of strain 1071 to 70 C for 10 min destroyed its ability to kill susceptible cells. The same treatment was sufficient to kill the few remaining cells in a supernatant fluid of strain 416 but did not diminish the lethal character of its bacteriocin.

Serological cross-reactions among the phages. Normal rabbit sera had no effect on any of the phages. Each of the antisera prepared against phages 1, 14, 15, 16, 17, 18, 21, 22, S2, and F1 completely neutralized all of the other phages of this group at dilutions of 1:2 and 1:10. None of these sera appreciably neutralized phages M and K under the same conditions. Thus, phages 1, 14, 15, 16, 17, 18, 21, 22, S2, and F1 seemed to be serologically related to each other but not to phages M and K.

Ability to lyse resistant bacterial mutants. Three phage-resistant mutants of *C. sporogenes* 213 were selected. Two of these, 213/18A and 213/18B, were selected by phage 18, and one, 213/21B, was selected by phage 21. Their sensitivity

TABLE 3. *Host ranges of the 12 phages on 25 strains of Clostridium sporogenes**

Host strain	Phages											
	1	14	15	16	17	18	21	22	S2	F1	M	K
174	—	—	—	—	—	—	—	—	—	—	—	—
175	C	C	C	C	C	C	C	C	C	C	T	C
212	C	C	C	C	C	C	C	C	C	C	T/—	C
213	C	C	C	C	C	C	C	C	C	C	T	C
225	T	T	C	C	C	C	C	C	C	T	T	C
361	—	—	—	—	—	—	—	—	—	—	—	—
371	—	—	T	T	T	T	T	T	—	C	—	C
379	—	—	T	T	T	T	T	T	T	T	—	T
386	—	—	T	T	T	T	T	T	—	—	—	T
396	C	C	C	C	C	C	C	C	C	—	—	T
397	—	—	—	—	—	—	—	—	—	—	—	—
416	—	—	—	—	—	—	—	—	—	—	—	—
420	—	—	C	C	C	C	C	C	C	T	—	C
423	—	—	—	—	—	—	—	—	—	—	—	—
613	T	T	C	C	C	C	C	C	C	C	—	C
815	C	C	C	C	C	C	C	C	C	C	T	C
845	C	C	C	C	C	C	C	C	C	C	T	C
935	C	C	C	C	C	C	C	C	C	C	T	C
1009	C	C	C	C	C	C	C	C	C	C	T	C
1010	C	C	C	C	C	C	C	C	C	C	T	C
1012	C	C	C	C	C	C	C	C	C	C	T	C
1050	C	C	C	C	C	C	C	C	C	C	T	C
1071	—	—	T	T	T	T	T	T	T	C	T	C
2835	—	—	T	T	T	T	T	T	T	T	—	T

* C indicates clear lysis; T indicates turbid lysis or discrete plaques on spot; and — indicates no apparent lysis.

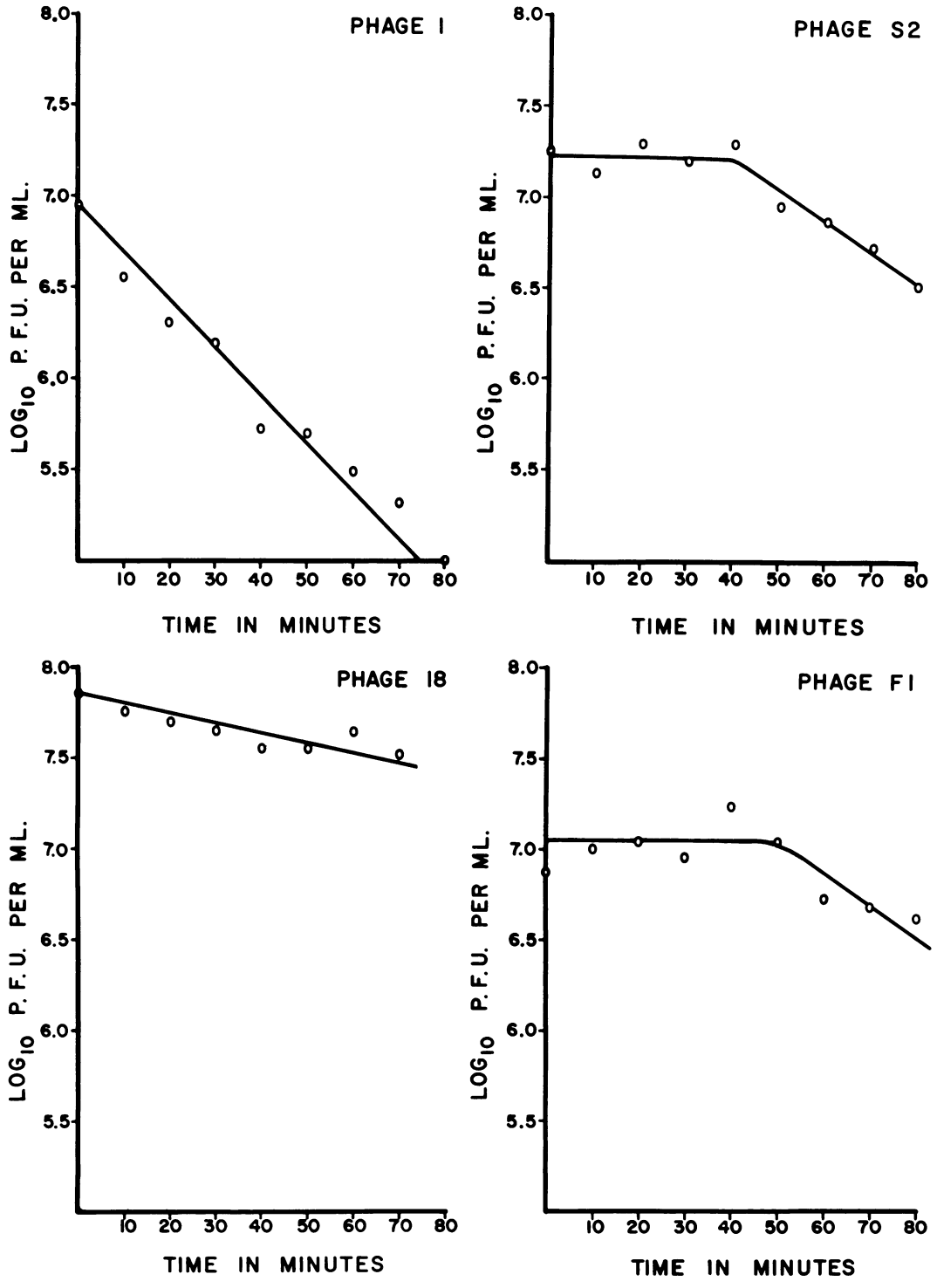

FIG. 3. *Patterns of thermal inactivation of four phages active on Clostridium sporogenes incubated in S broth at 60 C.*

to the other phages was determined (Table 2). Since these mutants lost the ability to adsorb phages 15, 16, 17, 18, 21, 22, S2, and F1 in the same mutational step, we assume that the host-cell receptor sites which they used were either identical or "overlapped" enough so that the same alteration could destroy all of them. Phages 1 and 14, which were serologically similar to these eight phages, apparently did not share the same receptor site with them. Phages M and K, which were serologically dissimilar to the other phages, also apparently differed from them in the receptor sites which they used.

Host range. Table 3 shows the host ranges of the 12 phages on the 25 strains of *C. sporogenes* tested. Phages 1 and 14 gave identical reactions with all the strains tested. Phages 15, 16, 17, 18, 21, and 22 also had identical host ranges. Phages S2 and F1 differed only slightly in host range from phages 15 through 22, and from each other. Phage K differed from these eight phages in the clearness with which it lysed certain strains. Phage M lysed fewer strains than did the other phages, and lysed none clearly.

Heat resistance. The patterns of thermal inactivation of phages 1, 18, S2, and F1 at 60 C are shown in Fig. 3. Phages 1 and 18 were both inactivated exponentially, but at markedly different rates. Phage 1 was 98.8% inactivated in 80 min, whereas phage 18 was only 65% inactivated in the same time. Phages S2 and F1 were almost completely resistant at 60 C for at least 40 and 50 min, respectively. Thereafter, both were inactivated exponentially. S2 was 82.4% inactivated in 80 min, and F1 was 60.5% inactivated in the same time.

DISCUSSION

Phages active on *C. sporogenes* were isolated from natural sources in which the host organism is commonly found in abundance. The initially temperate character of two of the isolates, S2 and M, suggests that these two phages may have originated from naturally occurring lysogenic strains of *C. sporogenes*. However, none of the 25 pure strains of this organism tested was found to be overtly lysogenic. This, of course, does not rule out the possibility of defective lysogeny, or the detection of overt lysogeny provided more suitable indicator strains were used.

	GROUP I				GROUP II	GROUP III
	SUBGROUP A 1, 14	SUBGROUP B 15, 16, 17 18, 21, 22	SUBGROUP C F1	SUBGROUP C S2	K	M
CROSS REACTS WITH ANTISERA AGAINST:	ALL OF GROUP I PHAGES				–	–
PLAQUE TYPE	CLEAR	BULLSEYE	BULLSEYE		BULLSEYE	TURBID
LYSES MUTANTS RESISTANT TO 18 AND 21	YES	NO	NO		YES	YES
ACTIVITY ON STRAIN NO.						
371	–	T	–	C	C	–
379	–	T	T	T	T	–
386	–	T	–	–	T	–
396	C	C	C	–	T	–
420	–	C	C	T	C	–
2835	–	T	T	T	T	–

(–) = NO LYSIS; (T) = TURBID LYSIS; (C) = CLEAR LYSIS

FIG. 4. *Classification of 12 phages for Clostridium sporogenes.*

The production of bacteriocin-like substances may be fairly common in this species. Of the 25 cultures tested, 9 produced bacteriocins and 20 were susceptible to one or more of these. Because of this incompatibility between certain strains, consideration should be given to which cultures are grown together in the mixed-culture technique for phage isolation. For example, strain 416 killed many phage-sensitive strains but was resistant to all the phages in our possession. In the mixed-culture technique, such an organism could kill a phage-sensitive strain before any phage enrichment could occur, thus rendering a time-consuming isolation procedure fruitless from the beginning.

Figure 4 gives a summary of data which are presented as a classification scheme for the phages. The phages are divided into three groups. The ten phages placed in group I were serologically related to each other, but not to phages M and K. These latter two phages differed from each other in their plaque morphology, host range, and degree of virulence. In group I, the two phages 1 and 14, of subgroup A, differed from the others in plaque morphology, cell receptor site used, and host range. They were identical to each other in all characteristics tested and in routine handling, and may represent two isolations of the same phage. Similarly, the six phages of subgroup B did not show any differences among themselves and are also probably six isolations of the same phage. The two phages of subgroup C were very similar to the phages of subgroup B and to each other. They could be distinguished from each other and from the subgroup B phages by their host ranges.

The heat sensitivities of four selected phages from group I were correlated with their grouping in this scheme. Phages 18, S2, and F1, which had similar plaque morphology, receptor sites, and host range, were rather resistant to thermal inactivation at 60 C. Phage 1, which gave different plaques, did not use the same receptor site, and was more restricted in host range than these phages, was also much more sensitive to thermal inactivation.

All of the phages were generally similar to most phages for aerobic bacteria with which the authors have had experience. The most notable difference was the strict requirement for anaerobic conditions in demonstrating or assaying the phages by their growth on susceptible cells. Further studies on the interactions of these phages with their host cells have been undertaken.

ACKNOWLEDGMENTS

The authors wish to thank L. S. McClung of Indiana University for his generous gifts of phage and bacterial stocks, and for sharing with us his great experience in dealing with anaerobic bacteria. John L. Worden of St. Bonaventure University, Francis W. Liegey of State Teachers College, Indiana, Pa., and Richard L. Sames of Bellarmine College, Louisville, Ky., also gave much helpful criticism and advice. One of us (J.V.B.) was greatly aided by predoctoral fellowships granted him by the National Science Foundation from June 1960 to August 1962.

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