ISOLATION AND PRELIMINARY CHARACTERIZATION OF BACTERIOPHAGES FOR *Bacillus subtilis*

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Received for publication February 13, 1961

ABSTRACT

Romig, W. R. (University of California, Los Angeles), AND A. M. Brodetsky. Isolation and preliminary characterization of bacteriophages for *Bacillus subtilis*. J. Bacteriol. 82:135–141. 1961.—A simplified procedure for direct isolation of phages for *Bacillus subtilis* from soil was developed. Phage enrichment was accomplished by growing streptomycin-resistant *B. subtilis* in medium previously inoculated with the soil sample. Contaminating soil bacteria were eliminated by adding bactericidal quantities of streptomycin and the phages were isolated by conventional agar layer techniques. By this method 1 or more subtilis phages were isolated from 15 of 18 soil samples tested. Several of these phages were unusually sensitive to chloroform and all of them were relatively unstable when stored at refrigerator temperatures. Of 6 phages retained for study, 1 was temperate for *B. subtilis*, but attempts to obtain stably lysogenic bacteria following infection with this phage were unsuccessful. All 6 phages had identical host ranges and were able to lyse all strains of *B. subtilis* tested, as well as several related species of *Bacillus*.

Although several investigators have published reports on bacteriophages for *Bacillus subtilis*, to date no comparative studies have been made on this group of bacterial viruses. Wahl and Lewi (1939) described methods for improving the antigenicity of subtilis phages; Wollman and Lacasagne (1940) made some size determinations on these bacteriophages using X irradiation; and Wahl (1946) studied the effect of visible light on subtilis phages. In addition, a subtilis phage was included in a study of the effects of antiseptics on viruses by Nicolle and Mimica (1947); and one of them was measured by electron microscopy by Giuntini et al. (1947). But none of these studies was directed toward the characterization of these bacterial viruses in a manner similar to the well studied T group of coliphages.

Recent discoveries have made it likely that *B. subtilis* will become increasingly useful for investigating genetic phenomena in bacteria. Spizizen (1958, 1959) demonstrated the deoxyribonucleic acid (DNA)-mediated transformation of biochemical requirements in *B. subtilis*, and his conclusions have since been confirmed in several other laboratories (e.g., Ephrati-Elizur and Zamenhof, 1959; Schaeffer, Ionesco, and Jacob, 1959). In addition, Kohiyama (1959) has reported the occurrence of a type of sexual recombination in this species. Since phages have proved quite valuable in a variety of genetic studies with bacteria, it was thought that a better knowledge of those capable of utilizing *B. subtilis* as host should enhance its value in studies of this nature. Accordingly we have devised a simple method for isolating phages active against various strains of this organism, and report some of the preliminary steps in their characterization.

MATERIALS AND METHODS

Bacteria. The bacteria used in this study and their sources are listed in Table 1. The streptomycin-resistant strains were selected as spontaneous one-step mutants to 1,000 µg per ml of streptomycin sulfate, which was either included in agar plates or added to heavy broth suspensions of the sensitive bacteria. The thymine-requiring auxotroph of ATCC 6633 has been described by Brabander and Romig (1960).

Media. The liquid medium used for growing the bacteria and for phage dilutions was similar to the L broth described by Lennox (1955). It is referred to as TY broth and contained 10 g tryptone (Difco); 5 g yeast extract (Difco); 10 g NaCl; and 1,000 ml deionized water. The pH was adjusted to 7.4 with NaOH before autoclaving; CaCl₂ (2.5 × 10⁻³ M) and MnCl₂ (10⁻⁵ M) were
TABLE 1. Source of species and strains of Bacillus and their susceptibility to phages isolated against the Marburg strain of Bacillus subtilis

<table>
<thead>
<tr>
<th>Groups, species, and strains of Bacillus</th>
<th>Source of culture</th>
<th>Lytic activity of phages*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. subtilis</strong> strains and mutants:</td>
<td>UCLA, Dept. of Bacteriology</td>
<td>++++++ + +</td>
</tr>
<tr>
<td>2C ....................................</td>
<td>C. Yanofsky, Stanford University</td>
<td>+++++ + +</td>
</tr>
<tr>
<td>Marburg (wild type) ..........................</td>
<td>C. Yanofsky, Stanford University</td>
<td>+++++ + +</td>
</tr>
<tr>
<td>168 (indole requiring Marburg) ..............</td>
<td>ATCC 6633 mutant</td>
<td>+++++ + +</td>
</tr>
<tr>
<td>23 (threonine requiring Marburg) ..........</td>
<td>ATCC 9372</td>
<td>+++++ + +</td>
</tr>
<tr>
<td>Thy-1 (thymine requiring) .................</td>
<td>College of Osteopathic Physicians and Surgeons, Los Angeles</td>
<td>+++++ + +</td>
</tr>
<tr>
<td>var. niger (black pigment not formed on L agar)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>var. niger (black pigment formed on L agar)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other Bacillus species of group I:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. cereus var. mycoides ..................</td>
<td>UCLA, Dept. of Bacteriology</td>
<td>+++++ + +</td>
</tr>
<tr>
<td>B. cereus var. alesti .....................</td>
<td>Hannay, Dept. of Agriculture, Canada</td>
<td>+++++ + +</td>
</tr>
<tr>
<td>B. firmus ................................</td>
<td>ATCC 8257</td>
<td>+++++ + +</td>
</tr>
<tr>
<td>B. megaterium ..........................</td>
<td>UCLA, Dept. of Bacteriology</td>
<td>+++++ + +</td>
</tr>
<tr>
<td>B. anthracis (2 strains; virulent and avirulent)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. thuringiensis var. sotto ..............</td>
<td>UCLA, Dept. of Bacteriology</td>
<td>+++++ + +</td>
</tr>
<tr>
<td>B. licheniformis ........................</td>
<td>Hannay, Dept. of Agriculture, Canada</td>
<td>+++++ + +</td>
</tr>
<tr>
<td>Bacillus species of group II:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. brevis ................................</td>
<td>UCLA, Dept. of Bacteriology</td>
<td>+++++ + +</td>
</tr>
<tr>
<td>B. polychromogenes ........................</td>
<td>Hannay, Dept. of Bacteriology</td>
<td>+++++ + +</td>
</tr>
<tr>
<td>B. licheniformis ................................</td>
<td>ATCC 6598</td>
<td>+++++ + +</td>
</tr>
<tr>
<td>Bacillus species of group III:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. sphaericus ..........................</td>
<td>ATCC 10206</td>
<td>+++++ + +</td>
</tr>
<tr>
<td>Miscellaneous Bacillus species</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. pumilus ................................</td>
<td>UCLA, Dept. of Bacteriology</td>
<td>+++++ + +</td>
</tr>
<tr>
<td>B. graveolens ................................</td>
<td>UCLA, Dept. of Bacteriology</td>
<td>+++++ + +</td>
</tr>
</tbody>
</table>

* (+) Indicates lysis; (-) indicates lack of lysis by phage.

added after sterilization. TY broth was solidified with 1.5% agar (TY agar) when used as basal layers for phage assays and other plating procedures; for soft agar, 0.7% agar was added. When used, streptomycin was added aseptically from stock solutions containing 5 mg per ml.

**Isolation of phage.** The phages reported here were selected from a group isolated from soil using essentially the method of Ivanovics and Lantos (1958). These phages were selected specifically for their ability to lyse the Marburg strain of *B. subtilis* and various of its auxotrophic mutants because it is this strain with which transformation reactions have been demonstrated. Unless otherwise stated, this organism was used as host for all phage experiments.

In the isolation procedure, 5 g of soil collected from grassy areas were suspended by vigorous shaking in about 15 ml of tap water and the mixture stored at room temperature for 2 days. At the end of this period the suspension was again thoroughly shaken and allowed to settle for 30 min. Five milliliters of the clearer top layer were then added to 5 ml of TY broth contained in a tube (22 by 200 mm) and incubated in a slanted position on a reciprocal shaker for 4 hr at 37 C. This preliminary incubation of the soil suspension allowed any lysogenic spore formers that

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might be present to germinate and subsequently release phage during their growth. Five milliliters of a 6 to 8 hr streptomycin-resistant culture of the appropriate strain of *B. subtilis* and a quantity of streptomycin solution sufficient to give a final concentration of 50 µg per ml were then added to the tube and incubation was continued for an additional 6 hr.

At the end of the final incubation period, the phage-enriched cultures were diluted directly in 10-fold steps and assayed by the agar layer technique (Adams, 1950), using the original streptomycin-resistant *B. subtilis* as indicator. In most instances well isolated plaques were obtained at dilutions from $10^{-3}$ to $10^{-4}$ depending upon the soil sample used. Plaques with different morphology were purified by at least three successive single plaque isolations. Stocks of each of the phage isolates were prepared by the plate lysis technique (Adams, 1959).

*Host range determinations.* The bacteria used in the host range studies were incubated in TY broth at 37 C on the reciprocal shaker until a cell concentration corresponding to an optical density of 0.26 to 0.27 at 600 mµ was obtained. This optical density represents a viable cell concentration of about $5 \times 10^7$ per ml. Four drops of this standard culture were added to 2.5 ml of soft agar and the mixture was poured onto previously prepared TY agar plates. Phage suspensions containing $10^9$ plaque-forming units per ml were spotted onto the surface of these indicator plates with a standard loop. After overnight incubation at 37 C, the plates were examined for the presence or absence of lysed areas in the lawn of confluent bacterial growth. A phage was considered to possess lytic activity against the test organism if the spotted area showed a well defined margin on its circumference, with the central area exhibiting either complete lysis or lysis with varying degrees of turbidity due to growth of resistant bacteria.

**RESULTS**

From a total of 18 soil samples, 15 have yielded at least 1 phage, and from several 3 different phages, as judged by plaque morphology, have been recovered. No major differences have been noted in the efficiency of phage isolation or in the host range of the phages recovered, regardless of the bacterial strains used in the primary isolation procedure. Streptomycin-resistant mutants (2C, 23, and 168) of *B. subtilis* have been used for this purpose.

The *subtilis* phages appear to be much less stable than many of those attacking other species of bacteria. Several were entirely inactive after storage at 5 C for 3 weeks. In addition, some were inactivated when sterilization of lysates by shaking with chloroform was attempted. Although most of these phages were relatively unaffected by chloroform, all subsequent lysates were sterilized by passage through UF sintered glass or membrane filters. The instability of this group of phages may be an inherent feature of the phages themselves, or may simply reflect the fact that optimal conditions for their preservation have not been found.

Six of the phages isolated from soil have proved more stable than the others, and it is these that were retained for further study. They have been designated SP, for *subtilis* phage, and numbered consecutively in the order of isolation. Those included in this study are SP5, SP6, SP7, SP8, SP9, and SP13.

**Preliminary classification of the phages.** The phages were first grouped on the basis of plaque morphology using the wild-type *B. subtilis* as host. During this process special precautions were taken to control plating conditions, adsorption times, incubation temperatures, and other factors known to influence plaque morphology.

SP5, SP7, SP8, and SP9 produced plaques that were very similar in appearance, having clear centers and well defined edges. Two sizes were seen; the larger varying from 1.0 to 1.2 mm in diameter, the smaller approximately one-half that size. An example of the plaques produced by these four phages is presented in Fig. 1 in which the plaque morphology of SP5 is shown.

Phage SP6 produced turbid plaques about 1 mm in diameter with small, clear centers and a wide heavy turbid halo separated from the main lawn of bacterial growth by a narrow line of clearing. Among the turbid plaques a small number of clear plaques was always found, some of which produced an effect resembling lysis inhibition, as shown in Fig. 2. The turbid plaques are shown in greater detail in Fig. 3. The phages that produce clear plaques, shown in Fig. 4, have never been observed to revert to the original turbid state.
FIG. 1 TO 5. Plaques formed by various subtilis phages on Bacillus subtilis Marburg strain.

FIG. 1. Plaques formed by SP5 on medium with added MnCl2. X 5.

FIG. 2. Plaques of SP6, effect resembling lysis inhibition. X 4.

FIG. 3. Turbid plaques formed by SP6. X 5.

FIG. 4. Clear plaques formed by SP8. X 5.

FIG. 5. Plaques formed by SP5 without added MnCl2. X 5.
The plaques produced by SP13 were about 1 mm in diameter and were almost completely turbid upon initial isolation. They have been observed to occasionally produce plaques with a clear center, a turbid halo, and a narrow line of clearing similar to SP6. Since the turbid plaques produced by this phage strongly resembled those formed by temperate phages of other bacterial species, the possibility that SP13 was also temperate was investigated.

Tests on lysogenizing ability of SP13. Young broth cultures of the wild-type *B. subtilis* were infected with SP13 at an input multiplicity of five phages per bacterium. After 15 min adsorption at 37 C, soft agar was added to samples of the infected bacteria and the mixture was poured onto TY agar plates. A heavy background growth developed on these plates after 24 hr incubation at 37 C which was presumed to consist of surviving lysogenized bacteria. They were again incubated until most of the bacteria had formed endospores. The spores were collected and washed five times with phosphate buffer (0.06 [M]) to reduce the free phage concentration. The washed spores were then heated in buffer at 80 C for 30 min to inactivate the remaining exogenous phages. Control experiments showed that SP13 lysates were completely inactivated within 15 min at this temperature, as was the plaque-forming ability of artificially prepared mixtures of free phages and normal spores. It was therefore concluded that the heating procedure efficiently eliminated free phages from the spore suspensions, so that any phages recovered from heated spore preparations must have been included within the spore.

The heated spores were diluted and spread with glass rods on TY agar to give well isolated colonies after overnight incubation. These were transferred by the replica plating technique (Lederberg and Lederberg, 1952) onto the surface of TY agar plates overlaid with a suspension of *B. subtilis* in soft agar. About 50% of the colonies that appeared on the confluent lawn of indicator bacteria were surrounded with a turbid halo. Bacteria from the corresponding colonies on the master plates produced phages when grown in liquid medium and were not lysed when SP13 suspensions were spotted onto indicator plates prepared from such cultures. Since these responses are typical of lysogenic bacteria, SP13 was concluded to be temperate.

Attempts to maintain pure cultures of these lysogenized bacteria revealed, however, that they rapidly lost the properties of hereditary phage production and immunity to superinfection. This fact was demonstrated as follows: The lysogenic cultures derived from heated spores were streaked onto TY agar and 40 to 50 randomly chosen colonies were transferred to TY broth and incubated for 4 to 6 hr. Less than half of these subcultures retained the ability to produce lysis when they were spotted onto indicator plates of the original wild-type bacterium. Such cultures were also shown to have lost their immunity to the lytic activity of SP13. Despite repeated single colony isolations from the cultures that retained their phage producing capacity, it was not possible to obtain a stable lysogenic culture of *B. subtilis* by this method.

In other experiments, bacterial survivors picked from the center of turbid plaques formed by SP13 were isolated on TY agar and a colony that proved able to produce phage was inoculated into broth containing phage antiserum (K = 140). Samples of this broth culture were removed at various intervals during growth, suitably diluted, and plated onto TY agar. The colonies that arose were tested for phage production, and those retaining this ability were scored as lysogenic. It was found that although lysogenic bacteria persisted for as many as three serial transfers in serum-containing broth, the ratio of lysogenic to sensitive bacteria decreased very rapidly.

Host range of the phage. A total of 23 strains was tested by the spotting method for susceptibility to the 6 phages. The results are presented in Table 1. The bacteria used here are divided into the following categories: the 7 strains and mutants of *B. subtilis*; the other species of *Bacillus* of group I according to the classification of this genus in *Bergey's Manual of Determinative Bacteriology* (Breed, Murray, and Smith, 1957); the species in groups II and III of the above classification; and 2 miscellaneous species. The 2 miscellaneous species are so indicated because Smith, Gordon, and Clark (1952) identified 2 cultures named *B. pumilus* as *B. subtilis*, and they placed 2 of 3 cultures labeled *B. graveolens* in the synonymy of *B. megaterium* and the other in that of *B. subtilis*.

From these data it may be seen that all of the subtilis phages thus far tested are either uniformly active or uniformly inactive on a given bacterial species. In no case have we isolated a
phage possessing unique host range characteristics.

Addition of MnCl₂. McCloy (1958) noted that addition of MnCl₂ to the medium used for assaying phages for B. cereus influenced plaque formation and promoted better growth of the indicator bacteria. Huybers (1953) showed that development of phage in lysogenic B. megaterium does not occur after ultraviolet induction in media deficient in manganese. The effect of added manganese was clearly evident with our phages and the indicator bacteria used. Assays of all 6 phages on media with and without added MnCl₂ disclosed that in the absence of this compound the plaques were smaller, less well defined, and in some instances so faint as to be scarcely discernible. This effect is not due to lack of adsorption of the phage to the indicator bacteria in the absence of MnCl₂, but seems to be a function of the growth of the bacteria. Fig. 5 shows plaques formed by SP5 under conditions of MnCl₂ deficiency, and should be compared with Fig. 1, which shows plaques formed in the presence of MnCl₂.

DISCUSSION

The method described above for the isolation of phages for B. subtilis from soil has proved to be a relatively simple procedure. The soil from most grassy areas contained one or more phages active against this organism, and this probably reflects the fact that B. subtilis itself can be readily isolated from many grasses.

The uniformity of the host ranges of these phages was an unexpected feature of this investigation. Their common host range might be due to the method of isolation and selection, or to some other cause such as the manner in which phage receptor sites are gained or lost by the bacilli.

It is conceivable that despite the fact that the phages were isolated from different soil samples, certain ecological factors in this area permit the recovery of only a single phage "species." If such was the case one would have to further assume that the differences observed in plaque morphology among our phages arises from the fact that the isolates exhibiting different properties represent stable mutants of the hypothetical single phage species. This interpretation is rendered unlikely on the basis of some qualitative experiments on the inactivation of different phage isolates with the antiserum prepared against a single isolate. From work thus far accomplished, there seem to be at least two unrelated groups among our SP phages.

Experiments in which mutants resistant to virulent phages (e.g., SP5) were used showed that bacteria that became resistant to one phage simultaneously gained resistance to the others. In addition, several of these resistant mutants when cultivated in the absence of phage for several transfers, lost their resistance to lysis at a rate similar to the rate of prophage loss from bacteria infected with SP13. These observations suggest that phage attachment in B. subtilis is much less specific than in the Enterobacteriaceae, but at present not enough data have been collected to adequately test this hypothesis.

The host range of the phages shows a fair correlation with the degree of relatedness of the members of the genus Bacillus as outlined in Smith, Gordon and Clark's (1952) classification in that those species closely related to B. subtilis on morphological and physiological grounds are generally susceptible to the subtilis phages, whereas the more distantly related species are generally resistant to these phages.

The inability to obtain stably lysogenic strains of B. subtilis cannot be explained at the present time. Some other observations with different Bacillus phages indicate the phenomenon of prophage loss which we have described is not an isolated case. Lantos, Varga, and Ivanovics (1960) reported a similar phenomenon of rapid loss of prophage from B. anthracis lysogenized with phage A5. They speculated that the loss of prophage from this bacterium might be due to an induction brought about by heating the spores of lysogenic bacteria. A similar explanation would satisfy the results we obtained with heated spore preparations, but would not explain the loss of prophage from cultures picked from turbid plaques and purified on agar plates. In B. megaterium, also, the prophage attachment seems to be more easily disturbed than in the enteric bacteria. De Carlo, Sarles, and Knight (1953) reported that lysogenic cultures of B. megaterium reverted to the sensitive state when grown in medium containing 1.5% glucose. Loss of lysogenicity and complete reversion to the sensitive state was also reported to occur when these bacteria were cultivated serially in medium devoid of calcium (Clarke, 1952), or in medium containing oxalate (Lwoff, 1953). All of the preceding observations are compatible with the conclusion that loss of
phage determinants from members of the genus *Bacillus* occurs more readily than in other lysogenic bacteria, but do not offer a clue to explain the nature of the phenomenon.

ACKNOWLEDGMENTS

This study was aided by grants from the Faculty Research Committee and the Cancer Research Coordinating Committee, University of California, Los Angeles.

The authors wish to thank those who provided α3 with the cultures used in this study and to express our appreciation to R. Epstein and M. J. Pickett for helpful criticisms during preparation of the manuscript. Some of the phage isolations were performed by J. J. Pene and by Mary Ellen Riepe.

LITERATURE CITED


