A BACTERIOPHAGE FOR B. ANTHRACIS

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There occur in the literature of bacteriophagy several descriptions, such as that by Pesch (1924), of pseudolytic reactions with the anthrax bacillus, but to the writer's knowledge no true bacteriophage active against this organism has ever been obtained. It is the object of this paper to report some brief studies of a lytic filtrate which, in all of its manifestations, displays the characteristics of the bacteriophage as we know it.

The following anthrax cultures were used in the investigation:

Strasbourg—obtained from Professor L. F. Rettger.

Thomas—freshly isolated from a case of malignant pustule in a tannery employee at Peabody, Mass., and obtained through the courtesy of Dr. J. E. Sullivan.

American Type Culture strains Nos. 7, 8, 9, 10, 240, 241, 242, 937, 938, 4229, 4230. Atypical Cultures Nos. 10 and 242.

All cultures were grown in alkaline extract broth of pH 7.8, or on nutrient extract agar of pH 7.0, and all were incubated at 30°C. L_3 and L_5 Chamberland candles were used for filtrations.

As a source of bacteriophage, there was used crude sewage, filtered through paper only and enriched in 100 cc. quantities with the proper amount of beef extract and peptone. To this mixture was added the growth from an eighteen-hour agar slant culture of the Strasbourg strain,—the strain which was routinely used throughout these experiments. After overnight incubation the mixed culture was filtered and the usual process of alternate feeding and filtration was pursued. One-tenth of a cubic centimeter of the third-passage filtrate exhibited some ability to clear broth cultures, and, on agar plates heavily seeded with culture,

formed numerous tiny plaques of partial lysis. After ten passages the filtrate had a titer of 2×10^{-9} as determined by plaque counts, and caused complete lysis of broth cultures in three to four hours.

The lytic principle thus obtained was highly active against the Thomas strain of *B. anthracis* as well as against all of the American

TABLE 1

Characteristics of anthrax strains used

STRAIN	MOTILITY	GELATIN LIQUEFACTION	PINE-TREE GROWTH	PATHOGENICITY FOR MICE*	SUSCEPTIBILITY TO ANTHRAX BACTERIOPHAGE	SUSCEPTIBILITY TO ATYPICAL ANTHRAX BACTERIOPHAGE
Strasbourg	−	−	+	+	+	−
Thomas	−	−	+	+	+	−
A. T. C. C. 7	+	−	+	−	−	−
A. T. C. C. 8	−	−	+	−	+	−
A. T. C. C. 9	−	−	+	−	+	−
A. T. C. C. 10	−	−	+	+	+	−
A. T. C. C. 240	−	−	+	+	+	−
A. T. C. C. 241	−	−	+	−	+	−
A. T. C. C. 242	−	−	+	+	+	−
A. T. C. C. 937	−	−	+	+	+	−
A. T. C. C. 938	−	−	+	+	+	−
A. T. C. C. 4229	−	−	+	+	+	−
A. T. C. C. 4230	+	−	+	−	−	−
A. T. C. C. atypical 10	+	+	−	−	−	+
A. T. C. C. atypical 242	+	+	−	−	−	+

* The mice were injected at the root of the tail with 0.1 and 0.2 cc. amounts of eighteen-hour broth cultures. A strain was considered non-pathogenic if mice failed to succumb to it within five days.

Type Culture strains with the exceptions of nos. 7 and 4230. These two strains, however, showed active motility in hanging-drop preparations, so that their authenticity is open to question.

It seems pertinent to include here the following observations. Before the complete series of Type Culture strains had been obtained, the bacteriophage was tried against two cultures which had come to us through two other laboratories and were supposed

to be strains 10 and 242. The two strains were completely resistant, and as a result another bacteriophage active against them was obtained by the method described above. This second lytic principle, however, had no effect on any of the other thirteen cultures of *B. anthracis*. A study of the two strains showed that the organisms were motile and liquefied gelatin rapidly, while 0.2 cc. amounts of twenty-four-hour broth cultures failed to kill mice in five days. In these respects the cultures were similar to *B. cereus*. The same strains obtained from the Type Culture Collection were typical *B. anthracis*. Whether the two atypical strains were contaminants or had undergone an extreme dissociation is open to question, but the fact that strains 7 and 4230 also showed motility when obtained directly from the Collection suggests that the latter possibility should receive consideration. Nungester (1929) has described the dissociation of *B. anthracis* in detail, but none of his variants were motile. Haag (1927), however, believes that motility is possible under some circumstances. Incidentally, it may be stated that the bacteriophage for the atypical strains 10 and 242 was active against *B. mycoides* as well as against an atypical culture of *B. mesentericus*. The true anthrax bacteriophage was active against the latter form also. *B. cereus* was susceptible to neither principle.

THERMOLABILITY OF THE ANTHRAX BACTERIOPHAGE

In contrast to many other lytic filtrates which can, as d'Herelle (1926) has shown, withstand temperatures up to 72° for half an hour and more, this particular principle is killed or inactivated by heating at 60° for ten minutes, although it survives 55°. On numerous occasions such treatment has been followed by as many as six serial passages against sensitive cells without the appearance of any regeneration in lytic power as evidenced by action in broth or by plaque formation.

SECONDARY GROWTH AND RESISTANCE TO LYTIC ACTION

No secondary growth has been observed in bacteriophage suspensions filtered through L_3 or L_5 Chamberland candles even

when these filtrates have stood at room temperature for over three months. In broth cultures which have undergone apparently complete lysis, secondary growth may appear. Such growth, however, seems to bear a definite relation to the number of organisms present in the original suspension as is shown by the following experiment, typical of numerous others that have been made.

Duplicate dilutions of organisms were made by adding 0.5 cc. of an eighteen-hour broth culture of *B. anthracis* to 4.5 cc. of broth and making 0.5 cc. transfers through ten tubes. One-tenth cubic centimeter of potent bacteriophage was then added to each tube of one series, while the other series served as a control. In the following table the numbers indicate the days on which turbidity was first observed. At the end of three weeks no further change was seen.

TABLE 2

	DILUTION									
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
B. anthracis	1	1	1	1	1	1	1	1	–	–
B. anthracis + Bp	1	6	–	–	–	–	–	–	–	–

In another experiment the dilutions were divided into four equal parts, each of which was added to a tube of broth. One-tenth cubic centimeter of bacteriophage was added to all of the tubes except the controls. Three of the portions from the 10^{-1} dilution containing bacteriophage showed secondary growth after one day. The fourth portion became turbid on the tenth day, but all of the other tubes were clear at the end of three weeks. The controls through high dilutions showed growth after one day.

These results suggest that this particular strain of *B. anthracis* may contain a very small percentage of cells resistant to lytic action, and that it is from these that secondary growth develops.

When secondary growth is subcultured in series on agar slants, the appearance in eighteen hours does not suggest the presence of any lytic agent. After further incubation, however, the culture tends to become glassy, and in these glassy areas further

growth develops. This behavior has been observed through a series of eight agar-slant cultures and, from the eighth, a potent bacteriophage was recovered, as was to be expected.

Such secondary growth produces abundant spores. If these spores are heated at 80° for ten minutes to inactivate the bacteriophage and to kill the vegetative forms, the resulting growth on agar is similar to that of normal cultures, but the organisms are resistant to the lytic action. One of these resistant strains has been carried through four cycles in which spores have been allowed to develop on agar, have undergone heating at 80° for ten minutes, and have then been allowed to germinate in broth. At the end of this series the strain had apparently retained its complete resistance to lysis. It is of interest that such resistance is carried through the spore stage and repeated heating, which indicates a rather deep-seated change in the characteristics of the strain. There seems to be no marked relationship between this resistance to bacteriophage action and virulence for mice as 0.1 cc. amounts of 18-hour broth cultures of either the susceptible or the resistant strain were able to kill in from twenty to thirty hours. The strains recovered from the mice at autopsy had retained their specific behavior to the lytic principle.

The value of the bacteriophage in immunization against anthrax as well as in therapy is under investigation.

THE VALUE OF SEWAGE AS A SOURCE OF LYTIC PRINCIPLES

Numerous investigators have made use of sewage in obtaining bacteriophages. However, in view of the success which workers in this laboratory have had in utilizing this source for lytic filtrates, it may be well to reëmphasize its value for such a purpose. Aside from bacteriophages for such common organisms as *B. coli*, principles have been secured active against the following species: *C. diphtheriae*, *Bact. pneumoniae*, *B. megatherium*, *B. petasites*, *B. tumescens*, *B. anthracis*, *B. mycoides*, and several other spore-forming bacilli of doubtful classification.

SUMMARY

A bacteriophage highly potent for *B. anthracis* has been obtained from sewage. All of the typical anthrax strains studied, eleven in number, were susceptible to its action.

It is possible to develop resistant strains of *B. anthracis* in which the resistance is carried through the spore stage and maintained through repeated pasteurizations.

The occurrence of secondary growth with the strain of *B. anthracis* used in these experiments, at least, seems to be dependent upon the number of organisms subjected to the lytic principle.

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