THE ACTION OF PASTEURELLA PESTIS BACTERIOPHAGE ON STRAINS OF PASTEURELLA, SALMONELLA, AND SHIGELLA

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Bacteriophages active against *Pasteurella pestis* have been described by a number of workers, including Flu (1927), Sugino (1932), Advier (1933), and Girard (1942). The subject has been reviewed by Harvey (1933) and Wu *et al.* (1936). This report describes certain relationships of *P. pestis* to *Pasteurella pseudotuberculosis* and to *Salmonella* and *Shigella* strains, as shown by bacteriophage action and confirmed to a limited extent by agglutination tests.

BACTERIOPHAGE EXPERIMENTS

Materials. The strain of phage used in this study was obtained from the Pasteur Institute of Dakar, Senegal, in 1945. It was originally isolated from a positive blood culture prepared from a clinical case of bubonic plague (Advier, 1933). The phage was passed several times in contact with liquid and solid cultures of *P. pestis*, avirulent strain A1122 (Jawetz and Meyer, 1943), using single plaque procedures as well as lysis in broth. A batch of phage, sufficient for the present study, was prepared in a meat infusion broth. This phage will be referred to throughout this paper as the "parent strain." At a later date, to rule out the possibility of contaminants, the phage was further purified by picking isolated plaques to broth and incubating in the presence of *P. pestis* A1122. This procedure was repeated six times. The resulting phage was considered to be free from any contaminants and is referred to below as the "purified phage."

The standard medium used was Difco proteose no. 3 agar. A liquid medium was prepared using the same formula without agar.

Methods. Standard agar plates, dried for several days at 37 C, were spread with from 0.1 to 0.25 ml of a young broth culture of the strain under investigation. The plates were held at room temperature until the liquid was completely absorbed, usually 5 to 10 minutes. The phage was then applied to marked areas, using a standard platinum loop having an inside diameter of 3.0 mm. Care was taken to avoid excessive disturbance of the film of organisms on the surface of the agar. It was possible to apply up to nine such phage areas on a single plate. The plates were incubated at 37 C after the phage was absorbed sufficiently to permit inversion.

All cultures found susceptible to lysis by the parent strain of phage, or by any phage adapted from the parent strain, were tested for lysogenic ability. The methods of Fisk (1942) were used for unmasking lysogenic strains. Except as otherwise noted, no evidence of lysogenesis was found.

Results

P. pestis. Twelve strains of human and animal origin were studied. Ten of these strains were avirulent for animals, two were fully virulent. All strains tested were susceptible to the parent strain of phage. Advier (1933), using the same phage, found that all of 47 strains of *P. pestis* were lysed; therefore no effort was made to test additional cultures. The properties of the parent phage were as follows:

On agar plates, a phage dilution of 10^{-6} gave an area of confluent lysis with all strains after 24 hours at 37 C. Higher dilutions, up to 10^{-8}, showed isolated plaques. All strains were similar in susceptibility, the only variations being within the limits of experimental procedures.

In liquid media, all strains were lysed in contact with a 10^{-8} dilution of the parent phage after 24 hours at 37 C.

On solid media, isolated plaques were about 3 mm in diameter in 24 hours. Plaques continued to enlarge with prolonged incubation at 37 C, or at room temperature, until the entire plate might be cleared.

No secondary growth was observed in liquid or solid media after lysis of normal strains of *P. pestis.* Lysates were sterile on subculture. On three occasions phage-resistant strains were obtained by passing cultures daily in broth for a number of transfers and then exposing the organisms to undiluted phage on agar plates. The phage-resistant strains showed no envelope substance microscopically, formed tiny (0.1 mm in diameter) colonies, produced a very granular growth in broth, and died out in a few months on storage at +4 C on blood agar slants. Advier (1933) reported no secondary growth following lysis of 47 strains of *P. pestis* using the same phage.

The thermal death range of the parent strain of phage was 61 to 63 C in 30 minutes.

The minimum time required for lysis was 40 minutes, when a broth culture was used showing turbidity barely visible to the eye (approximately 5×10^7 bacteria per ml) and when undiluted phage was added in an amount equal to 2 per cent of the volume of the culture. Older cultures and smaller amounts of phage prolonged the time required for lysis. On agar plates, plaques were visible within 2 hours after the addition of phage to a plate spread with 0.15 ml of a 24-hour culture of *P. pestis.*

The stability of the parent phage was marked. No appreciable loss in titer against the homologous strain of *P. pestis* was observed after 2 months at room temperature. Dilutions prepared in broth and stored at +10 C showed no loss in potency after 2 months.

P. pseudotuberculosis. Sugino (1932) reports absence of lytic action of *P. pestis* phage in contact with *P. pseudotuberculosis.* Advier (1933) made similar observations on three strains of *P. pseudotuberculosis,* using the same *P. pestis* phage employed in the present study. On the other hand, Girard (1942) reports a *P. pestis* phage able to lyse some strains of *P. pseudotuberculosis* to the same titer as *P. pseudotuberculosis* phage.

In the present study, 27 strains of *P. pseudotuberculosis*, at least 3 of which were from human cases, were investigated for sensitivity to the parent and purified *P. pestis* phages. All cultures were further examined for susceptibility to an adapted phage produced from a single plaque. The plaque was picked

TABLE 1

Lysis of P. pseudotuberculosis strains by phage

(Highest dilution showing confluent lysis on plates)

P. PSEUDOTUBERCULOSIS STRAIN	PURIFIED P. PESTIS PHAGE	P. PESTIS PHAGE ADAPTED TO P. PSEUDOTUBERCULOSIS
NIH 8-295	10^{-1}	10^{-3}
NIH K-1	10^{-3} R	10^{-5}
NIH B-111	10^{-4}	10^{-6}
NIH B-519	10^{-4}	10^{-6}
NIH 274	–	10^{-3}
Berk. C1903	10^{-4}	10^{-6}
Berk. D1774	–	10^{-5}
Berk. IRL	10^{-3} R	10^{-4} R
NCTC 1102	10^{-4}	10^{-6}
NCTC 2200	±R	10^{-6}
NCTC 2476	10^{-1} R	10^{-6}
NCTC 3570	10^{-3} R	10^{-5}
Jawetz 4	–	10^{-1}
Jawetz 5	10^{-1} R	10^{-1} R
S-274	10^{-1} R	10^{-2}
S-275	–	10^{-3}
K. Meyer 1	±R	10^{-6}
Seattle	–	10^{-2}
Spokane	–	10^{-3} R
Hobmaier	10^{-3} R	10^{-6}
New Orleans	–	10^{-5}
San Francisco	10^{-1}	10^{-1}
C. T. Snyder	–	10^{-2} R
Saranac	10^{0} R	10^{-5}
Moss	10^{-5}	10^{-5}
Mason	10^{-1} R	10^{-1} R
Topping	±R	10^{-4}
P. pestis A1122	10^{-6}	10^{-6}

± = isolated plaques, without confluent lysis, using undiluted phage.
– = no lysis with undiluted phage.
R = resistant growth.

from a plate spread with *P. pseudotuberculosis* (Spokane strain) to which had been added undiluted *P. pestis* phage of the parent strain. After 12 passages in contact with broth cultures of *P. pseudotuberculosis* (Spokane), this adapted phage showed a definite increase in ability to lyse most strains of *P. pseudotuberculosis*, without loss of action on *P. pestis*. A comparison of results is shown in table 1.

The phage adapted to *P. pseudotuberculosis* showed the same general properties as the parent strain of *P. pestis* phage, except that an occasional small plaque, about 1 mm in diameter, was noted. It was felt that this discrepancy in plaque size might have indicated a lysogenic strain, but no evidence of lysogenic cultures could be obtained by the use of the methods of Fisk (1942) for unmasking lysogenic strains. It seems reasonable to assume that the results in table 1 were due to adaptation and not to the presence of contaminating phages.

The marked variation in susceptibility of different strains of *P. pseudotuberculosis* to the two phages used is of considerable interest. This point is discussed below.

It was hoped that the differences shown in table 1 might supply a means to differentiate *P. pestis* from *P. pseudotuberculosis*, a problem not always readily solved by cultural methods. Although such a differentiation is possible in most cases, it is felt that the observed reactions are not sufficiently reliable and reproducible to constitute a means of clearly distinguishing between the two organisms. Further studies are being conducted in an effort to establish a reliable method for differentiation.

It was of interest to note that most strains of *P. pseudotuberculosis* showed a definite increase or decrease in sensitivity to phage after prolonged storage on agar slants under oil. The relationship of this altered sensitivity to changes in antigenic structure will be studied further, in an effort to determine whether the sensitivity to *P. pestis* phage might be a quantitative means to measure antigenic relationships between *P. pestis* and *P. pseudotuberculosis*.

P. septica. Eleven strains of *P. septica* isolated from domestic fowl and one strain isolated from a gray squirrel were tested by the plate method for sensitivity to both the parent phage and the phage adapted to *P. pseudotuberculosis*. No evidence of lysis was observed. Five of these 12 cultures carried phage acting on themselves.

Salmonella. Forty-two strains of *Salmonella*, representing 25 species, were tested by the plate method against the parent strain of *P. pestis* phage. Three strains showed lysis, namely, *S. schottmuelleri* (no. 6), *S. hirschfeldii* (58G, no. 31), and *S. rubislaw* (phase I, no. 159). These three strains were lysed only by the undiluted phage, and secondary growth occurred on further incubation. In broth cultures, the addition of phage resulted in partial lysis with resistant growth. Repeated attempts to transfer the phage by serial subculture in broth were unsuccessful.

The 3 strains of *Salmonella* lysed by the parent phage, as well as many of those not lysed, had been maintained on agar for several years. The 3 strains susceptible to the phage were predominantly in the rough phase. At least 12 of the strains that were not affected by the parent strain of *P. pestis* phage were also in the rough phase. Cultures which could not be lysed included 3 strains of *S. schottmuelleri* and 2 of *S. hirschfeldii*. The phage action, therefore, was not related to the age, the species, nor the state of dissociation of the cultures studied.

Shigella. Thirty-seven strains of *Shigella* were tested for sensitivity to both the parent strain and the purified *P. pestis* phage. Included were 26 strains of

S. paradysenteriae, 4 of *S. sonnei*, 3 of *S. dysenteriae*, 2 of *S. ambigua*, 1 of *S. alkalescens*, and 1 of *Shigella* sp., Newcastle type. Clear-cut lysis occurred with 4 strains, namely, *S. paradysenteriae* type 103, no. 12, *S. sonnei* no. 7, *S. dysenteriae* no. 44, and *S. ambigua* no. 33. Partial lysis occurred with *S. sonnei* cultures no. 5 and no. 6. All these 6 cultures showed secondary growth after 8 to 24 hours at 37 C. There was little difference between sensitivity to the parent phage and to the purified strain.

The 6 *Shigella* cultures which showed lysis were old stock cultures with the exception of *S. paradysenteriae* no. 12, which had been isolated from a dysentery case within a year. The *S. sonnei* cultures were all in the rough phase. Those strains of *Shigella* which were not sensitive to the phage included both recently isolated and old stock strains in both the rough and smooth phases. Five cultures of *S. paradysenteriae*, type 103, were not susceptible to the phage.

TABLE 2

Lysis of P. pestis and Shigella by parent phage and adapted phages

(Highest dilution showing confluent lysis on plates)

ORGANISM TESTED	PHAGE				
	Parent strain	Parent strain after 6 to 8 transfers on			
		S. paradysenteriae	*S. sonnei*	*S. shigae*	*S. ambigua*
P. pestis A1122	10^{-6}	10^{-6}	10^{-5}	10^{-5}	10^{-6}
S. paradysenteriae	10^{0}	10^{-3}	10^{-1}	10^{-2}	10^{-1}
S. sonnei	10^{0}	10^{0}	10^{-2}	10^{-1}	10^{0}
S. shigae	10^{-1}	10^{-3}	10^{-2}	10^{-3}	10^{-3}
S. ambigua	10^{-3}	10^{-3}	10^{-2}	10^{-3}	10^{-5}

Hence the parent phage failed to act on all members of a given species or even of a given serologic type.

Transfer of phage in broth cultures of Shigella. The parent strain of *P. pestis* phage was transferred serially on young broth cultures of each of the 4 *Shigella* cultures showing clear-cut lysis on plates. In the first two or three transfers the lysis in broth was slight and secondary growth developed within 4 hours. Later transfers gave complete lysis within 2 hours, and secondary growth was either absent or delayed for at least 18 hours. The phage was transferred in this manner for 25 subcultures on *S. paradysenteriae* no. 6, *S. ambigua* no. 33, and *S. sonnei* no. 7. Several attempts to transfer the phage beyond the ninth subculture on *S. shigae* no. 44 were unsuccessful. The purified strain of *P. pestis* phage was similarly transferred on *S. ambigua* no. 33.

The titer of the phage after transfer on *Shigella* was tested by the plate method and compared with the titer of the parent strain. The results are summarized in table 2.

Table 2 shows a 100-fold to a 1,000-fold increase in the activity of the phage after transfer on a particular *Shigella* species. The activity of the adapted

phage for other sensitive *Shigella* strains sometimes increased, but not to the same degree as for the species on which the transfers were made. There was no increase in titer for *S. ambigua* except when the phage was transferred on that culture. No significant change in the titer for *P. pestis* occurred, which agrees with the results following adaptation of the parent phage to *P. pseudotuberculosis*. Results similar to these were obtained with the purified phage.

The phage apparently became adapted to the *Shigella* cultures so that it lysed them more readily than did the parent strain, but without any alteration of its ability to lyse *P. pestis*. This increased action on *Shigella* reached a maximum after four or five transfers, and subsequent subcultures up to 25 did not enhance its ability to lyse *Shigella* strains. The same observations were made regarding the phage adapted to *P. pseudotuberculosis*, which reached its maximum ability to lyse that organism in a few transfers and did not increase after additional passages.

The 41 cultures of *Shigella* which were not lysed by the parent strain of phage were tested again in contact with phage adapted to susceptible *Shigella* strains. Two additional strains of *S. paradysenteriae* and one of *S. sonnei* were lysed, but other *Shigella* cultures remained resistant.

Three *Salmonella* strains which were susceptible to the parent *P. pestis* phage were tested for sensitivity to the phage adapted to *Shigella*. Sensitivity was the same as for the parent phage.

Fourteen strains of *P. pseudotuberculosis* were tested against the phage adapted to *Shigella*. Sensitivity was essentially the same as for the parent strain of *P. pestis* phage. Adaptation of the parent phage to *P. pseudotuberculosis* did not alter its ability to lyse susceptible *Shigella*.

After transfer on *Shigella*, the adapted phage was again subcultured for six passages on *P. pestis* A1122. There was no change in its activity against either *P. pestis* or susceptible *Shigella* strains.

Transfer of phage on plate cultures of Shigella. Isolated plaques from plate cultures of the parent strain of phage on the different susceptible *Shigella* cultures were transferred for several generations, using the method of picking isolated plaques to broth described above. These "purified" phages were then tested against *P. pestis*. Size and number of the plaques were unchanged, and it was apparent that cultivation of the phage on *Shigella* did not alter its action on *P. pestis* in any observable way.

Both the parent strain and the purified strain of *P. pestis* phage produced plaques varying in size from 0.5 to 4.0 mm on susceptible *Shigella* cultures. The smaller plaques tended to increase in size on further incubation, but not so rapidly as did plaques on *P. pestis*. Small plaques (0.5 to 1.0 mm) and large plaques (3.0 mm or more) were isolated and tested for several generations. On plates with susceptible *Shigella* cultures, neither the large nor small plaques bred true, forming both large and small plaques. On plates of *P. pestis*, both large and small plaques produced uniformly large plaques identical with those produced by the parent strain of phage.

The *S. ambigua* culture was lysogenic for 20 strains of *Shigella*, but not for *Pasteurella*. This phage carried by *S. ambigua* was readily distinguished from *P. pestis* phage because of the minute plaques (0.1 to 0.3 mm) produced on *Shigella*.

Miscellaneous bacteria. The parent strain of *P. pestis* phage was tested by the plate method against 77 cultures, which included 37 species. The following 17 genera were represented: *Aerobacter*, *Alcaligenes*, *Bacillus*, *Chromobacterium*, *Corynebacterium*, *Eberthella*, *Escherichia*, *Gaffkya*, *Klebsiella*, *Micrococcus*, *Neisseria*, *Proteus*, *Pseudomonas*, *Serratia*, *Staphylococcus*, *Streptococcus*, and *Vibrio*. There was no lysis of any of these cultures. Since only one to six strains of each species were tested it cannot be assumed that other strains might not be susceptible to lysis.

SEROLOGICAL EXPERIMENTS

Burnet (1927) found a close correlation between the sensitivity of different bacteria to a phage and the distribution of surface somatic antigens. Numerous observations have supported the belief that sensitivity to phage is closely related to antigenic structure. The subject is well summarized in Topley and Wilson (1946).

In this study an attempt has been made to correlate the sensitivity of certain *Salmonella* and *Shigella* strains to *P. pestis* phage with a demonstrable antigenic relationship as shown by agglutination tests. It was considered unnecessary to investigate *Pasteurella* strains extensively, since the relationship in this genus has been thoroughly studied, especially by Schütze (1928, 1932) and by Bhatnagar (1940).

Methods. Standard macroscopic agglutination tests were made, using 0.3 per cent salt solution as the diluent. All tests were incubated overnight at 37 C.

Results

P. pseudotuberculosis. An antiserum prepared in rabbits by repeated injections of living *P. pestis* A1122 agglutinated both *P. pestis* and *P. pseudotuberculosis* (Saranac and Spokane strains) to about the same titer. These two strains of *P. pseudotuberculosis* were also agglutinated in dilutions up to 1:320 by antisera for *S. dysenteriae* and for *S. schottmuelleri*.

Salmonella. Considerable difficulty was experienced in serological studies owing to the roughness of the three *Salmonella* strains sensitive to *P. pestis* phage. However, there was definite indication of agglutination of *S. schottmuelleri* (no. 6) and *S. hirschfeldii* (58G, no. 31) in low titer by serum prepared against living, intact plague organisms, with satisfactory controls. No agglutination was observed using an antiserum for the carbohydrate fraction of *P. pestis*.

An antiserum prepared against *S. schottmuelleri* agglutinated *P. pestis* A1122 antigens in significant titer. The highest titers, 1:640, were obtained using antigens without envelope substance. Antigens prepared under conditions permitting the maximum development of envelope were not agglutinated by this

Salmonella antiserum. The antigenic factor in common may be considered, therefore, to be present in the soma of *P. pestis*, a reasonable hypothesis, since phage action is probably dependent on the surface antigens of the bacterial cell.

It seemed of value to determine whether the antigen or antigens held in common by *P. pestis* and *Salmonella* organisms could be identified. Efforts were unsuccessful, using alcoholized, heated, and live antigens of *P. pestis* A1122 with purified *Salmonella* "O" and "H" typing antisera. However, confirmation of agglutination of *P. pestis* by unpurified *Salmonella* typing antisera was supplied by the Division of Laboratories of the California State Department of Public Health. It seems likely that the common antigen is some minor somatic fraction as yet unrecognized.

Shigella. Antisera for *S. dysenteriae*, *S. ambigua*, and the W, X, Y, and Z types of *S. paradysenteriae* agglutinated *P. pestis* A1122 in dilutions of 1:40 to 1:320. Antiserum for the V type of *S. paradysenteriae* did not react with *P. pestis*. All of these antisera agglutinated the homologous *Shigella* strain in a titer of at least 1:2,560.

Antiserum for living, intact *P. pestis* agglutinated the majority of the 37 *Shigella* antigens in dilutions of 1:20 to 1:80, and 12 of the antigens in dilutions of 1:160 to 1:320. The results were identical with living and formalin-killed antigens. Antiserum for the carbohydrate substance of *P. pestis* agglutinated most of the *Shigella* cultures in 1:20 to 1:40 dilutions and three of the strains in 1:160 dilution. The titer of both these antisera for *P. pestis* was 1:1,280.

The slight cross reactions in agglutination tests between *P. pestis* and *Shigella* suggest that they possess some common antigenic factor, although the majority of the *Shigella* strains tested were not acted upon by *P. pestis* phage. There was no correlation between the agglutination titer with *P. pestis* antiserum and lysis by *P. pestis* phage. For example, strains of *Shigella* agglutinated in 1:320 dilution might not be lysed by *P. pestis* phage, whereas others agglutinated only in 1:20 dilution might show lysis. This may be interpreted as a lack of correlation between phage sensitivity and agglutination, or as the result of an antigenic variation causing temporary loss of the minor relationship apparently existing between the two genera.

MISCELLANEOUS OBSERVATIONS

It was determined that lysates of *P. pestis* cultures were toxic for mice, producing death a few hours after intravenous inoculation. Similar observations were made with lysates of *P. pseudotuberculosis*, which were shown to be lethal for mice, guinea pigs, and rabbits. However, some strains of *P. pseudotuberculosis* showed a markedly greater toxigenic ability than others. Investigations are now being conducted to determine the properties of the toxin present in lysates of this organism and the factors responsible for the variation in toxigenic ability of different cultures.

Lysates of *P. pestis* were shown to be efficient vaccines, protecting mice against lethal doses of virulent plague bacilli. Flu (1929) made similar observations regarding the ability of such lysates to protect rats against many times

the normal lethal dose of plague organisms. The use of lysates of *P. pestis* offers a number of interesting possibilities for human vaccination.

DISCUSSION

Flu (1927) observed that a phage isolated from canal water in Leyden, Holland, was able to lyse *P. pestis*, *E. coli*, and *S. dysenteriae*. Girard (1943) confirmed Flu's results, using several *P. pestis* phages. No other observations of this type have been recorded, although Girard (1942) has reported lysis of some strains of *P. pseudotuberculosis* by *P. pestis* phage.

If the assumption is made that the antigenic structure of the bacterial cell is one of the main factors permitting phage action (Topley and Wilson, 1946), the findings reported here apparently demonstrate a minor relationship between *P. pestis* and some strains of certain species of the *Salmonella* and *Shigella* groups. However, all strains of a given species, and even all strains of a given serological type within a species, were not equally susceptible to *P. pestis* phage. The organisms susceptible to *P. pestis* phage were not shown to be any more closely related to *P. pestis* than phage-resistant organisms, by the use of direct agglutination tests. Schütze (1928) has shown a close similarity between one of the somatic antigens of *P. pseudotuberculosis* and the "O" antigen of *S. schottmuelleri* and related salmonellas. No such relationship seems to have been described for *P. pestis*.

In view of known antigenic factors common to *P. pseudotuberculosis* and *P. pestis* (summarized by Schütze, 1932, and Bhatnagar, 1940), the action of *P. pestis* phage on *P. pseudotuberculosis* is not unexpected. The fact that strains of *P. pseudotuberculosis* differ so markedly in susceptibility to *P. pestis* phage (table 1) indicates an antigenic flexibility that may account for the wide variation in results obtained by different workers who have attempted to immunize animals against plague by the use of *P. pseudotuberculosis* antigens (summarized in Topley and Wilson, 1946). The action of *P. pestis* phage on certain *Shigella* and *Salmonella* organisms may possibly be accounted for by the presence of some unstable minor antigenic fraction held in common. The agglutination tests reported in this study give some basis for assuming the existence of such a relationship, but adequate proof is lacking.

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SUMMARY

A strain of *Pasteurella pestis* phage which lysed 12 out of 12 strains of *P. pestis* has been shown to lyse, in varying degree, 19 out of 27 strains of *Pasteurella*

pseudotuberculosis. After adaptation to *P. pseudotuberculosis*, the phage lysed all 27 strains of that organism, most of them in higher dilution than originally. Other *Pasteurella* species were not susceptible to either phage.

Three out of 42 *Salmonella* strains and six out of 37 *Shigella* cultures were susceptible to *P. pestis* phage. Seventy-seven cultures from 17 other genera were not affected by the same phage. After adaptation to *Shigella* species, the phage showed an increased potency toward the six susceptible *Shigella* strains.

After adaptation to *P. pseudotuberculosis* or to *Shigella*, the phage retained completely its ability to lyse *P. pestis*.

Minor serological relationships have been shown by agglutination tests to exist between *P. pestis* and certain strains of *Salmonella* and *Shigella*. These relationships were not clearly correlated with susceptibility to phage.

The significance of these findings is discussed. The use of *P. pestis* lysates as vaccines and the study of the lethal properties of *P. pseudotuberculosis* lysates are suggested as worthy of further investigation.

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