THE ANTIGENIC RELATIONSHIPS OF BACTERIOPHAGES ACTIVE AGAINST *XANTHOMONAS PRUNI*

by

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INTRODUCTION

The inherent characteristics of bacteriophages have made them excellent tools for the investigation of biological phenomena. Serological reactions of the bacteriophage, i.e., the production of immune serum and inactivation of the antigen with this serum, is one of the most specific of all phage characteristics. The specificity of the immune responses provides an invaluable tool for the determination of antigenic relationships; closely related antigenic substances demonstrate cross reactions, the strength of the cross reactions being an indication of the degree of relatedness. For this reason, the relationship of phages to one another can be determined on an antigenic basis.

Several workers (Burnet, 1933 and Delbruck, 1946) have demonstrated distinct serologic groups in the T series of phage active against Escherichia coli. The T series comprising seven bacterial viruses, T_1 through T_7, after serologic analysis formed four unrelated antigenic groups: (1) T_1, (2) T_2, T_4 and T_6, (3) T_3 and T_7, and (4) T_5, as indicated in Table 1 (Luria, 1953).

Table 1. Cross-neutralization by representative antiserum against bacteriophages T_1 - T_7.

Serum	K = Fractional Rate of Inactivation						
	T_1	T_2	T_3	T_4	T_5	T_6	T_7
Anti-T_1	80	0	0	0	0	0	0
Anti-T_2	0	400	0	150	0	80	0
Anti-T_3	0	0	120	0	0	0	30
Anti-T_4	0	75	0	250	0	20	0
Anti-T_5	0	0	0	0	80	0	0
Anti-T_6	0	200	0	40	0	500	0
Anti-T_7	0	0	75	0	0	0	120

Although the antiserum of any one of these groups showed no detectable serological cross reaction with any other group, phages within a group demonstrated cross reactions. For example, T_2, T_4, and T_6, when neutralized with T_2 antiserum, had serologic or K values of 200, 50 and 90 respectively, while T_3 and T_5 treated with the same antiserum had zero K values (neutralization constants). Since the immune response was controlled by the protein antigen, the serologic or K values are indicative of the antigenic relatedness of the phages. Therefore, T_6 was more closely related to T_2, than to T_4, and T_4 was more closely related to T_2 than to T_6.

Eisenstark and Thornberry (1950) observed differences in the plaques of the bacteriophages active against Xanthomonas pruni and suggested the possibility of distinct genetic varieties. Mandell (1950) and Mandell and Eisenstark (1953) isolated five distinct genetic varieties of pruniphage: XP_1 through XP_5. A few years later another variety, XP_8, was isolated from a lysogenic strain by Eisenstark, et al. (1954).

Mandell (1950) presented evidence of distinct serological variation of the viruses active against X. pruni; indicating differences in the antigenic structure. The evidence was insufficient to demonstrate any antigenic relationships of the virus or to correlate their serological specificity with the morphological characteristics of plaques as demonstrated by Delbruck (1946a) for the T series active against E. coli.

The purpose of this study was to determine if an antigenic relationship, as demonstrated with the T series of E. coli phages, existed between the six viruses, XP_1 through XP_5 and XP_8, that are active against Xanthomonas pruni. However, during the course of investigation two other phenomena were observed which were deemed to be important and these were also taken into consideration.

The first of the two phenomena taken into consideration was the effect

of the dilutent on the rate of the reaction of immune serum and phages. Previous studies had shown that various factors such as, temperature, pH, and the concentration of antiserum, can influence the rate of inactivation of bacteriophage by antiserum. Jerne and Skovsted (1953) demonstrated the rate of inactivation was also dependent on the dilutent and concentration of NaCl in dilutents. They obtained the highest rate of inactivation in distilled water. Since broth was originally used as the dilutent in the present investigation it seemed worthwhile to investigate the possibility of substituting distilled water for broth.

The second interesting side phenomenon was concerned with a sudden increase in the plaque count, accompanied by a change in plaque morphology (Table), following 99 percent inactivation of phage XP_3 by heterologous antiserum. These new plaques appeared larger than plaques of the parent phage and possessed a very turbid halo as compared to the clear halo of the parent phage, XP_3. This phenomenon opened to speculation the possibility the the antiserum had induced a mutation of the bacteriophage. Preliminary studies were made with some of the plaques demonstrating the new morphological characteristics in an attempt to explain this unusual phenomenon.

REVIEW OF LITERATURE

Shortly after the discovery of phage by Twort (1917) and D'Herelle (1926), Bordet and Cuica (1924) demonstrated the antigenicity of the bacteriophage by their ability to stimulate antibody production in experimental animals. The antigen has been shown by Burnet et al. (1937) to be one of the phages' most definite characteristics, regardless of the host at whose expense they propogate, each phage antigen remains the same. Burnet, et al.

(1937), Pollard (1953), and Lanni (1953) stated that the antigenic unit consists of two portions. The larger part of the antigenic unit was responsible for the antigenic specificity, the smaller portion being non-specific. Any variation or change in the activity of the small fraction would not change the antigenic specificity. For instance, Hershey (1946) could not detect any antigenic difference between the host range mutants of T_2R type coli phage mutants which form large plaques with clear halos and its T_2R ancestors which form small plaques with turbid halos. So far, an antigenic mutant has not been discovered for bacterial viruses.

The antiserum produced by the phage inactivates the virus by the deposition of the antibody on the surface of the bacteriophage, according to Kalmanson and Bronfenbrenner (1943) who established this concept by reactivating the phage after digestion of the antibody coat with papain. Similarly Anderson and Doermann (1952) demonstrated reactivation using sonic vibrations. Although the bacterial virus may be reactivated by special treatment, Hershey (1943) has shown that the reaction of the antigen with the antibody was irreversible by demonstrating no increase of infectivity of neutralized phage when the mixture stood for six weeks after diluting antiserum 1000 times beyond the concentration causing neutralization. The mode of inactivation had been thought to be the prevention of attachment of the bacteriophage to the surface of the bacterium. However, Hershey (1943a) demonstrated by direct and quantitative methods an instance in which the inactivated phage was as readily adsorbed to bacteria as an active particle, indicating that the inhibition of some step in the growth cycle subsequent to the attachment of the phage may also be responsible for the inability to multiply.

Burnet, et al. (1937) observed that the results of the physical conditions evidenced in the reaction of the immune serum combining with the

antigen was typical of a bimolecular reaction, provided that one substance (the antiserum) was in large excess. The rate at which this bimolecular reaction took place could be influenced by various factors. Andrewes and Elford (1933) showed that the antibodies combining with the virus particles in a given interval of time would neutralize only a certain percentage of these virus particles regardless of the concentration of the phage; this was termed the "percentage law". Deviation of the "percentage law" occurred when 99 percent of the phage was inactivated due to the existence of resistant types of phage. However; the resistance was not genetically controlled, for it did not give rise to resistant progeny. The greatest percent of inactivation took place with the homologous system, i.e., the phage and the immune serum produced by that particular phage. Temperature has been shown by Hershey (1943) to influence the rate of inactivation directly; for every 10 degree elevation in temperature the rate of inactivation increased by a coefficient of two. Another factor, the type of dilutent used, was demonstrated by Jerne and Skovsted (1953) to be important; the greatest rate of inactivation being evidenced when triple distilled water was used.

In consideration of these variables, temperature, "percentage law", time and the bimolecular reaction, Adams (1950) mathematically expressed the reaction of the immune serum with virus particles by the formula $K = 2.3D/t$ times $\log po/p$. K represented the empirical rate of virus inactivation, D the dilution, t the time in minutes the antigen and antibody were allowed to react, and po/p the survival ratio; po represented the number of active bacteriophage particles at the start of inactivation and p the number at the end. The equation held for 90-99 percent inactivation of the active phage present in the original mixture.

Adams (1950) used the K values to demonstrate the antigenic relatedness

of bacteriophages. If several viruses were antigenically related, the antiserum from any one would inactivate the ability of the phage to produce progeny, the most marked inactivation, as previously mentioned, occurring with the homologous system. The degree of relatedness was determined by the magnitude of the differences in K values; for example, the E. coli phages T_2, T_4 and T_6 when neutralized by T_2 antiserum had K values of 200, 50 and 90 respectively. Therefore, T_6 was more closely related to T_2 than to T_4, and T_4 more closely related to T_6 than to T_2.

Phage active against Xanthomonas pruni was first described and isolated by Anderson (1928). The host organism, X. pruni, has been observed to be widespread in the United States and to be of considerable economic importance as the cause of bacterial rot of peach and other seed trees. Thornberry, et al. (1948) demonstrated the specificity of the phage against X. pruni, and noted that all X. pruni cultures isolated from natural sources were lysed while closely related species X. compestris variety amoraciae and S. lactucaescariolae were not lysed. Eisenstark and Thornberry (1950) observed the existence of three distinct plaque formations; the differences suggested the existence of genetic varieties of phage active against X. pruni. Crownover, et al. (1950) in preliminary investigations determined the effects of some physical and chemical agents on the growth of the bacteriophage. Mandell (1950) and Mandell and Eisenstark (1953) isolated five genetically distinct viruses, XP_1 through XP_5, active against X. pruni. Several years later another variety, XP_8, was isolated by Eisenstark, et al. (1954) from a lysogenic strain of Xanthomonas pruni.

From a review of the literature, it may be seen that serology offers a good tool for determining relationships of phages.

MATERIALS AND METHODS

Determination of Antigenic Relationships

Preparation of Antiphage Sera. For each phage, XP_1 through XP_5 and XP_8, two rabbits were injected intravenously (marginal ear vein) with a viable suspension of high titer phage (10^{10}-10^{11} virus particles/ml). The phage suspensions were prepared by the method described by Bernstein and Eisenstark (1954). A bacterin of the host, Xanthomonas pruni, was also prepared and injected intravenously into another rabbit. Injections were given every 4-5 days for a period of three weeks, a week later a booster injection was given. Seven days following the booster injection, the rabbits were bled by cardial puncture, the serum harvested and stored in the deep freeze.

Medium. The medium employed (Mandell, 1950) to maintain host cultures of Xanthomonas pruni (American Type Culture, No. 10016) and for the plating technique was:

Glucose	5 grams
NaCl	5 grams
Beef Extract	3 grams
Peptone	5 grams
Agar	15 grams
Water	1 liter

The medium was adjusted to pH 7. The bacterial viruses (XP_1 - XP_5 and XP_8) and the host were suspended in broth having all the constituents of the above medium except the agar.

Assay of Antiphage Sera. The procedure used was essentially the method proposed by Adams (1950). Each serum was pre-tested to determine the dilution giving 90-99 percent inactivation in 5-10 minutes with the homologous system. These limits were used so the results would be applicable to the

formula for determining the antigenic relatedness. The phage stock was diluted to a titer of 10^7/ml of which 0.1 ml was added to 0.9 ml of the serum dilution previously determined; all phage dilutions were made in distilled water. One-tenth ml of the serum-phage mixture was removed every five minutes for 30 minutes, starting from the time at which the serum was added to the phage. To stop the action of the antiserum against the bacteriophage, 0.1 ml of the serum-phage mixture was added to 9.9 ml of the dilutent. One ml of this dilution was then added to 4 ml of broth containing 10^8 host bacteria per ml. The 5 ml of phage-host suspension was then added to 2 ml of melted agar cooled to 45° C, mixed and immediately poured over a sublayer of previously solidified agar. The plates were incubated at 24° C and examined 24 hours later.

Calculation of K Values (Neutralization Constants). The formula used to determine the K values of the assayed antisera was proposed by Adams (1950): $K = 2.3D/t$ times $\log po/p$. K represented the empirical rate of the phage inactivation, D the dilution of the antiserum, t the time in minutes the antiserum had reacted with the phage and po/p the survival ratio. Po was the titer of viable phage at zero time and p the titer at the subsequent time intervals. The formula held only for the inactivation of the 90 to 99 percent of the original amount of phage. The degree of relatedness was determined by the magnitude of the differences in K values.

Effects of Dilutents on the Rate of Inactivation

The bacteriophage XP_3 and its antiserum was used in all experiments pertaining to this phase of the work. Four dilutents were used: distilled water, broth, 1.0 N NaCl, and 0.1 N NaCl. The mixture of reacting agents

were prepared by placing 1.0 ml of XP_3, titer of 10^7 phage particles/ml, in the bottom of a two liter flask and the same volume of undiluted antiserum in the second flask. One liter of the dilutent was poured into the first flask and immediately transferred to the second flask. The point at which the contents of the first flask were added to the second flask was designated as zero time. Every five minutes beginning with zero time 1.0 ml of the serum phage mixture was removed and added to 4 ml of the host suspension which was plated and incubated in the same manner as previously mentioned.

Preliminary Investigation of Suspected Antigenic Mutants

Isolation. A few plaques showing the altered morphological characteristics were picked from a plate originally containing XP_3 phage which had been inactivated by XP_2 antiserum (Table). The isolated plaques were placed in 100 ml of host suspension and incubated at 22° C for 24 hours. The host-phage suspensions were centrifuged at 20,000 r.p.m. to obtain cultures of the isolates free of host bacterium and debris from lysis. One ml of each isolate was titered and the morphological characterictics of plaques were compared with those of the original isolates.

Determination of Host Range. Two cultures of host, *X. pruni*, which were not lysed by any other of the pruni phage series were used to test for the host range of the suspected mutants. Three spot inoculation plates were made for each isolate, incubated at 22° C and examined 24 hours later.

Table 2. K values (serum inactivation constant) after neutralization with XP_1 antiserum.

Phages	Po count	P count	% inactivation	Time Minutes	K
XP_1	1.67×10^7	1.7×10^5	98.0	15	43
XP_2	7.62×10^7	1.13×10^6	98.5	10	34
XP_3	3.67×10^7	6.4×10^5	98.3	10	25
XP_4	1.38×10^8	3.5×10^6	98.9	20	13
XP_5	1.16×10^7	8.0×10^5	93.3	20	13
XP_8	1.16×10^6	7.0×10^4	93.4	15	14

Po = Phage assay at zero time.
P = Phage assay at time (T).
Time = Length of exposure of phage to antiserum.
K = Serum inactivation constant.

Table 3. K values (serum inactivation constant) after neutralization with XP_3 antiserum.

Phages	Po count	P count	% inactivation	Time Minutes	K
XP_1	1.05×10^6	3.5×10^4	95.6	24	40
XP_2	2.04×10^6	1.4×10^5	96.2	24	30
XP_3	5.75×10^5	3.5×10^4	90.0	8	68
XP_4	1.7×10^6	3.0×10^4	98.4	16	50
XP_5	2.4×10^6	6.0×10^4	96.5	24	42
XP_8	1.06×10^6	4.0×10^6	96.7	15	53

Table 4. K values (serum inactivation constant) after neutralization with XP_4 antiserum.

Phages	Po count	P count	% inactivation	Time Minutes	K
XP_1	1.83×10^6	8.0×10^4	95.6	15	37
XP_2	3.23×10^6	1.25×10^5	96.2	20	32
XP_3	1.02×10^6	1.05×10^5	90.0	5	91
XP_4	1.56×10^6	2.5×10^4	98.4	10	102
XP_5	3.17×10^6	1.10×10^5	96.5	10	67
XP_8	1.06×10^6	3.5×10^4	96.7	15	45

Table 5. K values (serum inactivation constant) after neutralization with XP_5 antiserum.

Phages	Po count	P count	% inactivation	Time Minutes	K
XP_1	1.09×10^6	9.5×10^4	91.0	10	240
XP_2	2.92×10^6	2.4×10^5	91.8	15	155
XP_3	9.4×10^5	1.25×10^5	87.0	5	219
XP_4	9.55×10^5	4.5×10^4	95.3	10	305
XP_5	3.29×10^6	7.5×10^4	95.6	10	401
XP_8	1.06×10^6	5.0×10^4	97.9	20	152

Table 6. K values (serum inactivation constant) after neutralization with XP_8 antiserum.

Phages	Po count	P count	% inactivation	Time Minutes	K
XP_1	4.4×10^6	8.15×10^5	91.3	30	3.1
XP_2	1.45×10^7	1.69×10^6	88.8	30	3.3
XP_3	1.01×10^7	3.35×10^5	96.7	30	6.0
XP_4	5.75×10^6	5.50×10^5	91.0	17	6.9
XP_5	1.05×10^7	7.0×10^5	93.3	17	8.4
XP_8	5.16×10^7	5.15×10^5	99.0	17	11.2

EXPLANATION OF PLATE I

Figures 1, 2, 3. Plaque morphology of XP_1.

Figures 4, 5, 6. Plaque morphology of XP_2.

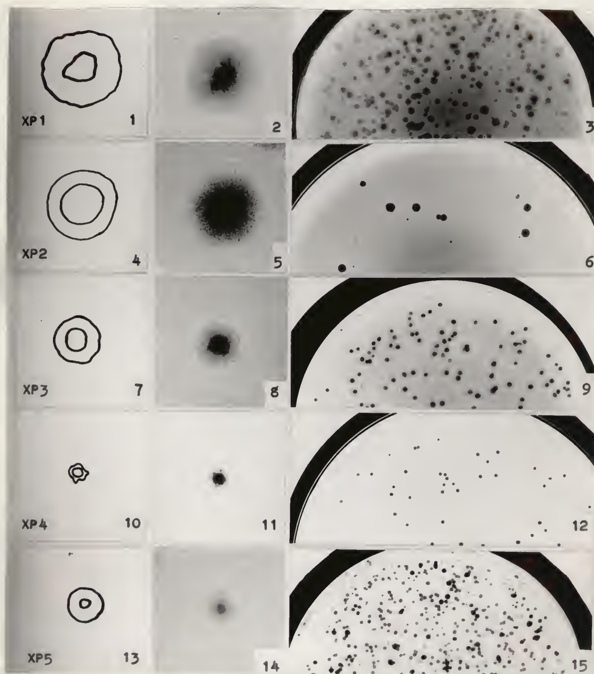
Figures 7, 8, 9. Plaque morphology of XP_3.

Figures 10, 11, 12. Plaque morphology of XP_4.

Figures 13, 14, 15. Plaque morphology of XP_5.

Antigenic relationships: (XP_2, XP_1) (XP_3) (XP_4, XP_5), therefore, XP_1 was more closely related to XP_3 than XP_2, and XP_4 more closely related to XP_3 than XP_5. The antigenic groups correspond to the plaque morphological groups.

PLATE I



The K value obtained from the effect of different dilutents upon the rate of reaction of immune serum and phage are listed in Table 7. The highest value was obtained when distilled water was used as the dilutent, broth, 0.1 N NaCl and 1.0 N NaCl followed respectively.

Table 7. K values (serum inactivation constant) with XP_3 antiserum in determination of the effect of dilutents upon the rate of inactivation.

Phage XP_3	Po count	P count	% inactivation	Time Minutes	K
Dilutents					
Water	1.4×10^7	2.4×10^5	98.3	10	201
Broth	1.23×10^7	1.55×10^5	98.7	20	109
0.1 N NaCl	1.2×10^7	1.55×10^5	98.7	20	109
1.0 N NaCl	1.16×10^7	2.25×10^5	98.1	20	99

Table 8. Jump in inactivation curve after exposure to antiserum beyond the 99 percent end point.

Phage XP_3	Minutes of exposure to XP_2 antiserum				
	0	5	10	15	20
Plate count	336	24	4	0	42
	Minutes of exposure to XP_5 antiserum				
Plate count	188	25	1	7	46

Table 9. K values of the original pruni phages and the possible mutant with XP_8 antiserum.

Phages	Po count	P count	% inactivation	Time Minutes	K
XP_1	4.4×10^6	8.15×10^5	91.3	30	3.1
XP_2	1.45×10^7	1.69×10^6	88.8	30	3.3
XP_3	1.01×10^7	3.35×10^5	96.7	30	6.0
XP_4	5.75×10^6	5.50×10^5	91.0	17	6.9
XP_5	1.05×10^7	7.0×10^5	93.3	17	8.4
XP_8	5.16×10^7	5.15×10^5	99.0	17	11.2
XP_x	1.01×10^7	4.45×10^5	98.0	17	7.2

Table 10. Survival ratio and calculated K values of the original pruni phages and the possible mutant with XP_5 antiserum.

Phages	Po count	P count	% inactivation	Time Minutes	K
XP_1	4.42×10^6	3.75×10^5	82.0	17	246
XP_2	1.56×10^7	1.60×10^6	90.0	17	227
XP_3	1.15×10^7	1.2×10^5	90.0	4	455
XP_4	5.75×10^6	8.0×10^4	98.6	10	428
XP_5	1.16×10^7	7.0×10^4	94.0	4	510
XP_8	3.0×10^7	2.37×10^6	92.1	10	253
XP_x	9.62×10^6	7.5×10^4	92.1	4	485

After propogation of the phage (XP_x) isolated from one of the plaques which suddenly appeared after 99 percent inactivation during the determination of the K values, it demonstrated the same plaque morphological characteristics as the plaques which were originally isolated. Namely, an increase in size and a very turbid halo as compared to the smaller plaque and clear halo of the parent phage, XP_3 (Plate 2). The new phage (XP_x) lysed the host range mutant that was resistant to infection by the other phages. The K values obtained with two antisera, XP_8 and XP_5, showed a significant difference from the parent phage and are listed in Tables and

EXPLANATION OF PLATE II

Figure 1. Plaque morphology of XP_3.

Figure 2. Plaque morphology of XP_x.

PLATE II

RESULTS

The K values obtained from the assay of the antisera are listed in Tables 2-6. Each phage demonstrated greater inactivation for the homologous system except XP_2. The values which were obtained were not included in the tables. The erratic behavior of XP_2 could not be explained. The bacteriophage used for injections was checked after the erractic results and found to be free of contaminating phage or bacteria. In spite of the erratic results, in the case of XP_2, the same group relationships were found to exist with the other antisera, namely, (XP_2, XP_1) (XP_3) (XP_4, XP_5, XP_8).

The relationships between the phages for each of the other antisera were as follows. The first represents the phage which had the highest K value with that particular antiserum and the others are arranged in descending order.

XP_1 antiserum, XP_1 XP_2 XP_3 XP_8, XP_4, XP_5;

XP_3 antiserum, XP_3 XP_8, XP_4 XP_5, XP_1 XP_2;

XP_4 antiserum, XP_4 XP_3 XP_5 XP_8 XP_1, XP_2;

XP_5 antiserum, XP_5 XP_4 XP_1 XP_3 XP_2, XP_8;

XP_8 antiserum, XP_8 XP_5 XP_4 XP_3 XP_1, XP_2.

The antigenic relationships found, (XP_2, XP_1) (XP_3) (XP_4, XP_5, XP_8), could be correlated with the plaque morphologic characteristics of the bacteriophage (Plate I). This indicated that either method, serology or plaque morphology, could be used for phage identification. The host antiserum had no effect on the phage, nor did normal rabbit serum.

DISCUSSION

The results indicated that the bacteriophages of *X. pruni* could be grouped into three distinct antigenic groups even though the phages formed a very closely related group of antigenic entities. The individual and group relationships evidenced in the experimental work were: (XP_2, XP_1) (XP_3) (XP_4, XP_8, XP_5). The lack of response of the phage to the host antiserum indicated no antigenic relationship between the host and the phages. Unlike the T series active against *E. coli*, the pruni phage exhibited no unrelated antigenic groups. On the other hand, the pruni series resembled the coli series with regard to correlation of serological properties with plaque morphology; i.e., when two phages had K values similar to each other, the plaque morphologies were similar. The correlation of plaque morphology and antigenic relationships is an interesting one.

The highest rate of inactivation of the phage by antiserum was observed when distilled water was used as the dilutent; broth, 0.1 N NaCl and 1.0 N NaCl gave respectively lower values. The K value obtained when distilled water was used was approximately twice that of the other dilutents. The difference in K values between broth and 0.1 NaCl were insignificant, however, the difference between 1.0 N NaCl and the two dilutents previously mentioned were significant. Although a higher value was obtained with 0.1 N NaCl as compared with 1.0 N NaCl, the difference between the two values did not approach that which was demonstrated by Jerne, et al. (1953). Upon the basis of this experiment the broth originally used as the dilutent was replaced with distilled water.

The observation of an increased plaque count with plaque morphologic change after inactivation of A5 with heterologous antiserum has opened to

speculation the possibility of antiserum inducing a mutation on the bacteriophage. The possibility of the new plaque type existing as a contaminant was eliminated by the lack of such morphological plaque types on the plates of the previous time intervals of the special phage, XP_3 in this case, from which the plaque variant was isolated. The lack of the new phage type on the previous plates would also eliminate adaptation as the causative factor, for if adaptation was responsible the new types would appear in greater numbers as inactivation approached the maximum value. As may be seen from Table , this did not occur. Neither could one phage produce this effect by multiplication since the host-virus suspension was plated immediately after mixing, thus preventing propagation. It also was unlikely that the increased plaque formation was caused by partially inactivated phage belatedly infecting a bacterium since the plaque size was larger than its predecessor, XP_3. A partially inactivated phage would produce a smaller plaque as the immune serum would inactivate the portion to which the antibody was adsorbed, thereby, partially inhibiting the propagation process. The K values obtained also indicated a difference in antigen structure. Taking the various factors into consideration, it seems plausible that the antigen in some manner was altered by the action of the antiserum, and that this change could be carried from parent to offspring phage.

SUMMARY

Antiserum was prepared for each virus type and the host, Xanthomonas pruni, and assayed by prescribed methods to determine if an antigenic relationship existed between the phages. The assay of the antisera demonstrated that the phages constituted a group of closely related antigens which could

be separated into three groups. The host and the phage were antigenically dissimilar as the *X. pruni* antiserum had no effect on the phage. In cases where the phage types resembled each other serologically, they also resembled each other with respect to plaque morphology.

An experiment on the effect of the dilutents on the rate of phage inactivation was performed and the results obtained showed that distilled water gave the highest rate of inactivation while the presence of salt inhibited the rate of inactivation.

During the determination of the antigenic relationships, a sudden increase in the plaque count and plaque size was observed after 99 percent inactivation of the phage by antiserum. Preliminary studies indicated that the new phage was a new genetic variety since, when it was subcultured, the new type produced plaques identical to the plaque originally isolated. The new variety had a different host range than any other of the phage types. Serologic methods suggested that the new type was a distinct antigenic entity. Taking these various factors into consideration it appeared as if the antiserum could induce the phage to mutate forming a new type of phage with distinct antigenic properties. Further investigation is necessary to verify and characterize this phenomenon.

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REFERENCES

Adams, M. H.
Methods in Medical Research. Vol. II, Yearbook Publishing Company, Chicago, 1950.

Anderson, H. W.
Bacteriophage of Xanthomonas pruni. Abst., Phytopath., 18: 144, 1928.

Anderson, T. F., and A. H. Doermann.
Sonic reactivation of antiserum-neutralized bacteriophage T_3. J. Bact., 63: 291-292, 1952.

Andrewes, C. H., and W. J. Elford.
Observation on antiphage sera. I. The "percentage law." Br. J. Exp. Path., 14: 367-376, 1933.

Andrewes, C. H., and W. J. Elford.
Observations on antiphage sera. II. Properties of incompletely neutralized phage. Br. J. Exp. Path., 14: 376-383, 1933a.

Bernstein, L. B., and A. Eisenstark
Production of high titer phage. In preparation for publication, 1954.

Bordet, J., and M. Cuica.
Exudats leucocytaires et autolyse microbiene transmissible. Compt. rend. Soc. de biol., 1924, 90: 96.

Burnet, F. M.
The serological classification of Coli-dysentery phages. J. Path. Bact., 36: 307-318, 1933.

Burnet, F. M., E. V. Koegh, and Dora Lush.
The immunological reactions of filterable virus. Australian J. Exptl. Biol. and Med., 15: 227-368, 1937.

Clifton, C. E., Elizabeth Mueller, and W. Rogers
Neutralization of the bacteriophage. J. Immunol., 29: 377-388, 1935.

Crownover, Ruth.
Studies on bacteriophage with particular reference to Phagus pruni. Master's thesis, Oklahoma A. and M. College. 1950.

Crownover, R., J. D. Mandell, and A. Eisenstark.
Studies on bacterial viruses active against Xanthomonas pruni (Holmes). Proc. of Oklahoma Acad. of Science, Vol. XXXI, 1950.

Delbruck, M.
The growth of bacteriophage and lysis of the host. J. Gen. Physiol., 23: 643-660, 1940.

Delbruck, M.
The effects of specific antiserum on the growth of bacterial viruses. J. Bact., 50: 137-150, 1945.

Delbruck, M.
Bacterial viruses or bacteriophages. Biol. Rev., 21: 30-40, 1946.

Delbruck, M.
Experiments with bacterial viruses (bacteriophages). The Harvey Lectures Series, XLI, 161-187, 1946a.

D'Herelle, F.
The bacteriophage and its behavior. Williams and Wilkins Co., Baltimore, 1926.

Eisenstark, A., and H. H. Thornberry.
Plaque formation by Xanthomonas pruni bacteriophage. Phytopath., 40: 876-877, 1950.

Eisenstark, A., S. Goldberg, and L. B. Bernstein.
Study of Lysogenicity in Xanthomonas pruni. Bact. Proc., 1954.

Hershey, A. D.
The absolute rate of phage antiphage reaction. J. Immunol., 41: 299-319, 1941.

Hershey, A. D.
Experiments with bacteriophage supporting the Lattice-Hypothesis. J. Immunol., 47: 77-87, 1943.

Hershey, A. D.
Immunological reaction of bacteriophage. J. Bact., 45: 74-75, 1943a.

Hershey, A. D.
Mutation of bacteriophage with respect to type of plaque. Genetics, 31: 620-640, 1946.

Hershey, A. D., G. M. Kalmanson, and J. Bronfenbrenner.
Quantitative methods in the study of the phage-antiphage reaction. J. Immunol., 46: 267-279, 1943.

Hershey, A. D., G. M. Kalmanson, and J. Bronfenbrenner.
Quantitative relationships in the phage-antiphage reaction: unity and homogencity of the reactants. J. Immunol., 46: 281-299, 1943a.

Jerne, N. K., and Lis Skovsted.
The rate of inactivation of bacteriophage T_4R in specific antiserum. Annales de l'Institut Pasteur, Tome 84: 73, 1953.

Kalmanson, G., and J. Bronfenbrenner.
Evidence of serological heterogencity of multi-valent "pure lime" bacteriophage. J. Immunol., 45: 13-19, 1942.

Kalmanson, G., and J. Bronfenbrenner.
Restoration of activity of neutralized biologic agents by removal of antibody with papain. J. Immunol., 47: 387-407, 1943.

Kalmanson, G., A. D. Hershey, and J. Bronfenbrenner.
Factors influencing the rate of neutralization of bacteriophage by the antibody. J. Immunol., 45: 1-12, 1942.

Landsteiner, K.
The specificity of serological reactions. Harvard University Press, Cambridge, 1945.

Lanni, F., and Y. T. Lanni.
Antigenic structure of bacteriophage T_2. Fed. Proc., 12: 45-451, 1953.

Luria, S. E.
General Virology. John Wiley and Sons, Inc., New York, 1953.

Mandell, J. D.
Studies on bacterial viruses active against Xanthomonas pruni. Master's Thesis, Oklahoma A. and M. College, 1950.

Mandell, J. D. and A. Eisenstark.
Bacterial viruses active against Xanthomonas pruni. Monthly Research Report of the Office of Naval Research, April issue. pp. 1-4, 1952.

Mandell, J., and A. Eisenstark.
Factors affecting plaque formation by Xanthomonas pruni bacteriophage. Phtopath., 43: 27-29, 1953.

Pollard, E.
Physics of viruses. Academic Press, Inc., New York, 1952.

Pollard, E., and J. Setlow.
Action of heat on the serological affinity of T_1. Archives of Biochemistry and Biophysics, 43: 136-142, 1953.

Rivers, T. M.
Viral and Rickettsial diseases of man. J. B. Lippincott Co., Philadelphia, 1952.

Roundtree, Phyllis M.
The serological differentiation of staphylococcal bacteriophages. J. Gen. Microbiol., 21: 1-16, 1949.

Roundtree, Phyllis M.
A complement-fixing antigen of Bact. Coli bacteriophage T_5: Its behavior during growth. Brit. J. Exp. Path., Vol. XXXII, No. 4, August, 1951.

Thornberry, H. H., A. Eisenstark, and H. W. Anderson
Studies on the bacteriophage of Xanthomonas pruni. Phytopath., 38: 907-911, 1948.

Twort, F. W.
The bacteriophage: The breaking down of the bacteria by associated filtered-passing lysins. British M. J., 1922, ii 23.

THE ANTIGENIC RELATIONSHIPS OF BACTERIOPHAGES ACTIVE AGAINST *XANTHOMONAS PRUNI*

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The inherent characteristics of bacteriophage have made them excellent tools for the investigation of biological phenomena. One of the most specific of all phage characteristics is its serological reactions; the production of immune serum and inactivation of the phage with this antiserum. The specificity of these reactions enables determination of antigenic relationships; closely related antigens demonstrate cross reactions, the degree to which the cross reactions approach each other being an indication of the relatedness of the antigens.

Several workers have demonstrated distinct serologic groups in the T series of phage active against Escherichia coli and correlated these serologic groups with the morphologic characteristics of the phage.

The purpose of this study was to determine if antigenic relationships existed between the six viruses (XP_1 through XP_5 and XP_8) which are active against Xanthomonas pruni. Included in this study were two interesting phenomena which were observed during the course of investigation. These phenomena were the effect of the dilutent upon the rate of reaction of immune serum and the phage, and the sudden increase in the plaque count after 99% inactivation of the phage accompanied by a change in plaque morphology of the phage.

Antiserum was prepared for each virus and the host, Xanthomonas pruni, by intravenous injection (marginal ear vein) of a high titer stock (10^{10}-10^{11} organisms/ml.) every 3 or 4 days for a month, after which the rabbits were bled by cardial puncture, the serum harvested and stored in the deep freeze until used.

The antisera was assayed by prescribed methods. The phage, diluted to 10^7 virus particles/ml., was added to 0.9 ml. of a dilution of antiserum, previously determined, giving 90-99% inactivation of the phage in 5-10

minutes. Every five minutes for 30 minutes 0.1 ml. of the serum phage mixture was removed and added to 9.9 ml. of distilled water to stop the action of the antiserum. One ml. of this dilution was added to the 4 ml. of host and plated by the agar layer technique. The plates were incubated at 24° C and examined 24 hours later.

The formula used to determine the K values (neutralization constants) was K = 2.3D/t times log po/p. K represented the imperial rate of phage inactivation, D the dilution of antiserum, t the time in minutes the antiserum had reacted with the phage and po/p the survival ratio. Po was the titer of the phage at zero time and p the titer at subsequent time interval. The formula held only for inactivation of 90-99% of the original amount of phage. The degree of relatedness was determined by the magnitude of the differences in K values.

The effect of the dilutent on the assay of antiphage serum was determined by placing 1.0 ml. of XP_3, titer of 10^7 phage particles/ml., in the bottom of a 2 liter flask and the same volume of undiluted antiserum to a second flask for each dilutent used. One liter of each dilutent, distilled water, 1.0 N NaCl, 0.1 N NaCl, and broth was poured into the first flask and immediately transferred to the second flask. Every five minutes starting with zero time, 1.0 ml. of the serum phage mixture was removed and added to 4 ml. of host and plated by the agar layer technique and incubated in the same manner as previously mentioned.

To make preliminary investigation on suspected phage mutants, a few plaques showing the altered characteristics were isolated and placed in 100 ml. of host suspension and incubated at 22° C for 24 hours. The host-phage suspension was centrifuged to obtain a phage culture free of bacteria and debris from lysis. The phage obtained from this procedure was titered and

the morphological characteristics compared to the original isolates. The host range of the phage was tested against two host range mutants.

The assay of the antisera demonstrated that the phages active against Xanthomonas pruni constituted a group of closely related antigens which could be separated into three groups: (XP_2, XP_1) (XP_3) (XP_4, XP_8, XP_5). The host and the phage were antigenically dissimilar. In instances where the phage types were similar serologically, they also resembled each other with respect to plaque morphology.

An experiment on the effect of dilutents was carried out and the results obtained agreed with those of previous workers: distilled water gave the highest rate of inactivation while the presence of salt inhibited the rate of inactivation.

Preliminary studies on the suspected phage mutant isolated from a plate showing a sudden increase in plaque count and change in plaque morphology after 99% inactivation indicated the new phage was a new genetic variety having a different host range than any of the other types of phage. Serologic methods indicated that the new type was a distinct antigenic entity. Taking these various factors into consideration, the possibility exists that the antiserum could induce the phage to mutate forming a new type of phage with distinct antigenic properties. Further work must be done to verify and characterize this phenomenon.