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Inhibition of Bacterial Growth by Bacteriophage as distinct from Lytic Action¹

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SUMMARY: Certain races of streptococcal phage with a normal lytic action on some strains of streptococci were found to be adsorbed on, and to inhibit the growth of, other strains, without the occurrence of lysis or multiplication of the phage. One race of phage inhibited the growth of several streptococcal strains which had hitherto been considered unrelated to one another on the basis of their reactions to lytic phages. Cultures which recovered from temporary inhibition by phage contained variants which were resistant both to inhibition and to lysis by phages which attacked the original strain. This resistance is, however, short-lived as compared with the resistance of variants obtained through the action of a lytic phage.

The typical interaction between bacteriophage and bacterium is that in which adsorption of phage is followed after an interval by lysis of the bacterium and a many-fold increase in number of phage particles. The majority of workers now visualize phage as a bacterial parasite which grows in or on the bacterial cell, ultimately causing lysis with liberation of phage particles. There are, however, other types of association between phage and bacterium to which this picture does not apply. For instance there are the cases where bacteria are lysogenic, or where resistant organisms support the growth of an associated phage. Another association which is not followed by lysis or phage reproduction has also been observed. Rakieta & Rakieta (1937) and Rakieta & Tiffany (1938) observed that staphylococcus phages were absorbed on strains of *Bacillus subtilis* and of enterococci without apparent effect on the growth of these organisms. Andrewes & Elford (1932) and Adams (1949) reported observations on 'citrate sensitive' coli phages which, in the absence of calcium, attached themselves to bacteria, prevented bacterial multiplication, but did not cause lysis. Felix (1949) reported that with certain salmonella adsorption of phage was not always followed by lysis.

We have encountered in the group of lactic streptococci some instances of phage adsorption without subsequent lysis. Certain phage races which exert a lytic effect on some strains of streptococci are adsorbed by other strains without any lytic effect and when present in relatively high concentration the adsorbed phage inhibits the growth of the adsorbing organisms. It appears that adsorption takes place as usual but that subsequent growth of the phage, normally leading to lysis, is blocked. This blockage also results apparently in interference with the metabolism of the bacterium.

MATERIALS AND METHODS

Cultures. The eight bacterial cultures used were strains of *Streptococcus cremoris* as used in commercial cheese manufacture. They had been isolated in this Institute over about 17 years, and were selected on the bases of acid-

producing activity in milk and lack of relationship to one another in lytic phage reactions. The stock streptococcal cultures were maintained at 22° in sterilized milk and transferred daily. Although these strains grew more rapidly between 30° and 35° during one or two subcultures, they rapidly lost their acid-producing power when maintained any longer at such temperatures and died out after transfer for a few days at 37°.

Demonstration of phage action. The stock phages, originally isolated from cheese whey, were maintained as whey filtrates (Seitz) at 5–7°. The demonstration of phage action on a bacterial culture (*a*) and the estimation of the strength of phage preparations (*b*) respectively, were carried out as follows. (*a*) Standard loopfuls (0.004 ml.) of 1/10 dilutions were added to tubes (9 ml.) of sterilized skim milk inoculated with two drops (0.1 ml.) of a 24 hr. culture of the required streptococcal strain and incubated at 30° or 37°. All tubes which showed coagulation in 24 hr. were subcultured once. (*b*) By means of a standard loop a drop of each 1/10 dilution was placed on the surface of an agar plate previously spread with a mat of the streptococcal culture, and incubated at 30°.

The phage titre is expressed as the highest dilution which caused failure to coagulate in method (*a*) or which gave plaques in method (*b*). Frequently method (*a*) gave a titre higher by one dilution than method (*b*). This was to be expected since streptococcal phages form small plaques which when present in small numbers, can easily be overlooked. In spite of this we generally used method (*b*), which was accurate enough for our purposes, had the advantage of revealing anything characteristic about plaque form or size, and lent itself to diagrammatic presentation of results.

Phage adsorption. Five ml. of a 24 hr. clotted milk culture of the streptococcal strain under test were measured into a sterile $4 \times \frac{1}{2}$ in. tube fitted with a rubber bung. Two drops (0.1 ml.) of undiluted phage preparation were added and the mixture allowed to stand at room temperature for 5 hr. with frequent shaking. It was then centrifuged, the clear supernatant fluid drawn off, a series of 1/10 dilutions prepared from it, and the phage titre determined by the plate method (*b*). A control series of dilutions starting from two drops of the phage preparation in 5 ml. saline was also spotted on the plate. (This control gave the same titre as a control prepared from two drops of phage in 5 ml. of acid-clotted skim milk). Where a series of cultures was tested for ability to adsorb a certain phage, and the supernatant fluids were spotted on a plate spread with the culture which the phage normally lysed, adsorption of phage was made evident by a significant decrease (at least by two dilutions) in titre of the supernatant fluid from the adsorbing cultures. In this test the homologous culture always showed adsorption of phage. Cultures not lysed by the phage showed adsorption of various degrees or gave a result identical with that of the control, indicating no adsorption.

Observation of lysis or inhibition under the microscope. Tubes of sterilized milk inoculated with streptococcal strains and with various amounts of phage were incubated in a water-bath at 30° or 37°, the temperature selected depending on the characteristics of phage race and bacterial strain. At

intervals, smears from the tubes were made on a slide, dried, and stained with methylene blue. By examination under the microscope an estimate was made of extent of growth, progress of lysis, and any other effect which the phage might have on the morphology or arrangement of the bacteria. It was adequate for our purpose to use an estimate based on inspection of a series of fields and expressed by signs ranging from - to + + + +.

RESULTS

A strain of *Streptococcus cremoris*, designated D4, was received from Australia during a search for active acid-producing cultures for use in cheese manufacture. After the strain had been in use for 6 weeks in a commercial cheese factory, a phage capable of lysing the organism was regularly found to be present in the cheese whey. This was in accordance with normal expectation and, we believe, implies that the phage, present in dormant form in infinitesimal amount in the factory surroundings, gradually increased in concentration by multiplication on the susceptible organism which was grown daily as a large exposed culture in the cheese vats. Phage 51 isolated in this way had a normal lytic action on *Strep. cremoris* strain D4. Tested on agar against seven other 'unrelated' strains of *Strep. cremoris*, a preparation of phage 51 which had a titre of 10^{-8} against streptococcus D4 did not show clear areas of lysis or plaques on any of the cultures; but on some of them it produced foggy areas of decreased growth where the drops of undiluted phage had been placed. Trial of the action of this phage on the cultures in a skim milk medium showed that there was inhibition of growth of those cultures which had shown the foggy areas in the plate test.

Table 1 gives the results of an experiment in which the seven *cremoris* strains were treated with varying amounts of phage 51, the extent of growth being estimated under the microscope as described. The results indicate that four of the strains were markedly inhibited by phage 51, two were only slightly affected and one was not influenced by the largest amount of phage used. The inhibitory action was usually only temporary; with smaller amounts of phage, cultures tended to recover within 6 hr. from the start of the experiment, and milk was usually coagulated overnight as with a normal culture. With larger amounts of phage, growth sufficient to cause coagulation of milk occurred only after two or three days. This inhibitory action of a single phage on several of the strains of *Strep. cremoris* in this group was all the more unexpected since these strains had been selected because of their apparent complete lack of relationship to one another in respect of sensitivity to lytic phages. In no instance, over the course of years, had a phage with lytic action against one of these strains been found capable of lysing any of the others. It should be noted, however, that the amounts of phage used in the above test were much higher than would normally be used in testing a phage for lytic power; subsequent trials, mentioned later, showed that among the lytic phages active on these seven strains were some which had inhibitory activity against heterologous strains.

Table 1. *The effect of phage 51 on the growth after 5 hr. at 30° of seven strains of Streptococcus cremoris*

Strain of <i>Strep. cremoris</i>	Vol. of phage 51 preparation added to 9 ml. milk culture (ml.)				
	Nil	0.1	0.25	0.5	1.0
	Relative amount of growth at 5 hr.				
HP	++++	+++	—	—	—
K	++++	+++	+	—	—
R1	+++++	+++	+	—	—
R6	+++++	—	—	—	—
ML1	+++++	+++++	+++++	+++++	+++++
KH	+++++	+++++	+++++	+++++	++
E8	++++	+++	+++	++	+

Nature of the inhibitory principle

The question arose whether the inhibitory action of the phage preparation was due to the phage itself or to some antibiotic substance formed during the growth or lysis of streptococcus D4. The question was answered in favour of the first alternative by the following observations. (i) The inhibitory principle was destroyed by heat coincidentally with the phage. Exposure to 66° for 5 min. (pH 5.0) was sufficient to destroy both lytic and inhibitory power in the whey filtrate. (ii) Phage 51 prepared by lysis of a strain of *Strep. lactis* (the only other organism apart from the streptococcus D4 so far found to serve as host) had exactly the same inhibitory power as phage 51 prepared by lysis of *Strep. cremoris* strain D4. (iii) Another phage race active against streptococcus D4 in a normal lytic manner did not show any inhibitory action against the other strains of *Strep. cremoris*. It seems most probable therefore that the inhibitory action was due to phage 51 itself and did not depend on a property of streptococcus D4.

Appearance under the microscope

Tubes containing 9 ml. of sterilized skim milk were inoculated with 0.1 ml. of a 24 hr. milk culture of streptococcus HP, and amounts of a preparation of phage 51, ranging from 0.05 to 1 ml. in the various tubes, were added. The tubes were incubated at 30° and smears made at intervals for staining and microscopical examination. A minimal amount of phage sufficient to have an observable inhibitory effect within the first 5 hr. of incubation (c. 0.05 ml. of a preparation with a titre of 10^{-8} on streptococcus D4) caused a decrease of growth as compared with a control and a shortening of the chains of streptococci. With increasing proportions of phage there was progressively less growth in the cultures and, as well as the shorter chains, there was an appearance as though individual cocci detached from the chains and lost their normal spherical shape. Tubes to which 0.5 ml. of phage preparation had been added frequently showed after 5 hr. incubation little more than a few of these 'spots', which gave the impression of being dead cocci in process of dissolution. The picture under the microscope thus

suggested a drastic interference with the metabolism of the bacteria, the degree of interference depending on the number of phage particles present. On continued incubation the cultures containing the smaller proportions of phage showed complete recovery within 24 hr. Those containing larger proportions showed little growth after 24 hr., but they grew sufficiently to cause coagulation of the milk after a few days.

Adsorption of phage on bacteria

When a culture of streptococcus HP, after being initially inhibited by phage 51, recovered and finally clotted the milk, it was possible to show that some adsorption of phage had taken place. The supernatant fluid obtained by centrifuging such cultures, containing originally up to 0.5 ml. of phage, did not contain enough phage to give plaques on a plate spread with streptococcus D4. Cultures to which more than 0.5 ml. of phage had originally been added showed some phage in the supernatant fluid. A demonstration of phage adsorption by various strains of *Strep. cremoris* was more conveniently carried out, however, by the method described earlier. Clotted milk cultures of the seven streptococcal strains used were investigated for their capacity to adsorb phage 51. The results are shown diagrammatically in Fig. 1. The results indicate that the strains most powerfully inhibited adsorbed the phage most readily. There were some anomalies with the three strains ML1, KH, and E8. Strain ML1 was not significantly inhibited by 1 ml. phage preparation per 9 ml. of culture, yet it adsorbed phage as strongly as strain E8 which suffered moderate inhibition. Strain KH adsorbed more strongly than strain E8 yet was inhibited to a lesser extent. In general, however, it is reasonable to conclude that adsorption of phage and inhibition of growth were linked.

When the test was applied to organisms and phages whose combination resulted in lysis, adsorption was always evident, as with streptococcus D4 in Fig. 1. The adsorption was always less marked, however, than it was with organisms which were strongly inhibited. It cannot be deduced from the adsorption tests with clotted milk cultures whether an adsorbing organism will show lysis or inhibition by the phage.

The results obtained in an adsorption with a phage race which had no inhibitory action on the *cremoris* strains used are illustrated in Fig. 2. which should be contrasted with Fig. 1. Phage race 2 had a lytic action on streptococcus HP only, and the results show that adsorption to a significant degree occurred only with streptococcus HP.

Adsorption tests carried out with the *cremoris* strains and the various phage races which attacked them disclosed two more instances where phages had an inhibitory action on strains other than those which they lysed. Phage 4 which lysed streptococcus K was adsorbed on and inhibited streptococcus R6. Conversely phage 9 which lysed streptococcus R6 was adsorbed on and inhibited streptococcus K. These reactions had been overlooked when tests for phage action had been carried out with only small amounts of phage and adsorption tests had not been done. It seems evident that the phenomenon

of adsorption and inhibition of growth without lysis is not uncommon among phages for the lactic streptococci, although the action of phage 51 on unrelated strains may be unusual.

Fig. 1

Fig. 2

Production of phage-resistant variants

The bacterial growth which eventually occurred in a milk culture in which streptococcus HP had temporarily been inhibited by phage 51 differed from a normal culture of HP in that it frequently contained phage-resistant variants. Tubes of skim milk inoculated with HP, and various amounts of phage 51 were incubated at 30° until the milk clotted; the resulting cultures were streaked on agar. Colonies picked at random from the incubated plates into sterile milk yielded a series of subcultures, some of which were apparently identical with the original strain HP while others differed in appearance under the microscope and in reaction to phage. These variants were much less inhibited by phage 51 than was a normal culture of HP and were completely resistant to phage 2, a race which lysed HP. The proportion of phage-resistant variants in the cultures was not constant from experiment to experiment; the maximum proportion was estimated at 20 %.

Variants obtained as a result of the action of phage 51 were compared with phage-resistant variants obtained in the usual way by continued incubation of a culture of streptococcus HP lysed by phage 2. The latter treatment yielded a large proportion (often 100 %) of resistant substrains which are considered (Luria & Delbrück, 1943; Delbrück, 1946) to have originated from a few resistant individuals present in the culture as a result of a normal

process of variation. The two kinds of resistant streptococcus, namely that produced during inhibitory action by phage 51 and that developed after total lysis of a culture by phage 2, appeared to be similar. They differed from the normal streptococcus HP in appearance under the microscope, the cocci being smaller in size and the chains being much longer. The appearance was indeed so characteristic in cultures incubated at 30° that it was possible to predict, from appearance alone, which of the substrains derived from cultures grown under the influence of phage 51 were resistant to lysis by phage 2 and which were not. The results of growth tests of representative substrains of the two kinds of streptococci subjected to the action of phages 2 and 51 are given in Table 2.

Table 2. *Effect of lytic and inhibitory phage on resistant variants of streptococci at 30°*

Addition		Streptococcus strain					
		HP		HP/2		HP/51	
		Periods of growth					
		4 hr.	6 hr.	4 hr.	6 hr.	4 hr.	6 hr.
Phage	Vol. (ml.)	Relative amounts of growth					
	0	+++	++++	+++	++++	+++	++++
No. 2	0.005	—	—	+++	++++	+++	++++
No. 51	0.10	+	+	+++	++++	+++	++++
No. 51	0.25	—	—	++	+++	++	+++
No. 51	0.50	—	—	++	++	++	+++
No. 51	1.00	—	—	+	+	+	+

HP/2 Resistant substrain prepared by action of lytic phage 2.

HP/51 Resistant substrain prepared by action of inhibitory phage 51.

It is clear from the results that both types of variants were completely resistant to lysis by phage 2 and that both, although slightly affected by phage 51, were much less inhibited by that phage than was the original strain HP. At first it seemed that the two resistant strains were identical, and that a close linkage existed between the action of the two phages, one lytic and the other inhibitory. But it soon became evident that there was a difference between the streptococcal strains HP/2 and HP/51. Whereas most strains of HP/2 retained their resistance to phage 2 at least for several months and sometimes for years, only gradually reverting to sensitivity, all the strains of HP/51 so far investigated reverted to a state sensitive to both phages within 14 days of daily subculture in milk. This fact, coupled with the early appearance and large proportion of HP/51 forms found in a culture temporarily inhibited by phage 51, strongly suggests that HP/51 variants are the result of a temporary change impressed on the bacteria by the action of phage 51, whereas HP/2 forms are probably variants normally present in the culture and made evident by the removal, by lysis, of all sensitive forms.

DISCUSSION

The inhibitory action of phage 51 on several strains of streptococci seems to be bound up with the formation of a host-virus complex in which further action leading to lysis is blocked. We have no evidence to indicate whether the blockage results from the presence of an inhibitory factor or the lack of some essential factor in the medium (cf. the 'citrate sensitive' *coli* phages), or whether lysis is impossible under any circumstances. The fact that relatively large amounts of phage (as compared with the amounts commonly used with a lytic phage) must be added to a culture to produce marked inhibition accords with expectation, since it is reasonable to suppose that the attachment of at least one phage particle is necessary to inhibit growth of each bacterium. With a lytic phage, lysis of the whole culture could, under ideal conditions, result from the presence of one phage particle by a series of waves of lysis following multiplication of phage and infection of further bacteria. In the inhibition phenomenon, where no multiplication of the phage takes place, the quantitative relationships are different.

The observation that phage 51 is adsorbed on and inhibits the growth of several strains of *Strep. cremoris* which are susceptible to attack only by distinct lytic phages raises an interesting point. The apparent lack of relationship between the lytic strains seems to indicate that the points of attachment on the bacteria for the lytic phages are distinct and unrelated; a lytic phage for one strain finds no point of attachment on the others and hence is not adsorbed. Yet several of the strains evidently have some point of attachment for adsorption of phage 51, which finds points of attachment on streptococcus D4 and on a strain of *Strep. lactis* which lead to lysis. It is possible, therefore, that the organisms which suffer inhibition of growth have more than one point of attachment for phages because they have points for the specific lytic phages and a common point for the inhibitory phage 51.

Another question concerns variants of streptococcus HP which become able to grow in the presence of phage 51 after having overcome its inhibitory effect and which are also resistant to the lytic phage, race 2, which normally attacks streptococcus HP. Also, variants of streptococcus HP resistant to phage 2, isolated from a lysed culture of HP, are resistant to the inhibitory action of phage 51. This suggests that the point of attachment on streptococcus HP for the lytic phage 2 is also involved in the attachment of inhibitory phage 51. This finding needs to be reconciled with the findings detailed in the previous paragraph. The fact that resistant forms of streptococcus HP, after the action of phage 51, rapidly revert to sensitivity to both lytic and inhibitory phages still further complicates the picture. Resistant streptococci obtained after the action of a lytic phage are almost certainly variants normally present in the culture in small numbers and made apparent because of the complete elimination of all the phage-sensitive forms. Resistant streptococci obtained by the action of inhibitory phage 51 occur in relatively large numbers in a culture which has only been partially inhibited. This suggests that these streptococci are the result of a change impressed on individuals in the culture

by the action of phage. The change appears to be transient since the drift back to sensitivity is usually complete in 10–14 days.

In this relationship between variants produced by the action of lytic and inhibitory phage there is a suggestion that adsorption of an inhibitory phage, which obviously results in interference with some metabolic activity of the adsorbing bacterium, is related to the primary action of a lytic phage. This, if substantiated, would be evidence that the lytic phage acts by interposing itself at some point in the essential metabolic machinery of the organism before beginning to multiply.

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