PHAGE-RESISTANT AND PHAGE-CARRYING STRAINS OF LACTIC STREPTOCOCCI

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The relationships between the lactic streptococci and the many races of bacteriophage which attack them have a wide significance in the field of commercial dairying, hence the increasing interest in many countries in the 'problem' of bacteriophage in cheese and butter manufacture. The subject has also aspects of interest from a more fundamental bacteriological view-point because it may have a bearing on variation in bacteria and upon the origin of the enormous numbers of variant strains which occur in nature.

This paper deals with investigations on the several types of resistance which may be acquired by lactic streptococci as a result of phage action, and, in particular, with the intriguing observation that under certain circumstances where organisms and phage grow in symbiosis, the organism is protected from attack by other phages by the presence of the symbiotic phage.

I. PHAGE-RESISTANT STRAINS

Organisms and phage races

The organisms studied were all strains of *Str. cremoris* (Orla Jensen) selected for cheese-making purposes on the basis of their activity in the production of lactic acid from milk. The phage races which attack them have a tendency to be strain (or at least type) specific in contradistinction to *Str. lactis* phages which usually have a wide range of lytic activity (Hunter, 1946*a*). After lysis of a strain of *Str. cremoris* by a phage in milk, a secondary culture is in general readily obtained on continued incubation for a further 24 or 48 hr. The cultures so far studied in this laboratory fall into two broad groups according to the main type of resistance exhibited by the secondary culture as a whole:

(1) Those strains which form secondary cultures completely and permanently resistant to the phage used.

(2) Those which give rise to a secondary growth which is (*a*) partially resistant, showing reduced sensitivity to the original phage; or (*b*) temporarily resistant with loss of resistance on daily subculture in milk.

Evidence of a symbiotic association of organisms and phage was encountered among strains of the second group.

A culture, HP, may be described as an example of group two. It is a pure culture of *Str. cremoris* isolated in this laboratory some 12 years ago from a commercial mixed starter, and kept in daily subculture in milk. It has been purified frequently by plating. Under the microscope, after growth in milk for 5–6 hr. at 37° C. (i.e. at a temperature near the maximum for growth of the culture), the organisms are characterized by the formation of medium to long chains of normal round cocci with the terminal coccus swollen. The organisms are completely lysed by a specific phage race 2.

Table 1. *Phage-organism relationships*

Medium: lactose-yeast-phosphate agar.
Temperature 30° C.

	Phage race				
Organism	2	10	36	34	37
HP	+++	+	+++	+++	++
B	−	++	−	++	−
OP	+	±	+++	+++	+
R_9	++	−	+	+++	±
TM	±	−	−	+	++

Key: Highest decimal dilution of phage preparation showing plaques when a loopful is placed on the surface of a mat of organisms spread on the solid medium.

10^{-6} or over +++, 10^{-4} ++, 10^{-2} +, neat phage ±, no plaques with neat phage −.

Other cultures, viz. B, OP, R_9, TM, which show a morphological similarity to HP (chains, with the terminal coccus swollen) after treatment at 37° C., were subsequently isolated from material obtained from commercial cheese factories. The phage-organism relationships within this series are set out in Table 1. The tests were made on lactose-yeast-phosphate agar (Hunter, 1946*b*), clarified by centrifuging the tubes of melted medium prior to pouring into Petri dishes (a water-clear agar suitable for demonstration of phage plaques results).

Phage races 10 and 34 differed from the other races in the table in that they possessed a wider range of activity than is indicated here. They were able to lyse certain other strains of lactic streptococci, morphologically and biochemically different from the group of which HP may be considered representative.

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Cross-resistance tests were carried out in order to ascertain whether the phage races could be grouped into types or shown to be similar in any degree. The reactions between phages, susceptible strains, and resistant forms on the surface of agar are summarized in Table 2. Resistant cultures produced by phage action in milk cultures are indicated as follows: HP/2 is the resistant strain developed by the action of phage race 2 on strain HP in milk. A positive sign represents a frank action, a negative sign indicates no action with neat-phage preparation.

Table 2. *Cross-resistance tests*

Medium: lactose-yeast-phosphate agar.
Temperature 30° C.

	Phage race				
Organism	2	10	36	34	37
HP	+	+	+	+	+
HP/2	−	+	−	+	−
HP/10	+	−	+	−	+
HP/36	−	+	−	+	−
HP/34	+	−	+	−	+
HP/37	+	+	+	+	−
B	−	+	−	+	−
B/10	−	−	−	−	−
B/34	−	−	−	−	−
OP	+	+	+	+	+
OP/10	+	−	+	−	+
OP/36	+	−	−	+	+
OP/34	+	−	+	−	+
OP/37	+	+	+	+	−
R_9	+	−	+	+	+
R_9/2	−	−	−	+	−
R_9/36	−	−	−	+	−
R_9/34	+	−	+	−	+
R_9/37	−	+	−	+	−
TM	+	−	−	+	+
TM/2	−	−	−	+	+
TM/37	−	−	−	−	−

OP/2 and TM/34 were not obtained; no lysis occurred when OP and TM were grown in milk at 22 and 30° C. with phages 2 and 34 respectively.

The results indicate that:

(1) Races 2 and 36 show considerable similarity. They give identical results on resistant forms produced from HP and R_9 although there are differences with OP and TM.

(2) Races 10 and 34 show some degree of relationship on resistant forms of HP and B, but considerable difference with OP, R_9 and TM. The fact that races 10 and 34 also attack strains outside the group of organisms under consideration also links them together.

(3) Race 37 shows very little similarity with any of the other races.

Thus, while it is evident from Table 1 that no two of the races are identical, the results of cross-resistance tests provide some slight evidence that the races may be divided into three general types, 2 and 36, 10 and 34, and 37. Without further evidence this classification would be too slenderly based to be at all convincing, but, as will be seen later, it receives some confirmation from the results obtained on protective symbiosis.

A general consideration of the morphology of the five original strains (HP, B, OP, R_9, TM) and of the sensitivity of strain HP to all the phage races leads to the postulation that HP is the parent strain and that the other strains are resistant (B), or partially resistant forms (OP, R_9, TM), which have acquired this resistance in their natural habitat (or in a secondary habitat such as a dairy factory) before they were isolated in the laboratory. This will be assumed to be true in all the following discussion on results.

Varying phage sensitivity of organisms within 'pure' cultures

The populations of the various streptococcal cultures, viz., parent strain HP, resistant, and partially resistant strains, were examined for phage sensitivity by means of a methylene-blue indicator test. The cultures were streaked on the surface of a solid medium and the resultant colonies picked into sterilized skim milk. Actively growing substrains from each type of culture were subjected to the test.

10 ml. quantities of sterilized skim milk, coloured with methylene blue (1:300,000), were inoculated with two drops of a 24 hr. clotted-milk culture and one drop of phage preparation. The incubation temperature was 37° C. As the organisms multiplied, the blue colour was reduced. The colour returned as soon as the phage destroyed the organisms. This method for observing phage action permitted large-scale tests to be carried out without the need for frequent microscopic examination of smears.

The preparations of phage 2 used in the tests gave a titre of 10^{-6} to 10^{-8} on agar on the parent strain HP. Substrains obtained by plating HP were lysed in milk, as determined by the methylene-blue test, in approximately equal times, generally in about 3 hr. from the commencement of the test. The substrains from a resistant form, HP/2, were all completely resistant, i.e. the blue remained reduced and the milk clotted after 7–8 hr. incubation. With partially resistant forms, e.g. OP and TM, which showed only a few plaques on the areas containing the higher concentrations of phage on agar, a different picture was obtained. The substrains varied in reaction, some being more sensitive and some more resistant than the parent culture. Two examples may be quoted.

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(1) The partially resistant culture TM was lysed in milk culture by phage 2 in 6 hr.; of the substrains isolated by plating TM, 8% were lysed in 3–3½ hr., 21% in 5–5½ hr., 40% in 6–6½ hr. 31% of the substrains clotted the milk before lysis occurred.

(2) Partially resistant culture OP was acted upon by phage 2 on agar but not in milk before clotting took place. 4% of the substrains isolated from OP were lysed in 4–5 hr., 12% in 5–6 hr., and 84% clotted the milk.

With only minor variations, these substrains maintained their relative sensitivities for periods up to 3 weeks when they were discarded. In a few cases (e.g. with one OP substrain partially resistant to phage race 2) a culture originally resistant became gradually more sensitive when transferred daily in milk for over a year and when retested the organisms were completely lysed by the phage within one generation at the higher temperature (30 and 37° C.) and in two or three generations at 22° C. It is evident, therefore, that with partially resistant cultures the organisms tend gradually to change in their reaction to phage, and the relationship is to some extent a fluid one. One might thus expect the reactions listed in Table 1 to change over a long period of daily subculture of strains. Happily the changes do not take place so rapidly as to frustrate any effort to gain some idea of the relationships involved.

It is interesting to observe that where cultures free from phage are maintained under daily subculture in the laboratory, the trend is always towards increased sensitivity to a given phage, never apparently towards increased resistance. This suggests that related strains, which when isolated show more resistance, have acquired that resistance by suffering phage action in their natural habitat, and that lactic streptococci are in nature constantly subjected to the action of a variety of phages.

The origin of resistant forms

The mechanism by which resistant forms originate after lysis of a culture by phage has not yet been satisfactorily explained. The suggestion that they arise from filterable forms present in phage lysates does not appear to be substantiated by indisputable evidence. The alternative theory is that a few cells which have acquired resistance to the phage remain alive in the lysed culture and that these give rise to secondary growth. The results of the experiment described below seem to indicate that so far as the lactic streptococci are concerned the second hypothesis fits the facts.

A flask of sterilized broth inoculated with culture HP and one drop of diluted phage preparation 2 was incubated until lysis and clearing of the broth occurred. One-half of the lysed broth culture was filtered through a Seitz E. K. filter pad and the filtrate added to a flask of sterilized skim milk. The unfiltered portion was added to another flask of sterilized milk. On incubation of the flasks, the one containing the unfiltered broth readily developed organisms which were similar in morphology to the original HP culture but resistant to phage 2. The other flask remained sterile.

II. PHAGE-CARRYING STRAINS

The symbiotic association of phage and organism

The most interesting finding arising from the survey of resistant forms within the somewhat limited group of *Str. cremoris* strains studied, was that resistant cultures OP/2 and TM/34 could not be obtained by the general method employed, since no lysis resulted in mixtures of OP and 2 and TM and 34 respectively in milk. The races, however, gave plaques with the stronger preparations on a solid medium previously spread with the organisms. A difference in action on solid and liquid medium has been noted previously by other workers (Burnet, 1929; Burnet & Lush, 1935). In several instances, a phage active against a lactic streptococcus has given areas of confluent lysis or plaques on the surface of agar, but no corresponding activity could be induced in milk. In work dealing with lactic streptococci for use in butter and cheese manufacture the behaviour of phages in milk is obviously of far greater practical significance than their action on a solid medium. For this reason, as well as for ease of interpretation of results, subsequent discussion will be mainly confined to the action of phages in milk. Although phage race 2 did not bring about lysis of culture OP growing in milk, a strong development of phage was detectable when supernatant fluid from a clotted culture, to which a trace of phage 2 had originally been added, was tested against HP. A similar effect was apparent with TM culture and phage race 34, using R_9 as the indicator strain. Titres up to 10^{-4} to 10^{-5} were observed on many occasions although no adverse effect of the phages on the organisms OP and TM was evident in milk. Further, the titre was maintained on daily subculture of the 'mixture' for long periods. Thus some partially resistant forms of *Str. cremoris* are able to 'carry' phage, which does not destroy the culture as a whole. This is the first instance reported of a strain of *Str. cremoris* and a phage race living in symbiosis. The main features of the phenomenon may be summarized as follows.

(*a*) *Influence of growth conditions on phage-organism symbiosis.* The association of strain OP and phage race 2 is not materially modified by incubation temperature. The culture 'carries' the phage at any temperature suitable for growth of the organisms. Other factors such as size of inoculum and composition of the medium similarly

have no detectable influence on the association between organism and phage.

This distinguishes the phenomenon sharply from that in which a particular incubation temperature favours the bacterium but not the phage, with the result that clotting of the milk medium occurs before lysis of the organisms takes place. In such a case, a raising or lowering of the temperature (depending on the optimum for a particular phage) in a subsequent subculture results in lysis of the organisms. The reaction between OP and phage race 10 is of this type. At an incubation temperature of 22° C. a culture of OP contaminated with phage 10 shows no evidence of phage action. The culture clots normally through any number of subcultures. If, however, the phage-contaminated culture is incubated at 37° C. lysis occurs within a few hours. Thus the association between OP and phage 10 is of a very different type from the association between OP and phage 2.

(*b*) *Persistence of the phage in a phage-organism mixture.* Phage sometimes dies out of a phage-organism mixture and it is impossible to predict in any given culture how long the phage will persist. In a typical experiment ten tubes of sterilized skim milk (10 ml.) all received two drops of culture OP and one very small loopful of phage 2. The cultures were incubated at 22° C. and subcultured daily by the transfer of two drops. Supernatant clear whey from the top of clotted cultures was tested on HP every few days to determine whether phage 2 was still present. There was great variation in this respect between the ten similar cultures. One culture retained the phage (at a titre of 10^{-2} to 10^{-4}) for 6 weeks; three cultures lost the phage in less than a week and the remaining six cultures lost the phage at various intervals between 1 and 6 weeks. In another experiment culture TM 'carried' phage 34 without significant loss in titre for more than 12 weeks at which time the culture was discarded. Thus there is a general tendency for phage to die out of the phage-organism symbiotic mixtures, but in some instances the mixture is relatively stable for several months. The fact that exactly similar phage-culture mixtures give quite different results may indicate a sensitivity to some unknown factor in the conditions of maintenance.

(*c*) *Effect of plating and repurifying a phage-carrying culture.* A phage-carrying culture such as OP + phage 2 plated on the surface of lactose-yeast-phosphate agar yields normal colonies which coagulate milk readily. The resultant substrains show no evidence whatever of the presence of phage 2. This rather unexpected result was checked repeatedly and up to the present it has not been possible to define how the culture is freed from phage by the plating procedure, nor what happens to the phage. The result proves, however, that the phage-carrying phenomenon is quite distinct from the lysogenesis described by some workers because true lysogenesis implies a fixed phage-producing property associated with every cell in a culture.

(*d*) *Action of other phages on phage-carrying cultures.* The presence of the phage in a phage-carrying culture sometimes inhibits the action of a second phage race which would otherwise lyse the organism in milk. For instance, culture OP is susceptible to attack by phage race 36. When, however, OP is carrying phage race 2 in symbiosis, race 36 is unable to lyse OP in milk culture. On the other hand, culture OP + 2 (henceforward a culture-carrying phage is indicated thus, while a phage-resistant culture is indicated by HP/2 and a partially resistant culture by HP/2) is still susceptible to attack by races 10, 34 and 37. Thus race 2 interferes only with the action of a race which shows some degree of relationship with it (Table 2). There is no interference with different type phages. Similarly, culture TM carrying race 34 (i.e. TM + 34) is no longer susceptible to race 10 but is still susceptible to attack by races 2, 36 and 37. These facts thus strengthen the otherwise rather slight evidence that races 2 and 36 and 10 and 34 belong to two different groups.

Production of phage-carrying strains from pure cultures in the laboratory

The phage-carrying properties of cultures OP and TM having been demonstrated, the question arose whether similar strains could be prepared in the laboratory from stock strains of lactic streptococci.

Secondary growth was obtained from a lysed milk culture of HP by continued incubation at 22° C. The resultant resistant culture proved to be capable of carrying phage 2 for long periods (at least 6 months). The culture HP/2 + 2 when plated out on agar gave colonies free from phage as did culture OP + 2, but many of the substrains thus obtained would carry phage 2 if it was added again. There was considerable variation among the substrains, and only by a detailed process of selection was it possible to find those which were both active acid producers in milk, and capable of carrying the phage. There was a peculiarity about even some of these strains. They had the habit, when free from symbiotic phage of clotting the skim milk in the culture tube only in the bottom half within 24 hr. at 22° C. When carrying phage 2, however, they clotted the milk throughout the tube. The phage thus seemed to enhance their acid-producing activity.

The protective influence exerted by the symbiotic phage was more complete with phage-carrying strains derived from HP than with OP cultures. In all, eight phage races, acting on HP, but isolated on strains other than the original HP culture were

available for trial. Tests were carried out in milk at 30 and 37° C. (Table 3). When lysis did not occur within 6–7 hr., the cultures invariably clotted and showed no sign of being influenced by the phage.

All the phages have shown differences when tested against a sufficiently large number of streptococci and some have been shown to belong to different types. Culture HP/2 + 2 was not attacked by any stock phage in milk during one generation and thus in contrast with OP + 2 exhibited a more complete protection against 'like' and 'unlike' phage races.

The phage-carrying cultures from HP, when grown in milk for 5–6 hr. at 37° C., showed the chains twisted and clumped together, suggestive of agglutination. When the phage was removed, the chains reverted to the more normal 'straight' formation. It was therefore possible, within certain limits, to pick out the phage-carrying cultures by microscopic examination of smears after growth of the cultures at 37° C.

Dooren de Jong (1931) found that the lysogenic property of a culture of *B. megatherium* persisted in the spores even after exposure to 100° C. Fisk (1942) described a latent 'carrier state' in strains of *Staphylococcus aureus*.

The phenomenon described in the present paper is obviously not true lysogenicity as described by most of the workers mentioned above, since the phage is quite easily eliminated from cultures of lactic streptococci by a plating procedure. The association between phage and streptococcus seems to depend upon a partially resistant state of the bacterium which in each subculture permits a development of phage sufficient to ensure its survival in the culture but insufficient to lyse the bacterium before growth of both phage and organism is checked by accumulation of end-products. With each transfer to a new batch of medium the process is repeated although sometimes due to reasons at present unknown the phage gradually dies out of the mixture. In other instances it persists for a

Table 3. *Action of phages on original, resistant and phage-carrying cultures*

Culture	Type	Temperature (° C.)	Phage race 2	10	34	35	36	41	37	39	40
HP	Original	30	+	−	−	−	+	+	+	−	+
		37	+	+	+	+	+	+	+	+	+
HP/2	Resistant	30	−	−	−	−	−	−	−	−	−
		37	−	+	+	+	−	−	−	−	−
HP/2 + 2	Phage-carrier	30	−	−	−	−	−	−	−	−	−
		37	−	−	−	−	−	−	−	−	−

+ = lysis within 6–7 hr.; − = no lysis of organisms growing in milk.

DISCUSSION

Lysis of lactic streptococci by phage is followed usually, but not always, by secondary growth consisting of a phage-resistant form of the original strain. In certain instances the resistant culture will 'carry' the phage in symbiosis.

Constant association of phage and bacterium without the occurrence of lysis has been reported by several workers in various groups of bacteria. Lisbonne & Carrère (1922) first observed the phenomenon in cultures of *Bact. coli*, some of which regularly contained a phage which could be demonstrated by its action on a sensitive indicator organism. Flu (1926), working with a type culture 'coli 88', showed that in this instance the phage-carrying property was inherent in every bacterial cell, since each colony picked from a plate was lysogenic. Later, Burnet (1932) found that of 124 strains of Salmonella, ninety-three possessed some degree of lysogenicity towards one or other of two sensitive indicator strains. He showed further that with all these lysogenic forms phage and organism were permanently associated; the phage could not be eliminated by purification of the cultures. Den considerable time (maximum time observed up to the present is 6 months). The opposite effect (i.e. an increased rate of phage growth with final lysis of the bacterium) does not seem to occur. Possibly the constant presence of the phage suffices to eliminate immediately any individual bacteria which 'drift' back towards sensitivity and provide a medium on which the phage could grow more actively.

The partially resistant forms of streptococci which will 'carry' phage in the manner described are not obtainable from all strains of *Str. cremoris*. In some instances secondary growth after phage action consists of completely phage-resistant organisms which evidently provide no 'foothold' on which the phage could develop. If phage is added to such a strain it is merely diluted out on subculture.

When a phage-carrying strain is plated the subcultures prepared from individual colonies no longer contain phage. Moreover, the substrains are not homogeneous in type, so far as their reaction to phage is concerned. Some of them are sensitive enough to suffer phage lysis in a milk medium. Some of them will, however, still act as carriers if fresh phage be added to them. If, on the other hand,

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they are maintained as stock cultures quite free from phage, many of them 'drift' back to a phage-sensitive state so that in time they may become indistinguishable from the original culture from which the whole process was started. The presence of symbiotic phage seems to prevent this 'drift'.

The phenomenon whereby a symbiotic phage inhibits the action of other phages on a lactic streptococcus is extremely interesting. There are few parallels in the literature. Burnet & Lush (1936) described two phages (one a mutant of the other) for a non-pathogenic white staphylococcus. Secondary cultures were resistant to both phages. Infection of the staphylococcus with the original phage race protected the organism against the more virulent mutant. White (1937) found that certain feebly acting phages protected vibrio cultures in which they were present from the action of more potent phages. He suggested that the phenomenon was due to the combination of the feeble phage with the 'phage receptors' of the bacterium, thus preventing the access of the potent phage. In connexion with certain plant viruses there seems to be a similar 'interfering' action. The presence of one virus in the cells of a plant sometimes precludes attack by another related virus (Smith, 1945). The protection given to the lactic streptococci by a symbiotic phage is difficult to explain on the data at present available. The ease with which a phage-carrying culture is freed from the phage does not seem to indicate a very close association between phage and bacterial cell, yet in the absence of such an association it is difficult to visualize how one phage 'blocks' access by another. Further work is necessary before the mechanism can be elucidated.

The phenomenon has, of course, an important bearing on problems encountered in commercial dairy practice. It offers the possibility of a form of protection for 'starters' (cultures of lactic streptococci used in cheese manufacture) from phage attack, much simpler and more convenient than those based on exclusion of airborne phage infection from a phage-sensitive culture. Trials of phage-carrying starter cultures in cheese manufacture are at present being made. The results will be reported elsewhere.

It is interesting to speculate on the part that phage-organism symbiosis may play in the ecology of bacteria in nature and possibly in bacterial variation. Both the lysogenic power and the power to 'carry' phage are easily overlooked in bacteriological work because their demonstration is possible only where the appropriate sensitive indicator strains are available to the investigator. It is evident, however, that both phenomena are widespread and one imagines that under some circumstances there must be a complex interplay between phages and sensitive strains, phage-carrying strains, and lysogenic strains of bacteria.

One important consequence of the present observations on phage-carrying organisms is worthy of note. Since the isolation of an organism by plating from some 'raw material' commonly results in the elimination of any phage which may be present in symbiosis, the bacteria handled as stock cultures in the laboratory may differ in behaviour in important respects from the organisms in the original impure culture. The investigator may have no means of determining whether symbiotic phages are present in the original material and may thus be unaware of the alteration he has brought about by the purification procedure. Moreover, the purified laboratory stock culture, originally a resistant or partially resistant strain, tends under regular subculture to lose its resistance and become once more a phage-sensitive form, whereas the phage-carrying organism in its natural habitat tends to retain its resistant nature.

SUMMARY

1. The types of resistance acquired by certain related strains of *Str. cremoris* as a result of phage action were investigated.

2. Some partially resistant forms of *Str. cremoris* were able to 'carry' phage in symbiotic association when subcultured daily in milk for long periods of time.

3. The phage 'carrying' power of the cultures was of a non-inherent nature. 'Purification' of the phage-carrying cultures by plating resulted in the production of phage-free colonies.

4. Under certain circumstances, the presence of symbiotic phage in a culture gave a measure of protection against attack by other phage races.

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