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Serological Studies of a Host Range Mutant of a Lactic Streptococcal Bacteriophage

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A host range mutant was isolated from a bacteriophage that attacked *Streptococcus cremoris* 114. The mutant was able to adsorb and grow on *S. cremoris* 266, where the parent phage could not. The mutant phage was unable to adsorb to the original bacterial host, *S. cremoris* 114. The change in host range was accompanied by an alteration in the neutralization antigen as shown by a change in neutralization rate by an anti-phage serum. Serum-blocking experiments confirmed the difference in neutralization antigen between parent and mutant phages. The two phages nevertheless had similar complement fixation antigens, confirming that one was a mutant derived from the other. A distinction between complement fixation and neutralization antigens, similar to that found for the coliphages and staphylococcal phages, has therefore been demonstrated for two lactic streptococcal phages.

Lactic streptococcal phages are of considerable commercial importance since they are the most important single factor that inhibits starter cultures in most milk fermentations. Electron microscope studies (7, 13, 15) have shown that these phages belong to Bradley's group B (3), and they have been further differentiated by host range and serology (6, 15, 16). In an earlier investigation (6) it was shown that most morphologically similar phages were related serologically, in that they were neutralized by the same antiserum, but that they could be further subdivided according to K values (1). These subgroups showed a good correlation with host range.

Streisinger (12), working with the T-even coliphages, found that differences in host range and in susceptibility to neutralizing antisera were determined at the same genetic locus. This suggests that structural alterations of the phage tail that alter its ability to adsorb to a bacterial cell may also alter the neutralization antigen and be reflected by a change in K value. To determine whether such changes occurred in lactic streptococcal phages, a host range mutant phage was isolated, and a comparison of its adsorption pattern and serology was made with the parent phage.

MATERIALS AND METHODS

Bacterial strains. Five *S. cremoris* and one *S. lactis* (ML_8) strains from the Institute collection were used. Strains were maintained in reconstituted skim milk and subcultured once in M17 broth (14) before use.

Phages. Phages were isolated from cheese factory wheys or were mutants, as indicated. Phages are designated by the prefix ϕ and the number in the Institute collection, followed by the number of the propagating bacterial strain in parentheses. The host range mutant isolated on strain 266 was designated $\phi 1033h^+(266)$.

Phage propagation, concentration and purification. Phage stocks were prepared as follows. The host strain was grown in M17 broth to an optical density of 0.1 at 600 nm (Bausch and Lomb Spectronic 20 spectrometer). Phage obtained initially from single plaques was added to a final concentration of 10^6 plaque-forming units (PFU) per ml, $CaCl_2$ was added to a final concentration of 0.01 M, and the culture was incubated at 30°C until cleared (3 to 5 h). Bacteria were removed by centrifugation at 16,300 × g for 10 min. To obtain purified phage preparations, polyethylene glycol 4000 (10%, wt/vol) and sodium chloride (0.5 M) were added to the phage lysate, and after 1 h at 4°C the lysate was centrifuged at 10,400 × g for 10 min. The pellet was suspended in 5 ml of 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.0) containing 0.1 M NaCl and dialyzed against the same buffer overnight at 4°C. This phage suspension was purified on a 10 to 30% sucrose gradient, and the fractions containing $>10^9$ PFU/ml were bulked, dialyzed, and filtered through a 0.45-nm membrane filter (Millipore Corp.).

Antiserum. The antiserum used in these experiments was prepared against $\phi 936(158)$. Three injections, each containing 2 ml of purified phage preparation (10^{10} PFU/ml) and 2 ml of Freund adjuvant, were injected subcutaneously into three rabbits at weekly intervals, and each rabbit was bled 1 week after the last injection. The antiserum then had a K value (1) of 620 min^{-1} against its homologous phage and was stored at −10°C. Absorption of antiserum with cells of bacterial host 158 resulted in no loss of neutralizing or complement-fixing ability.

Neutralization tests. Phage neutralization tests were carried out and the rate of neutralization (K) was determined as described by Adams (1). Phages (10^7 PFU/ml) and antiserum dilutions of 1:100 to 1:10,000 were incubated for 15 min at 37°C, and the percentage of surviving phage was determined.

CF tests. Quantitative complement fixation (CF) tests were carried out basically as described by Lanni (9). Washed sheep erythrocytes were sensitized for 1 h at 37°C with hemolysin at optimal sensitizing concentration. Mixtures containing 0.2 ml of phage, 0.2 ml of antiserum, and 0.1 ml of complement (3× minimal hemolytic dilution) were incubated at 37°C for 1 h. Sensitized erythrocytes (0.2 ml) were added, and the mixture was further incubated for 30 min at 37°C, with shaking at 15-min intervals. Chilled buffer (2.5 ml) was added, and unlysed cells were removed by centrifugation at low speed. The degree of complement fixation was read as light transmission of the supernatants at 541 nm (Unicam SP 500 spectrophotometer).

Phage adsorption. Bacterial cells were grown to an optical density of 0.60 at 600 nm. Adsorption was carried out at 37°C by mixing 0.9 ml of bacterial culture containing 0.01 M Ca^{2+} with 0.1 ml of phage lysate to give a final multiplicity of infection of 0.005. After 10 min, 0.1 ml was transferred to 9.9 ml of chilled diluent and centrifuged at 10,400 × g for 10 min. The supernatant was assayed in duplicate, both undiluted and diluted 1:10, and the adsorption was expressed as a loss in phage titer as compared with the control (phage only) assay. Adsorption of less than 20% was not considered significant.

Electron microscopy. Carbon-coated Formvar grids were immersed for 3 min in the phage preparation, excess sample was removed by touching to filter paper, and the grids were immersed in stain (equal parts of 2% potassium phosphotungstate and 2% ammonium molybdate, final pH 5.0) for 3 min. Grids were air dried on filter paper and examined in a Philips 200 electron microscope. Photographs were taken at 128,000 magnification.

RESULTS

ϕ1033(114) was isolated on strain 114 from a cheese factory whey during the course of routine phage monitoring of wheys. A phage stock (2.7×10^8 PFU/ml) was prepared by replating five times from a single plaque and propagating on strain 114. This phage lysate produced 16 PFU/ml when plated on strain 266. From these plaques, it was possible to replate phage that would grow on strain 266 with a high efficiency. This phage was replated from single plaques five times on strain 266 and propagated to produce a phage stock, $\phi 1033h^+(266)$.

In this way, phage stocks of each of the two phages were prepared. Lysates of ϕ1033(114), which had titers of 2.7×10^8 to 2.4×10^9 PFU/ml on strain 114, produced 1 to 16 PFU/ml on strain 266. Lysates of $\phi 1033h^+(266)$ with titers of 1.6×10^9 to 5×10^9 PFU/ml on strain 266 produced 20 to 70 PFU/ml on strain 114. The isolation frequency of the mutant phages was therefore between 10^{-8} and 10^{-10}.

Electron microscopy. Electron micrographs showed that both phages were of type A as described by Jarvis (6), having an isometric head 52 to 54 nm in diameter, a noncontractile tail 148 to 154 nm in length, and no visible collar. There was no detectable difference in morphology between ϕ1033(114) and $\phi 1033h^+(266)$.

Phage neutralization. ϕ936(158) is of the same morphological type as ϕ1033(114) and $\phi 1033h^+(266)$, and it has been found that phages that grow on strains 158 and 266 are closely related serologically (6). Therefore, antiserum prepared against ϕ936(158), designated antiserum ϕ936(158), was suitable for testing the serological characteristics of the phages. Figure 1 shows the difference between neutralization curves for ϕ1033(114) and $\phi 1033h^+(266)$. ϕ1033(114) was not readily neutralized by the antiserum, 56% surviving incubation with antiserum diluted 1:100. In contrast, $\phi 1033h^+(266)$ was rapidly neutralized by the antiserum, only 2% surviving incubation with antiserum diluted 1:1,000. K values for the antiserum with each of these two phages were 1 for ϕ1033(114) and 52 for $\phi 1033h^+(266)$, respectively (K = 100 with homologous phage).

Phage adsorption. Adsorption experiments were carried out with cells of strains 114 and 266 and the two phages. ϕ1033(114) showed a high degree of adsorption to strain 114 (93%) and very little adsorption to 266 cells (0 to 13%). $\phi 1033h^+(266)$ adsorbed well to 266 cells (72 to 92%) and showed insignificant adsorption (0 to 12%) to 114 cells. This indicated that, with these two phages, the change in host range resulted from a change in the adsorption ability of the phage. To check whether lack of ability to adsorb was in general the limiting factor in the propagation of lactic streptococcal phages, adsorption tests were carried out for 40 phages on four bacterial strains (Table 1). These repre-

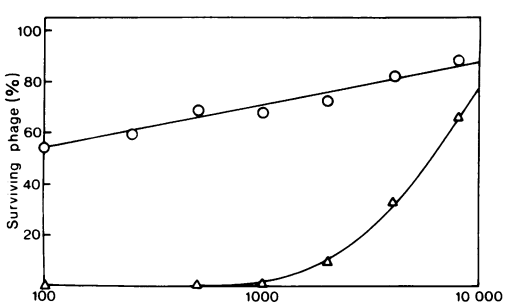

FIG. 1. *Neutralization of ϕ1033(114) and $\phi 1033h^+(266)$ by antiserum ϕ936(158). Symbols:* (○) *ϕ1033(114);* (△) *$\phi 1033h^+(266)$.*

sented 40 phages isolated, from factory wheys, on 16 different bacterial strains. The phages used did not include any more than two isolates similar both in morphology and in the bacterial strains which they attacked. In 87 out of 127 tests (68%), representing 37 different phages, the phage did not adsorb significantly to the bacterial host, indicating that in these instances inability to propagate on the bacterial strain was due, at least in part, to inability to adsorb. In the four instances where adsorption was less than 20%, but the phages grew on the indicator strains, they did so with a low efficiency.

CF tests. Purified preparations of ϕ1033(114) and ϕ1033h^+(266), having titers of 5×10^{10} PFU/ml, were obtained by polyethylene glycol–NaCl concentration and sucrose gradient centrifugation. These purified phages were used for CF tests with antiserum ϕ936(158). Almost identical results were obtained with the two phages (Fig. 2), indicating that they had similar CF antigens.

Antiserum used for CF experiments was heated at 56°C for 30 min. Since CF and neutralization data were to be compared with one another, the neutralization experiments were repeated by using the same antiserum preparation that was used in CF experiments and by using purified phage preparations instead of phage lysates. The results were similar to those obtained earlier, again showing a marked difference in neutralization rates for the two phages.

Serum-blocking power. Serum-blocking tests were carried out to determine whether the antigens in the two phages combined with the same antibody sites.

TABLE 1. *Adsorption and host range data for 40 phage isolates with four strains of lactic streptococci*

Bacterial strains	Phages able to propagate on strains		Phages not able to propagate on strains	
	Adsorption >20%	Adsorption <20%	Adsorption >20%	Adsorption <20%
AM_1	8	2	16	14
ML_8	7	0	13	20
112	8	2	8	22
158	6	0	3	31
Total tests	29	4	40	87
Number[a] of phages represented	21	4	33	38

[a] The number of phages in each category is less than the number of tests, some phages reacting with more than one host.

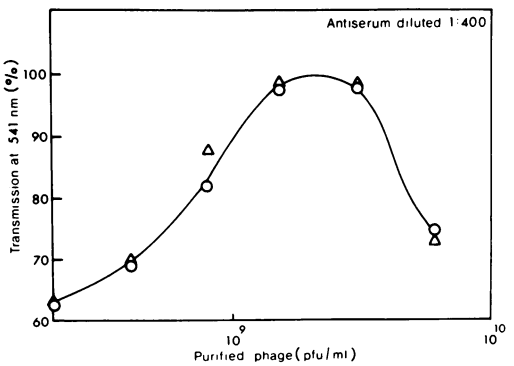

FIG. 2. *CF by ϕ1033(114) versus antiserum ϕ936(158) and ϕ1033h^+(266) versus antiserum ϕ936(158). Symbols:* (○) *ϕ1033(114);* (△) *ϕ1033h^+(266).*

Antiserum diluted 1:400 was absorbed with ϕ1033(114) or ϕ1033h^+(266) for 2 h at 37°C, then kept at 4°C for 16 h, and the phage-antibody complex was removed by centrifugation at 105,000 × *g* for 3 h. These absorbed sera were then used for CF and neutralization tests with ϕ1033h^+(266). Blocking antiserum with either phage completely blocked CF by ϕ1033h^+(266)-antibody complex, at two different antiserum dilutions, confirming that the CF antigens in the two phages were the same (Fig. 3). However, antiserum absorbed by ϕ1033(114) still neutralized ϕ1033h^+(266), although, as would be expected, antiserum absorbed with the latter phage lost neutralizing ability against the same phage (Fig. 4).

Preincubation of phage stock with antiserum. On four occasions stocks of ϕ1033(114), from which it was possible to isolate phages that attacked strain 266, were incubated with antiserum ϕ936(158) (1:400 dilution) for 15 min at 37°C, before being spotted on strain 266. The control phage stocks, without antiserum, gave 10, 5, 1, and 1 plaques, respectively, on strain 266 in four separate experiments. No plaques were detected on strain 266 after preincubation with antiserum, indicating that the phage that could propagate on strain 266 was in the phage preparation before growth on 266 and that the changes detected in ϕ1033h^+(266) had not arisen as a result of growth through strain 266.

DISCUSSION

Host range mutants of bacteriophages have been reported since 1929 (1), but in most cases serological data were not included as proof that the new phage was a mutant and not a contaminant. The mutant reported in this paper was isolated on numerous occasions after repeated purification by replating of the parent strain,

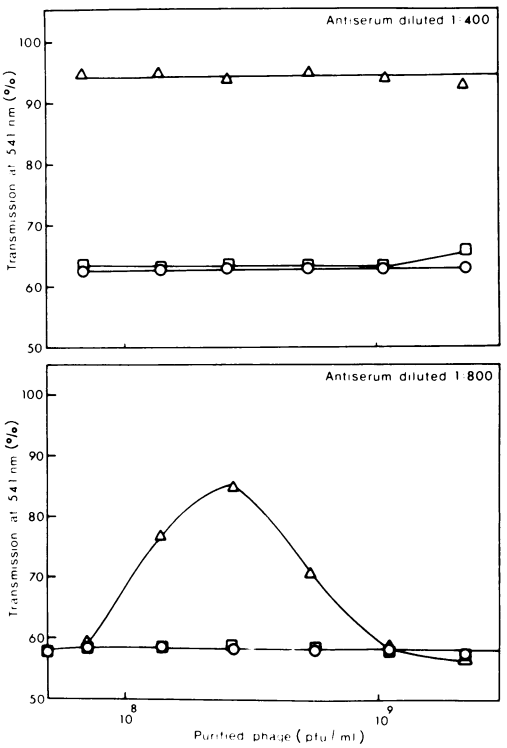

FIG. 3. *CF by $\phi 1033h^+(266)$ versus antiserum $\phi 936(158)$ at two different antiserum concentrations. Symbols: (△) antiserum not absorbed; (□) antiserum absorbed with $\phi 1033h^+(266)$; (○) antiserum absorbed with $\phi 1033(114)$.*

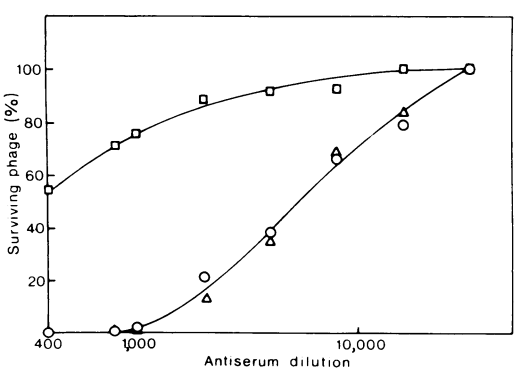

FIG. 4. *Neutralization of $\phi 1033h^+(266)$ by antiserum $\phi 936(158)$. Symbols: (△) unabsorbed antiserum; (□) antiserum absorbed with $\phi 1033h^+(266)$; (○) antiserum absorbed with $\phi 1033(114)$.*

and was shown by CF tests to be serologically identical to the parent strain. Electron microscopy showed that the two phages, $\phi 1033(114)$ and $\phi 1033h^+(266)$, were identical in morphology.

Host range mutants have been commonly found to arise through a change in the ability to adsorb to a bacterial strain, although phage mutants which are able to overcome immunity produced by lysogeny have also been reported (5). In the present study a host range mutant, $\phi 1033h^+(266)$, was isolated which was able to grow on strain 266 after an alteration in the parent phage $\phi 1033(114)$ allowed it to adsorb to strain 266. At the same time, it lost the ability to adsorb to strain 114. This change in adsorption pattern was accompanied by a dramatic change in the rate of neutralization of the phage by antiserum prepared against a morphologically similar phage, the *K* value increasing from 1 to 52% (compared with 100 for homologous phage). The difference in neutralization antigens was confirmed by serum-blocking tests. Preincubating either phage with the antiserum prevented the antiserum from subsequently neutralizing the same phage, but did not affect its ability to neutralize the other phage.

Unlike neutralization tests, which involve only the tail antigens, CF antigens are related to the total phage, and particularly to the head antigen (8). If $\phi 1033h^+(266)$ were derived from $\phi 1033(114)$ by a mutation which altered the structure of the tail antigen, it would be expected that the head antigen would remain unchanged. This was indeed the case. The two phages gave similar CF with antiserum $\phi 936(158)$. Blocking the antiserum with either $\phi 1033(114)$ or $\phi 1033h^+(266)$ prevented the antiserum from subsequently reacting with $\phi 1033h^+(266)$ as determined by CF; i.e., the CF antigens in the two phages were identical. A similar distinction between neutralization and CF antigens has been reported for coliphages (8) and staphylococcal phages (11).

The finding that a change in host range was accompanied by a change in neutralization antigen was in agreement with data from an earlier investigation (6), which showed that phages that could be propagated on a particular bacterial strain were similar to one another in their rate of neutralization by the same antiserum. Streisinger (12) concluded that differences in host ranges of T_2 and T_4 phages and their serological specificity as shown by neutralizing antisera are determined at the same genetic locus. Anderson (2) showed that the neutralization antigen is located in the phage tail. He also found that neutralization by antiserum may prevent a phage from adsorbing to its bacterial host, or may allow adsorption but prevent injection of phage DNA. Delbruck (4) reported phages were immune to antiserum once they had adsorbed to a bacterial cell, and this phenomenon is widely used in adsorption experiments (1). Thus, al-

though the neutralization antigen and adsorption site in a phage may not be identical, there is obviously a close relationship between the two. The simultaneous change of host range, adsorption, and serology as determined by neutralization tests reported here support this conclusion.

In this investigation, it was possible that the change in neutralization of the phage after propagation in strain 266 was due to a host-controlled alteration in the phage. However, preincubation of ϕ1033(114) with antiserum ϕ936(158), which neutralized ϕ1033h^+(266), and the subsequent failure to detect ϕ1033h^+(266) by plating ϕ1033(114) on strain 266, indicated that an alteration in the parent phage occurred before propagation on strain 266.

Rountree (11) found that an alleged phage mutation was in fact a phage released by a lysogenic indicator strain. However, in the present study repeated attempts to induce strain 114 by UV light and mitomycin C failed to produce phage that could be propagated on strain 266. In addition, no spontaneously released phage could be detected in the supernatants of cultures of strain 114, indicating that the mutant detected was not produced by the release of temperate phage.

The frequency of production of the mutant phage was 10^{-8} to 10^{-10}, in agreement with those reported for host range mutants (10). This frequency is much less than would be expected if a modification-restriction system were operating, when the efficiency of plating might be expected to be of the order of 10^{-3} to 10^{-6}. In addition, a change in efficiency of plating due to modification restriction would not be expected to be accompanied by structural or serological changes in the phage.

It can therefore be concluded that a host range mutant ϕ1033h^+(266) has been isolated from ϕ1033(114), and that the change in host range was due to a change in the ability of the phage to adsorb to a different indicator strain. It is suggested that the genetic change which resulted in the structural alteration in the adsorption site of the phage also resulted in a change in the structure of the neutralization antigen, and that these occupy closely related, if not identical, positions on the phage.

In cheesemaking, mutant phages with the ability to grow on starter strains other than those which propagated the parent phage are likely to arise if high numbers of phage are present. Electrophoretic protein patterns of *S. cremoris* 114 and 266 showed that these strains are very similar (Jarvis and Wolff, unpublished data), which presumably is the reason why the phage that grew on strain 114 was able to mutate readily to grow on strain 266. This suggests that the appearance of host range mutant phages is more likely to present a problem if the starter strains used in a cheese plant are closely related.

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