The Characterization of a Series of *Lactobacillus* Bacteriophages

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SUMMARY

The properties of four *Lactobacillus casei* phages and eleven *L. fermenti* phages are presented. Most of these phages are species specific; some have a limited intraspecies action. The two groups of phages are serologically distinct, but the members of each group are related serologically. All the phages have slow adsorption rates. The *L. casei* phages are citrate insensitive, while some of the *L. fermenti* phages are citrate sensitive. The *L. fermenti* phages have shorter latent periods than the *L. casei* phages and this correlates directly with the generation times of their hosts. The phages are large and have a tadpole appearance.

INTRODUCTION

The first phage active on lactobacilli was reported by Kopeloff (1934); this was isolated from sewage. No further details were provided apart from the statement that it attacked certain strains of *Lactobacillus acidophilus* and *L. bulgaricus* and did not attack R variants of these strains. Kiuru & Tybeck (1955) isolated two phages from starters used in the manufacture of Swiss cheese. These phages had very restricted host ranges. The one was active on a strain of *L. lactis* and the other on two strains of *L. helveticus*. Meyers, Walter & Green (1958) isolated from saliva a phage active on a strain of *L. casei*; this phage was characterized by Walter (1958). Coetzee, de Klerk & Sacks (1960) and Coetzee & de Klerk (1962) reported the isolation of 14 *Lactobacillus* phages from sewage; subsequently one more phage (no. 780) active on strains of *L. casei* was isolated, also from sewage. This paper reports the properties of these 15 phages.

METHODS

Media. The media used have been described (Coetzee *et al.* 1960; de Klerk & Coetzee, 1962).

Phages. The methods used for the assay of phage, the preparation and assay of antiphage sera, the determination of adsorption rates, latent periods and burst sizes and the isolation of phage-resistant mutants were those of Adams (1959). The phages are identified according to the number of the original host organism. These

are 41, 69, 206, 222, 222a, 276, 315, 514, 517, 535, 547 isolated on *Lactobacillus fermenti* hosts and 300, 316, 356, 780 isolated on *L. casei* hosts. Phage lysates were prepared by inoculating 40 ml. of a young broth culture from a plaque. After overnight incubation the cultures were clear and had plaque forming titres of about 1×10^9 /ml. Phage suspensions were stored above 0.1 vol. of chloroform at 4°. In some adsorption experiments the broth was supplemented with vitamin-free pancreatic casein digest (Nutritional Biochemicals Co., Cleveland, Ohio) 10 g./l. and/or 50% (v/v) of Hanks balanced salt solution (Weller, Enders, Robbins & Stoddard, 1952). In other experiments Tween 80 was omitted from all media. The influence of sodium chloride on the stability of the phages was tested by the method of Kiuru & Tybeck (1955).

Citrate sensitivity. The citrate sensitivity of the phages was expressed as their relative efficiency of plating (e.o.p.) on media supplemented with 2% (w/v) sodium citrate.

Heat inactivation. Heat inactivation constants were determined by maintaining the phages at 55° in broth and assaying samples at intervals. The samples were brought to 20° before plating.

Electron microscopy. Phage lysates were purified and concentrated by differential centrifugation (alternate cycles at 12,350g for 90 min. and 3090g for 10 min.) in a Servall centrifuge (model SS-4). Purified suspensions (plaque forming titre 1×10^{12} /ml.) were sprayed on to collodion-covered grids, air dried, shadowed with a gold + palladium alloy and examined with a Philips (Model EM 100) electron microscope.

Organisms. In addition to the host strains of lactobacilli used, 37 strains of *Lactobacillus acidophilus*, 14 of *L. casei*, 11 of *L. casei* var. *rhamnosus*, 12 of *L. salivarius*, 29 of *L. plantarum*, 27 of *L. brevis*, 35 of *L. fermenti* and one of *L. helveticus* strains were used in host range experiments; they were identified by the methods previously employed (Coetzee *et al.* 1960).

Generation times. The generation times of the lactobacilli were determined by total counts (de Klerk & Coetzee, 1962).

RESULTS

All the phages were stable in the presence of chloroform, thus differing from a series of *Clostridium perfringens* phages (Smith, 1959) and Brucella phages (McDuff, Jones & Wilson, 1962).

Electron microscopy. Electron micrographs were taken of the four *Lactobacillus casei* phages and four of the *L. fermenti* phages. These revealed tadpole morphologies. The *L. casei* phages had larger heads (average dimensions 105×87 m μ) than the *L. fermenti* phages (average dimensions 83×64 m μ). The tails of the former were slightly shorter and thicker than the *L. fermenti* phages (average dimensions 194×21 m μ and 210×18 m μ respectively). The average over-all length of the phages was nearly the same (299 and 293 m μ respectively.) All the phages showed terminal knobs like *Listeria monocytogenes* phages (Sword & Pickett, 1961).

Adsorption rates. The adsorption of all the *Lactobacillus* phages followed first-order reactions. The *L. fermenti* phages all adsorbed slowly (Table 1) and their adsorption rates could not be correlated with their other properties. The *L. casei*

phages (Table 2) also had slow adsorption rates, with the exception of phage 356, which differed in many respects from the other *L. casei* phages. The adsorption of all the phages was unaffected by the omission of Tween (White & Knight, 1958) or the addition of co-factors to the media. The optimal concentration of organisms was 1.0×10^9 colony forming units/ml.; smaller values than this slowed the adsorption rate.

Table 1. *Some properties of Lactobacillus fermenti phages*

Phage no.	E.o.p. on citrate media	Growth constants at 37°		Adsorption constants (ml./min.) at 37°	Heat inactivation constants at 55°
		Latent period (min.)	Burst size		
41	1	83	82	2.3×10^{-10}	0.044
315	1	77	60	1.5×10^{-11}	0.156
514	1	85	88	1.5×10^{-11}	0.620
222	1	85	63	4.0×10^{-10}	0.643
222a	1	97	30	2.8×10^{-11}	0.098
69	0.0001	99	35	5.7×10^{-12}	0.088
206	0.0002	72	100	5.7×10^{-11}	0.066
517	0.0001	72	90	2.6×10^{-11}	0.087
535	0.0001	85	73	1.6×10^{-11}	0.093
547	0.0003	87	60	1.6×10^{-11}	0.112
276	0.0001	72	70	1.2×10^{-11}	0.095

Table 2. *Some properties of Lactobacillus casei phages*

Phage no.	E.o.p. on citrate media	Growth constants at 37°		Adsorption constants (ml./min.) at 37°	Heat inactivation constants at 55°
		Latent period (min.)	Burst size		
300	1	220	20	5.0×10^{-12}	0.190
780	1	220	20	2.1×10^{-11}	0.204
316	1	220	6	3.3×10^{-11}	0.442
356	1	180	33	3.2×10^{-9}	0.104

Plaque morphology. The *Lactobacillus casei* phages formed minute clear plaques of average diameter 0.3 mm. and a shelving edge. The *L. fermenti* phages formed similar but larger plaques. Phages 41, 69, 276, 535 and 547 formed plaques of average diameter 0.5 mm. and phages 206, 222, 222a, 315, 514 and 517 formed plaques of diameter 1 mm. The plaques of individual phages varied somewhat in size and this variation may be explained (McDuff *et al.* 1962) as due to vagaries of adsorption. The phages are large and the smallness of the plaques was ascribed to this factor, but the slow adsorption rates may be a contributory factor (McDuff *et al.* 1962).

Serology. The inactivation of the phages by antisera followed first order kinetics; Table 3 shows the neutralization constants of the phage antisera. These constants are low. A second series of rabbits was immunized with the phages but their sera yielded lower K values. Table 3 shows that the *Lactobacillus casei* phages and *L. fermenti* phages are serologically distinct and are classified into groups I and II respectively.

Host range. The results of host range experiments have already been reported (Coetzee *et al.* 1960; Coetzee & de Klerk, 1962). These indicated that the phages were not only genus specific but practically species specific. The present results confirmed these findings. Individual *L. fermenti* phages productively lysed 25–40% of the 35 *L. fermenti* organisms and caused abortive infection with host killing in 14·3–66% of these organisms. The *L. casei* phages productively lysed 12–40% of the 25 *L. casei* spp. tested, and caused abortive infection with host death in 15% of these organisms. A few of the *L. fermenti* phages caused abortive infections with host killing of 3 *L. acidophilus*, 3 *L. casei* and 3 *L. brevis* organisms, and productive

Table 3. *Neutralization constants of Lactobacillus phage antisera at 37°*

Dilutions of homologous and heterologous phage antisera were mixed with the phages at 37°. Phage was assayed at intervals. The proportion of phage surviving was plotted against time. The survival was a first order reaction and from this neutralization constants were calculated.

Phage antiserum no.	Phage no.														
	41	69	206	222	222a	276	315	514	517	535	547	300	780	316	356
	Minutes ⁻¹														
41	4	3	2	4	4	4	4	4	4	4	4	0	0	0	0
69	8	11	3	10	10	6	9	8	10	10	10	0	0	0	0
206	8	5	9	6	6	7	2	3	4	3	2	0	0	0	0
222	12	12	5	11	11	7	12	11	13	11	12	0	0	0	0
222a	15	14	9	12	21	17	13	16	16	18	7	0	0	0	0
276	3	5	8	4	4	8	4	3	5	4	3	0	0	0	0
315	3	3	1	3	3	3	9	4	5	4	2	0	0	0	0
514	3	1	1	2	4	1	4	2	3	4	3	0	0	0	0
517	4	5	2	5	6	3	7	4	4	3	5	0	0	0	0
535	10	8	4	8	8	8	10	9	14	12	6	0	0	0	0
547	3	3	6	5	3	2	3	3	3	4	6	0	0	0	0
300	0	0	0	0	0	0	0	0	0	0	0	76	60	3·6	1·2
780	0	0	0	0	0	0	0	0	0	0	0	29	30	3·0	1·0
316	0	0	0	0	0	0	0	0	0	0	0	17	12	72	29
356	0	0	0	0	0	0	0	0	0	0	0	0·80	2·0	10	16

L. fermenti phages.
Serological group II

L. casei phages.
Serological group I

infections in 2 *L. casei* and 3 *L. brevis* strains. The *L. fermenti* host no. 296 on which *L. casei* phage 300 caused lytic infection (Coetzee *et al.* 1960) has been lost and extra-species activity of the *L. casei* phages is limited to the action of 3 of these on one or more of 3 *L. plantarum* organisms. The host ranges of the eleven *L. fermenti* phages differ from one another, while those of two of the *L. casei* phages (nos. 300, 780) are identical. With the exception of the few inter-species abortive and productive infections just mentioned, host range experiments support the serological grouping of these phages into two distinct groups.

Citrate sensitivity. The *Lactobacillus casei* phages did not require Ca²⁺ for adsorption (Table 2). *L. fermenti* phages 41, 222, 222a, 315 and 514 had an e.o.p. value of 1 in the presence of sodium citrate, while phages 69, 206, 276, 517, 535 and 547 had a plating efficiency of 10⁻⁴ (Table 1).

Heat inactivation. Heat inactivation of the phages at 55° followed first order

kinetics; the constants varied from 0.044 to 0.648 (Tables 1, 2). These values did not correlate with other properties of the phages.

Latent period. The latent periods of the *Lactobacillus casei* phages at 37° ranged from 180 min. (phage 356) to 220 min. (phages 316, 300 and 780); the burst sizes were below 50 (Table 2). The latent periods of all the *L. fermenti* phages were less than 100 min. (Table 1) and the burst sizes were between 50 and 100 (Table 1). The latent periods correlated directly with the generation times (McDuff *et al.* 1962). The generation time of *L. casei* organisms at 37° was 62 min. as compared with 50 min. for the *L. fermenti* organisms. By premature lysis of the host organisms with chloroform (Séchaud & Kellenberger, 1956) *L. casei* phage could be detected at 85 min. and *L. fermenti* phages were demonstrated in this manner after 60 min.

Effect of sodium chloride. Like the phages of Kiuru & Tybeck (1955) the *Lactobacillus casei* phages showed no significant decrease in plaque forming units when suspended in 25 % (w/v) NaCl solution for 2 days, while the *L. fermenti* phages were unstable. The titre of all the phages of this latter group decreased by more than 90 % under these conditions.

Table 4. *Cross-resistance grouping of citrate-insensitive Lactobacillus fermenti phages*

Phages were added to organisms and after adsorption for 15 min. unadsorbed phage was assayed. The contents of the adsorption tube were then diluted 1/100 in fresh pre-warmed broth and maintained at 37°. Samples were assayed at intervals.

Bacteria no.	Phage no.		
	41	315	514
41/ 41	—	—	+
315/315	+	—	—
514/514	+	+	—

+ = Productive infection; — = adsorption, but no phage production.

Table 5. *Cross-resistance grouping of citrate-sensitive Lactobacillus fermenti phages*

Phages were added to organisms and after adsorption for 15 min. unadsorbed phage was assayed. The contents of the adsorption tube were then diluted 1/100 in fresh pre-warmed broth and maintained at 37°. Samples were assayed at intervals.

Bacteria no.	Phage no.				
	69	206	517	535	547
69/ 69	—	+	+	+	+
517/517	—	+	—	+	—
547/547	+	+	+	+	—
206/206	—	—	+	—	—
535/535	—	—	—	—	—

+ = Productive infection; — = adsorption, but no phage production.

Cross-resistance tests. Cross-resistance tests afforded another method of distinguishing phages with similar properties. Phage-resistant mutants of *Lactobacillus casei* strains 300 and 780 and of *L. fermenti* strains 41, 69, 206, 514, 517, 535

and 547 were obtained on first attempt and are presumably one-step mutants. The phages adsorbed at the same rate to the resistant mutants, but no progeny phage was liberated and the bacteria were unaffected (Garen & Puck, 1951). All the mutants were of this type. No phage non-adsorbing mutants of these strains were obtained. Resistant mutants of *L. casei* strain 316 and *L. fermenti* strains 222, 222a, 276 were not obtained despite repeated attempts at selection. *L. casei* phages 300 and 780 could not be differentiated by cross-resistance tests and, as the other properties examined are similar, they are presumably identical. Table 4 shows that *L. fermenti* 41/41 is also resistant to phage 315. *L. fermenti* 315/315 is also resistant to phage 514 but is lysed by phage 41. *L. fermenti* 514/514 is lysed by phages 41 and 315. Table 5 presents the results of cross-resistance tests with a group of citrate-sensitive *L. fermenti* phages. Some of the mutants again show multiple resistance (Demerec & Fano, 1945) but the lytic pattern enables phages 69, 517 and 514 to be differentiated from one another. Phages 206 and 535 have the same host range on these mutants but can be differentiated by plaque size.

DISCUSSION

In recent years interest has been shown in phages other than those which attack organisms of the family Enterobacteriaceae. Smith (1959) described phages which attacked strains of *Clostridium perfringens*; Sword & Pickett (1961) described *Listeria monocytogenes* phages. Brucella phages were described by McDuff *et al.* (1962) and Bacillus phages have been characterized by Meynell (1962). A revival of interest has also been shown in the mycophages, Streptococcus phages and Staphylococcus phages (Groman, 1961). All these phages have properties of a similar nature to the T phages. The Lactobacillus phages are no exception. The characteristics of the four phages isolated on homofermentative hosts correspond to descriptions of the *Lactobacillus casei* phage characterized by Walter (1958). Thus Walter's phage has the same over-all length as our *L. casei* phages, although the tail is decidedly shorter and lacks a terminal knob. His phage also forms small plaques and has a host range limited to one strain of *L. casei*. The latent period (140 min.) is shorter than the latent periods of our *L. casei* phages but the burst size is similar. The long latent periods of the *L. casei* phages approach those of certain mycophages (Bowman & Redmond, 1959). Walter's phage had the same heat sensitivity as our *L. casei* phages. Apart from *L. casei* phage 356, the other phages investigated had slow adsorption rates which could not be increased. Walter (1958) encountered the same phenomenon with his phage. Our media contained Tween 80 and it was hoped to increase the rate by omitting it (White & Knight, 1958). This omission had no effect. Kiuru & Tybeck (1955) investigated their phages along different lines, but their two phages were relatively stable at 55° and like our *L. casei* phages were also not inactivated by 25% (w/v) NaCl solution.

The phages isolated on heterofermentative hosts differ in some respects from the *Lactobacillus casei* phages. The most striking properties of the former phages are the larger plaques, the very much shorter latent periods and their inactivation by 25% (w/v) NaCl solution. These eleven phages are serologically related but are not homogeneous as regards citrate sensitivity.

The Lactobacillus phages usually have only a limited intra-species host range.

No phage like phage α (Meynell, 1962), which lyses all strains of *Bacillus anthracis*, was isolated. The limited host-range of the phages may be helpful in devising a phage-typing scheme for lactobacilli; but, despite repeated attempts, no phages which productively lyse *L. acidophilus* or *L. salivarius* strains have been isolated from sewage, vaginal secretions or saliva. We have also not succeeded in primarily isolating phages on strains of *L. plantarum* and *L. brevis*.

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