APPLIED MICROBIOLOGY, Sept. 1971, p. 463-468

Vol. 22, No. 3
Printed in U.S.A.

Characterization of Two Psychrophilic *Pseudomonas* Bacteriophages Isolated from Ground Beef[1]

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Received for publication 13 May 1971

Characterization studies were performed on two psychrophilic phages which were isolated from ground beef samples. Phage inactivation by exposure to heat, low *p*H, osmotic shock conditions, and freezing showed that these two isolates were different. One-step growth experiments indicated that one isolate had a burst size five times as large (500) and a latent period two times as long (4 hr) as the other when tested at 7 C. Nucleic acid type was 2-deoxyribonucleic acid for both. Electron micrographs showed one to belong to Bradley's phage group A and the other to phage group C.

Spencer (16) reported the isolation of a bacteriophage specific for a luminous marine bacterium which formed plaques at or near 0 C. More than a decade later Olsen (12) also reported the isolation of phage of a psychrophilic *Pseudomonas*. Olsen et al. (13) later characterized this and other isolates obtained from sewage which grew on psychrophilic *Pseudomonas* hosts.

Bacteriophages capable of lysing psychrophilic hosts have been shown to be normally present within refrigerated food products (2, 7). The purpose of this study was to obtain basic information on these two phage-host systems preliminary to studies concerning phage-host interactions in refrigerated food products.

MATERIALS AND METHODS

Bacteriophage isolation. Methods involved have been published in a companion paper (18). The two phages selected, wy and ps_1, were felt to be representative of those obtained from the refrigerated food products examined.

Hosts. Both host bacteria were classified into group II of Shewan et al. (15). They were tentatively classified as *P. fragi* (18). Host WY was isolated from hamburger, and PS_1 was isolated from pork sausage. Cultures were held at 4 C on slants of Trypticase soy agar (TSA; BBL).

Quantitation. The agar layer method described by Adams (1) was one of the two counting procedures used (herein referred to as the double-layer method). During part of this research, the agar layer method was modified as described by Rizvi and Mora (14) by omitting the base layer of medium. The volume of the soft agar (TSA) layer was increased to 3.5 ml and that of the host culture was increased to 0.25 ml. This procedure was used only in the inactivation studies and was referred to as the single-layer method.

Propagation. Pure stock phage was diluted to a concentration that would produce overlapping plaques on plates when mixed with indicator cells. Diluted phage, 0.5 ml of host cells, and 7 ml of soft agar supplemented with calcium were poured into 15-cm petri plates and incubated for 12 to 15 hr at 21 C. The agar was scraped into a sterile beaker, and the remaining residue was rinsed from the plate into the beaker with 6 ml of Trypticase soy broth (TSB; BBL) before incubation for 3 to 5 hr at 4 C. Centrifugation at 10,000 × *g* followed, after which the supernatant fluid was sterilized by membrane filtration (0.22 μm). Sterile stock suspensions were stored at 4 C.

One-step and single-burst experiments. Host cells were grown in TSB for 20 to 22 hr while the medium was agitated at 7 C. Cell concentration thereupon was adjusted by optical density to 10^8 to 2×10^8/ml. A stoppered adsorption tube (10 by 75 mm), containing 0.9 ml of this host suspension and 0.1 ml of phage suspension [5×10^7 plaque-forming units (PFU)/ml], was agitated on a mechanical shaker (30 rev/min at 7 C for 20 min). A 0.5-ml sample was withdrawn and diluted 10^{-2} (dilution 1, D-1). It was further diluted to 10^{-4} (growth tube 1, GT-1) and 10^{-6} (growth tube 2, GT-2) in TSB at 7 C. Infected bacteria were removed from D-1 by membrane filtration (0.45 μm), and the filtrate was plated to determine the number of free phage after 20 min of adsorption; this figure was used to correct for free phage in GT-1. Samples were taken from GT-1 and GT-2 at either 30-min or 1-hr intervals and plated by the double-layer method for use in constructing the one-step growth curve.

Single-burst experiments were performed in the same manner except that a further dilution was made (10^{-1}) from GT-2 so that when 0.5-ml samples were dispensed into 50 individual sample tubes, each contained on the average 0.3 to 0.4 infected bacteria. These 50 tubes were incubated at 7 C until all bursts had occurred. Soft agar (3.5 ml) and the host (0.25

[1] Contribution from the Missouri Agricultural Experiment Station. Journal series no. 7099.

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ml) were added to each tube; the contents were plated and the plaques were counted.

Effect of temperature. The effect of time of exposure at various temperatures on plaque counts of plated phage was determined. Forty plates were prepared from a single dilution of phage wy at room temperature (23 C). Four were placed immediately at 20 C, whereas two groups of 18 each were placed at 10 and 29 C. At various time intervals up to 150 min, three plates from each group (10 and 29 C) were placed at 20 C. All plates were counted 18 hr after the start of the experiment.

Since it was known that ps_1 was heat-labile, the effect of plating temperature (45 C) on its survival was determined. A phage dilution, which would result in about 150 plaques per plate, was added to 12 tubes (10 by 75 mm) containing soft agar and these tubes were held at 45 C. Samples were plated in duplicate at 2-min intervals up to 10 min, with the host being added 20 sec before plating.

Survival of phages wy and ps_1 to exposure at 60 C over time periods up to 30 min was next determined. A sample of broth containing approximately 10^8 phage per ml was diluted 1:100 by addition to TSB which was being held at 60 C. Samples were withdrawn at time intervals of 1, 5, 10, and 30 min and then diluted into chilled TSB (4 C). Results of phage titrations were compared to titers of controls.

Effect of pH. TSB was adjusted to *p*H levels ranging from 3.0 to 7.0, and 100-fold dilutions of the phage suspension (approximately 10^8 per ml) were made into each. Samples were withdrawn after 1 hr, diluted with phosphate-buffered distilled water (*p*H 7.4), and then plated.

Effect of osmotic shock. Suspensions of about 10^6 phage per ml were exposed for 60 min to 2 M sucrose and 4 M NaCl solutions. Osmotic shock conditions were produced by 100-fold dilution into distilled water. Surviving phage were enumerated by the single-layer method with an additional 10^{-1} dilution being made in distilled water.

Effect of freezing. TSB, skim milk, and meat broth extract in 4.5-ml portions were inoculated with phage to obtain a final concentration of about 10^6 per ml. Preparation of the meat extract broth was as follows: 100 g of ground beef was blended with 200 ml of phosphate-buffered diluent, centrifuged at 5,000 × *g* for 10 min, sterilized by filtration (0.45 μm), and then stored at 4 C. Skim milk was sterilized by heating at 121 C for 10 min.

As a control to the frozen samples, a tube of TSB containing the same volume of phage stock was stored at 4 C. All samples were frozen within 20 min and stored at −20 C until sampled 24 hr later for surviving phage.

Effect of citrates. Citric acid was added at concentrations of 0.1 and 1% to soft agar unsupplemented with calcium. The *p*H was adjusted to 7.0 with 0.1 N NaOH. Soft agar, supplemented with calcium chloride (0.001 M), was used as a control. The phage stock, diluted to produce 100 to 150 PFU per plate, was plated with these media.

Electron microscopy. One-hundred milliliters of sterile high-titer lysate was centrifuged for 120 min at 40,000 × *g*. The pellet was resuspended in 0.1 M phosphate buffer to one-fifth its original volume and allowed to come back into suspension overnight. This suspension was centrifuged at 12,000 × *g* for 10 min. Supernatant fluid was recentrifuged at 40,000 × *g* for 120 min. This pellet was allowed to come back into suspension in 0.1 M ammonium acetate (*p*H 7.0) over an 8-hr period, after which it was filtered (0.45 μm) and stored at 4 C. Specimens of phage were air-dried onto carbon grids. Negative staining was performed on both phages by the method of Eisenstark (8).

TABLE 1. *Summary of results of one-step growth and single-burst experiments with phages ps_1 and wy at 7 C*

Determination	ps_1	wy
Per cent adsorption in 20 min	79	> 99
Latent period (hr)	1.5 to 2	3.5 to 4
Burst size		
One-step growth	118	529
Single burst	84	512
Single-burst experiment (range of PFU/sample tube)	6 to 115	203 to 1168

Type of nucleic acid. The fluorescent staining method of Bradley (4) was modified slightly as follows to determine nucleic acid type. Purified phage stock instead of phage lysate was digested for 2 hr at 37 C in a solution containing 30 μg/ml each of ribonuclease and deoxyribonuclease. To obtain the desired fluorescence, five to six drops of purified solution were needed per spot.

RESULTS AND DISCUSSION

Quantitation. The major part of this research was performed by using the double-layer method (1). However, since the modified method of Rizvi and Mora (14) omitted the foundation layer, thus providing economics in time and expense, this method was tested. No differences were observed in plaque counts (PFU/ml) when these two methods were compared by using phage-host systems ps_1-PS_1 and wy-WY (variations in plaque counts were within ±10%).

Propagation. When using broth methods in propagation with the two phages, the broth never completely cleared, and poor titers resulted. Much higher titers were routinely obtained by the plate method; therefore, this method was used throughout the research for the propagation of high-titer phage stocks.

One-step growth and single-burst experiments. The results of the one-step growth and single-burst experiments are shown in Table 1. The two phages differed greatly in their growth kinetics at 7 C. Phage wy had a latent period twice that of

TABLE 2. *Summary of the responses of phages ps1 and wy to stressful conditions*

Treatment	Percentage reduction as compared to control	
	ps1	wy
Heating at 60 C		
1 min	>99	14
30 min	>99	39
*p*H level		
6.0	0	0
5.0	41	11
4.0	>99	30
3.0	>99	99
Osmotic shock		
4 M NaCl	>99	26
2 M Sucrose	50	48
Freezing in		
Trypticase soy broth	7	0
Skim milk	70	6
Gound beef extract	0	0
Exposure to citrate (0.1%)	30	30

phage ps_1 and a burst size about five times greater. Preliminary one-step growth experiments, performed at 20 C, showed larger burst sizes and shorter latent periods for both phages than were later observed at 7 C. At 7 C, the latent period for ps_1 was approximately three times longer than at 20 C, whereas with phage wy it was twice as long. The rise period at 7 C was extended when compared to that at 20 C. Burst sizes at 20 C showed very large variations from experiment to experiment, although conditions were held constant in all five experiments.

Olsen et al. (13) worked with psychrophilic phages of *Pseudomonas* at a lower temperature (3.5 C). Of five phages, none had a burst size of over 25 or a latent period shorter than 6 hr, whereas at 25 C the burst sizes were much larger and the latent periods were shorter. Also, Olsen (12) observed that the burst size depended upon the temperature used for growing the host cells. Burst sizes were 300 to 350 when cells were grown at 3.5 C as compared to 10 to 40 when they were grown at 25 C. Temperature of infection was 3.5 C. In our experiments, host cells were grown and infected at 7 C, and we observed an unexpectedly large burst size for phage wy (Table 1).

Because of these large burst sizes, a single-burst experiment was used to validate the findings of the one-step growth experiments. This experiment was performed only once for each phage, but values averaged over all tubes in which bursts occurred agreed well with those of the one-step growth experiments for both phages.

Effect of temperature. Both phage ps_1 and wy produced plaques when plates were incubated at 2, 7, and 21 C. Both hosts grew at 32 C, but plaques were not formed. Neither host grew at 37 C in TSA. Diameter of plaques increased as incubation temperature was decreased.

Unexpected variations in plaque counts were observed with phage wy when plates were left at room temperature (25 to 27 C) for more than 1 hr before incubation at 20 C. Plaques were not observed if plates were incubated at 32 C. Therefore, an incubation temperature of 29 C was selected, and the time interval at this temperature was varied. The mean plaque count of the control was significantly higher ($P < 0.05$) than the mean for the treatment at 29 C, but means for the control, placed immediately at 20 C, and the treatment at 10 C were not significantly different. Significant differences were also shown between plaque count means of the treatments at 10 and 29 C. Plates incubated at 29 C for 1 hr or more showed significantly fewer plaques than untreated controls or those held for only 30 min at 29 C ($P < 0.05$). Plaques failed to develop when plates were held for 150 min at this temperature. Incubation for 150 min at 10 C had no effect on the plaque count as compared to the control (20 C).

Because of the heat lability of phage ps_1 at 60 C, it was suspected that plaque counts would be decreased when plates were poured in the usual manner with the medium tempered at 45 C. Our experiments showed that phage survival was markedly decreased by holding the broth suspensions at 45 C for at least 2 min. Decreases in plaques of 15 and 72% occurred when exposure times were 2 and 10 min, respectively. However, this would normally be of little consequence because phages were exposed to this temperature for only a few seconds during the plating procedure. These data and the observations of Olsen et al. (13), which showed that most psychrophilic phages are heat-labile, indicated that this step should be performed with dispatch. Phage ps_1 was very heat-labile when exposed to 60 C, whereas phage wy was relatively heat-insensitive (Table 2). Olsen et al. (13) concluded that thermal sensitivity correlated well with phage psychrophily. This appeared to be true with phage ps_1 but not with phage wy.

Effect of pH. Phage ps_1 was more susceptible to acid inactivation than was phage wy. The effect of low *p*H was exerted at *p*H 5 and below for both phages (Table 2). These values agree well with those of other workers (11, 13).

Effect of osmotic shock. Phage ps_1 showed 99% reduction when osmotically shocked by 100-fold dilution into distilled water from 4 M NaCl,

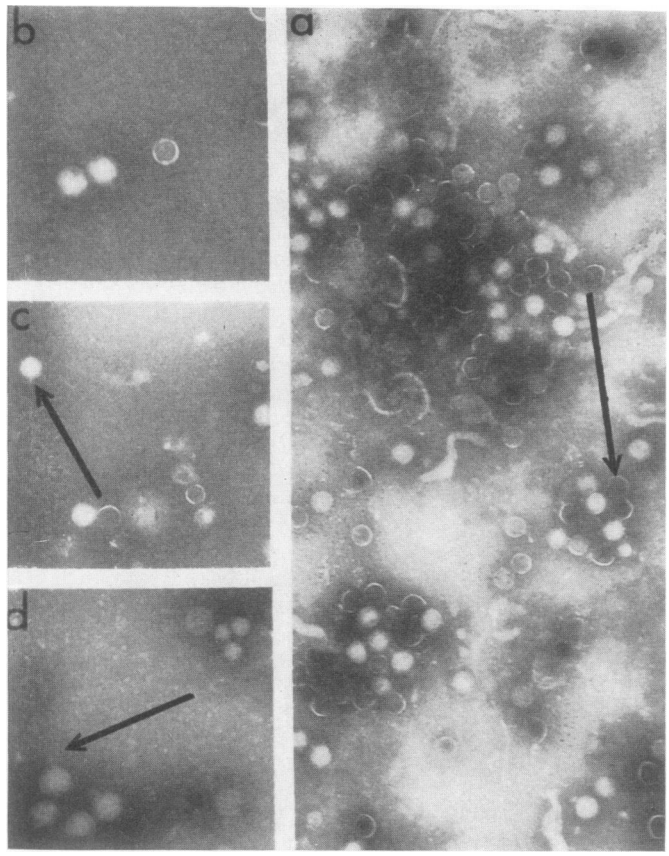

FIG. 1. *Electron micrographs of phage* ps_1. *(a)* × *79,000; (b, c, d)* × *112,000.*

whereas when diluted from 2 M sucrose only 50% of the phage population was inactivated (Table 2). Similar results (48% reduction) were observed for phage wy when it was diluted from sucrose, but dilution from NaCl resulted in only 26% reduction.

Olsen et al. (13) found only one of five psychrophilic phages was osmotically sensitive when diluted from 4 M NaCl, but all five were sensitive when diluted from 2 M sucrose.

Effect of freezing. Nearly 100% of the phages survived frozen storage at −20 C for 24 hr in TSB and in ground beef extract (Table 2). Phage wy survived well in skim milk, but a 70% loss in viability occurred when ps_1 was stored in skim milk. At first it was thought that the phages were inactivated by some constituent of the skim milk, as has been reported to occur when phages of streptococci and staphylococci are titered from skim milk (6, 17). This hypothesis was disproved by comparing titers of ps_1 obtained from skim milk and TSB, which had not been frozen after they received identical inocula of the phage. This procedure was replicated twice with the titers observed from each suspending mentruum differing by less than 5%.

Effect of citrate. Titers of both phages were reduced by 30% when suspensions were plated in agar containing 0.1% citrate. Growth of the host was inhibited by 1.0% citrate.

Olsen et al. (13) reported that the reduction in plating efficiency for five psychrophilic phages ranged from 69 to 100% when media contained 1.0% citrate, whereas at 0.1% the range was 14 to 100% for three phages. The other two were unaffected.

Electron microscopy. Electron micrographs of phage specimens were prepared by negative staining with phosphotungstic acid. Phage ps_1 (Fig. 1) appeared to have a hexagonal shape, especially those phage particles which were devoid of nucleic acid (Fig. 1a). Infectious intact phage particles (Fig. 1b) were also observed to have a hexagonal outline. Short tails appeared to be present (Fig. 1c, d), although resolution was poor.

Phage wy possessed a contractile tail (Fig. 2,

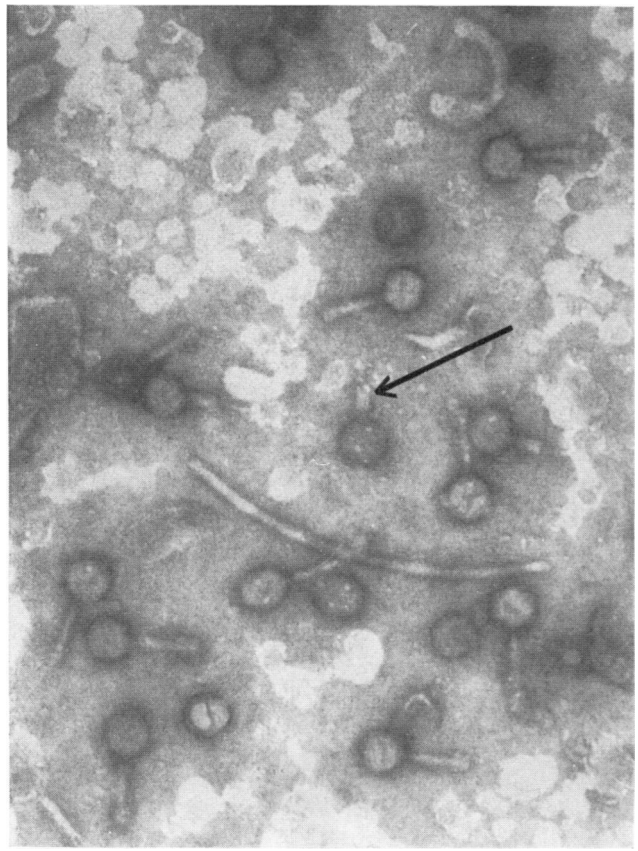

FIG. 2. *Electron micrograph of phage wy.* × *178,000.*

TABLE 3. *Morphology of phages ps*$_1$ *and wy*

Phage	Description	Dimensions (nm)	Bradley's[a] classification
ps$_1$	Short tail	18	Group C
	Hexagonal head	52	
wy	Contractile tail	93	Group A
	Hexagonal head	59	

[a] *See* reference 5.

arrow) attached to a hexagonal head. There appeared to be a base plate structure, visible on most of the noncontracted phage particles.

The morphological characteristics of phages ps$_1$ and wy are summarized in Table 3.

Pseudomonas phage wy was morphologically similar to three *Pseudomonas* phages reported by Olsen et al. (13) but was somewhat smaller than the *Pseudomonas* phage studied by Kropinski and Warren (9). It was much smaller than T2 of *Escherichia coli* B. Bradley (3), Olsen et al. (13), and Lee and Boezi (10) have described group C *Pseudomonas* phages, which are almost identical in size to phage ps$_1$.

Nucleic acid type. Color reactions, produced by fluorescent dye added to nuclear materials on glass slides, indicated the presence of 2-deoxyribonucleic acid (DNA) in both phages. Confirming evidence was obtained when spots of the phages were digested with deoxyribonuclease and then failed to take the stain when exposed to the fluorescent dye.

Only 2-DNA has been found within phages of groups A and C. These results give further evidence, therefore, that phages ps$_1$ and wy should be classified within groups C and A, respectively.

Thus, the two phages were distinctly different, but their characteristics were similar to those of other phages of *Pseudomonas*. Burst sizes, especially that of wy, were higher than expected and show that proliferation of these phages can proceed very rapidly even at 7 C.

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