CHARACTERISTICS OF BRUCELLAPHAGE[1]

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ABSTRACT

McDUFF, C. R. (University of Wisconsin, Madison), LOIS M. JONES, AND J. B. WILSON. Characteristics of brucellaphage. J. Bacteriol. **83**:324–329. 1962.—Methods of characterizing phage have been applied to a brucellaphage of Russian origin grown on its propagating strain, *Brucella abortus* R 19. Phage can be propagated by single plaque transfer. Phage titers of about 10^{10} particles per ml can be obtained by propagation on a young culture of R 19 in Albimi broth on a shaker at 37 C. After lyophilization, phage retains its activity during storage for at least 20 months at 4 C. Phage is stable in broth at pH values from 6 to 8 for 24 hr at 37 C. Some loss in activity results from heating for 1 hr at 60 C. All activity is lost in the presence of 10% chloroform. It has a slow adsorption rate ($K = 3.6 \times 10^{-11}$ ml/min), a latent period of 100 min, and a burst size of 121 particles. Electron micrographs indicate that the phage is approximately 65 mμ in diameter, polygonal in shape, with a short tail.

Although the existence of bacteriophage active against the brucellae was claimed by some workers (Pickett and Nelson, 1950; Carrère, Roux, and Mandin, 1956; Drozhevkina, 1957; Vershilova, 1957; and Parnas, Feltznowski, and Bulikowski, 1958), other workers were unable to isolate brucellaphage. Recently we obtained a bacteriophage of Russian origin, along with the host propagating strain *Brucella abortus* R 19, from W. R. Stinebring, University of Pittsburgh, who received the original preparation through the World Health Organization at Geneva. Stinebring and Braun (1959) reported that only smooth and intermediate strains of *B. abortus* were phage-sensitive; rough and mucoid strains and all of the *B. suis* and *B. melitensis* strains tested were refractory. Since then, however, several workers have shown that *Brucella* cultures (isolated from cattle in Britain) which have the cultural characteristics of *B. melitensis* are lysed by brucellaphage (Brinley-Morgan, Kay, and Bradley, 1960; Jones, 1960). These authors used brucellaphage from different Russian sources. Because brucellaphage is destined to assume an important role in species identification, we made a basic study of its characteristics and general properties.

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MATERIALS AND METHODS

Methods used in characterizing brucellaphage were based on those described by Adams (1959). The phage obtained from Dr. Stinebring had originally been prepared at Rostov on the Don and was labelled "*Brucella* bacteriophage, type *abortus*, strain 3." The host propagating strain which had accompanied the phage will be referred to as strain R 19. Standard methods of species identification, as outlined by Jones (1960), showed the bacterial culture to be a non-CO_2-requiring *B. abortus* of smooth-intermediate colonial morphology. As the culture was somewhat unstable, it was necessary to reisolate smooth-intermediate colonies to maintain phage susceptibility. A broth culture of R 19 was not found to be lysogenic before or after treatment with ultraviolet light (15 w "germicidal" General Electric lamp at a distance of 25 cm for 5 sec to 3 min). The culture was assayed for release of temperate phage on 14 other *Brucella* cultures.

Although Stinebring and Braun (1959) recommended the use of buffered beef-extract broth for propagation of the phage, we found that a two to three log increase in titer could be obtained with brucella broth (Albimi). No further increase in titer was observed with the addition of calcium (300 μg/ml) to the brucella broth. Brucella broth and agar (Albimi) were used for culturing and plating the host strain and for propagation and dilution of the phage. In later studies it was observed that, with some other *B. abortus* strains, better lysis was obtained on Trypticase soy agar (Baltimore Biological Laboratory) than on brucella agar.

The phage was propagated by the addition of approximately 10^5 phage particles per ml to a 6-hr broth culture of the host strain R 19 containing approximately 10^2 cells per ml. (In this paper, "phage particles" are used to mean plaque-forming units.) A growth curve of the host strain indicated that the log phase began 6 hr after the initial inoculation. After incubation at 37 C for 36 hr on a New Brunswick rotary shaker, the bacterial lysate was centrifuged at 2000 $\times$ g for 10 min to remove gross cellular debris. The supernatant was then filtered through a Millipore filter (type HA, 0.45 μ pore size), and the cell-free filtrate was stored at 5 C. Experiments had shown that there was no loss in phage titer by this method of filtration.

Phage titer was determined by the agar layer technique (Adams, 1959) modified as follows: 1 ml of the phage dilution and 1 ml of the host strain suspension containing 10^7 to 10^8cells per ml were added to 2 ml of molten brucella agar medium, gently mixed, and poured over brucella agar plates. Plaques were counted after 48 hr incubation at 37 C. Phage propagated in Albimi or Trypticase soy broth usually had a titer of 5×10^9 to 10^{10} plaque-forming units per ml. Phage stocks were also prepared from single plaques obtained from agar layer plates.

Phage antiserum was prepared by injecting a rabbit subcutaneously twice a week for 3 weeks with 2.5 ml of phage stock containing 5×10^9 particles per ml; 7 days after the last injection the rabbit was bled by cardiac puncture, and the serum was filtered and stored at -20 C in 1-ml amounts. The serum was assayed for antiviral activity by the method described by Adams (1959). Treatment of the bacterial host with serum did not affect the viable count of R 19 cells, and it was therefore assumed that it would not interfere with the ability of infected cells to produce plaques after the serum was diluted out

The rate of adsorption of brucellaphage to the host cell was calculated by determining the number of infected bacteria at varying time intervals in a mixture of phage and host cells. An input ratio of phage to bacteria approaching 1:1000 was selected, to obtain rapid adsorption and to assure single infection. Adsorption was allowed to occur in a shake flask at 37 C, and samples were removed at intervals and mixed with antiserum to neutralize the unadsorbed phage. The number of infected bacteria at each time interval was determined, and from these data the percentage of unadsorbed phage was calculated.

For the single-step growth experiment, the concentration of host cells necessary to insure maximal adsorption in 10 min was calculated from the data obtained by determining the rate of adsorption. After adsorption and after neutralization of free phage by treatment with antiserum, further dilutions in brucella broth were made so that the antiserum would not inactivate the phage liberated from lysing bacteria. The first and second growth tubes, representing 10^{-4} and 10^{-6} dilutions of the adsorption mixture, were held at 37 C for several hours, and samples were removed for phage assay at frequent intervals. The latent period and average burst size were determined.

To determine the heat stability of phage, six tubes containing 9 ml of broth and 1 ml of phage dilution were submerged for 60 min in water baths adjusted to 10, 20, 40, 50, 60, and 70 C, respectively. All tubes were brought to 20 C before plating.

Tubes of brucella broth were adjusted to pH values ranging from 3 to 10 by the use of 1 N NaOH and 1 N HCl. The actual pH of the adjusted media was determined with a Beckman pH meter. The phage dilution (0.1 ml) was added to 9.9 ml of the broth at each pH value, and tubes were incubated at 37 C for 24 hr. A 100-fold dilution in brucella broth (pH 7) to eliminate pH effects on adsorption preceded phage assay.

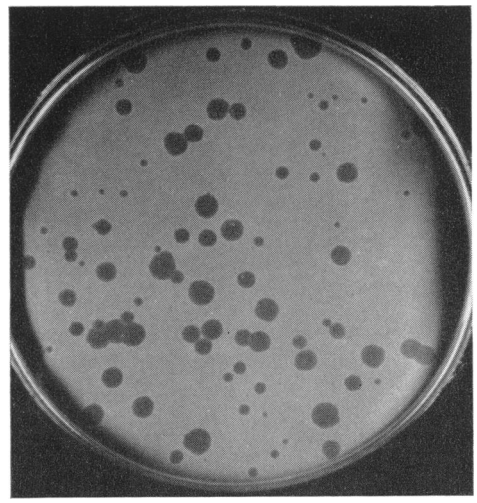

FIG. 1. *Brucellaphage plaque morphology*

The procedures employed in phage concentration for electron microscopy were based on those described by Williams and Fraser (1953) and Seto, Kaesberg, and Wilson (1956). Approximately 500 ml of a filtered phage preparation were centrifuged at 13,400 × *g* for 120 min. The pellets were resuspended in 1.8% ammonium benzoate and centrifuged at 5800 × *g* for 15 min. The supernatants containing the phage were centrifuged at 9000 × *g* for 90 min, and the phage pellets were resuspended in ammonium benzoate by placing in a refrigerator and gently rotating the tubes at varying time intervals. Phage preparations were mixed with 2% phosphotungstic acid, dropped on collodion-covered grids, and dried at room temperature. Specimens were examined in a Siemens ELMISKOP I electron microscope.

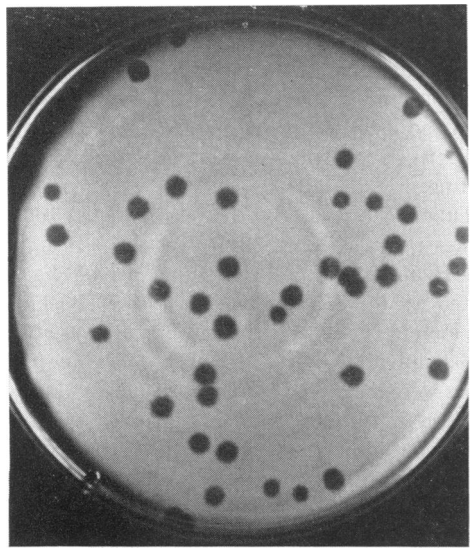

FIG. 2. *Brucellaphage plaque morphology when adsorption time is limited to 10 min.*

TABLE 1. *Percentage neutralization of 2.2 × 10^7 brucellaphage particles per ml after exposure to antiserum*

Time	Free phage remaining × 10^5 in serum diluted:		Neutralization* in serum diluted:	
	1:50	1:100	1:50	1:100
min			%	%
5	9.2	34	95.8	84.5
10	1.7	16	99.1	92.7
15	0.4	6.0	99.9	97.2
20	Not counted	2.1	—	99.0

* Serum neutralization constant (K value) = 31.7.

RESULTS

Plaque morphology. The clear, circular plaques, visible within 24 hr, reached their maximal size after 48 hr and ranged from 0.5 to 5 mm in diameter. Figure 1 suggests that a mixture of

TABLE 2. *Determination of rate of adsorption of brucellaphage to B. abortus R 19 cells**

Time	Number of infected bacteria per ml × 10^5	Unadsorbed phage
min		%
0	0	100
15	2.4	74
30	5.1	45
45	6.9	25
60	7.9	14

* Rate of adsorption (K value) = 3.6 × 10^{-11} ml/min. Input ratio: 9.2 × 10^5 phage particles to 7.5 × 10^8 bacteria.

TABLE 3. *Single-step growth experiment using a 12-hr broth culture of B. abortus R 19**

Time	Plaque counts from:	
	First growth tube	Second growth tube
min		
25	135	2
30	136	1
60	149	—
70	154	—
80	143	—
90	147	1
100	About 500	8
105	Confluent lysis	27
115		49
125		98
135		114
150		175
160		175
180		181
200		167
210		183
250		171

* Latent period: between 90 and 100 min. Rise period: approximately 40 min. Average burst size: 121 phage particles per cell. Input ratio: 1.4 × 10^6 phage particles to 3.9 × 10^9 bacteria.

brucellaphage may be present. Single plaques of the small and large size were picked and propagated on the host strain, but these preparations also produced plaques of varying size. When the adsorption time of the phage to a 12 hr broth culture of the host cell was limited to 10 min and unadsorbed phage was eliminated with antiserum, plaques of uniform size (up to 6 mm in diameter) were observed (Fig. 2).

Assay of phage antiserum. The percentage of neutralization of brucellaphage after exposure for varying times to antiserum diluted 1:50 and 1:100 is given in Table 1. The K value for serum diluted 1:50 was calculated to be 31.7.

Adsorption of the phage to the host cell. Table 2 gives the data used in calculating the rate of adsorption as 3.6×10^{-11} ml/min.

Single-step growth experiments. A typical single-

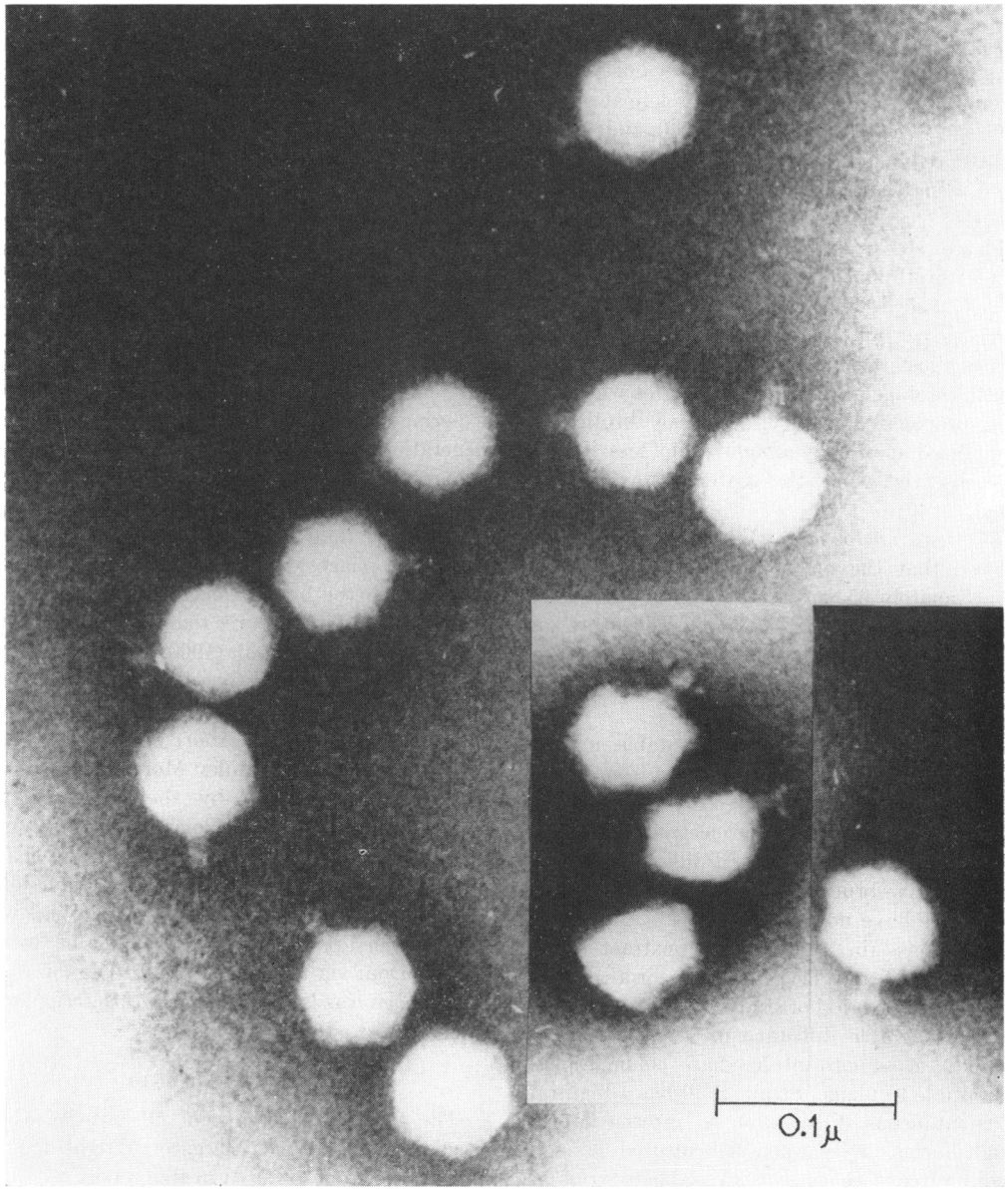

FIG. 3. *Electron micrograph of brucellaphage (500,000 ×).*

step growth experiment is shown in Table 3. A latent period of about 100 min and an average burst size of 121 particles were obtained with a 12-hr broth culture. In other experiments, a 16-hr broth culture showed a latent period of 150 to 160 min, and a 24-hr broth culture adsorbed the phage without any evidence of replication in a 4-hr period of observation.

Stability of phage. No inactivation of phage occurred at temperatures of 20, 40, or 50 C for 1 hr. At 60 C, 18% of the phage was inactivated; 100% was inactivated at 70 C. There was no loss in phage titer in broth at pH values of 6.2 through 8.1. Inactivation was complete at pH 3.1, 56% at 4.1, 24% at 5.0, 35% at 9.0, and 42% at 9.9. Gentle mixing of phage with 10% chloroform for 5 min resulted in 99% inactivation of the phage.

Phage stocks propagated in buffered beef-extract broth and tested after 20 months storage at 5 C and −20 C retained their titer of 10^7 particles per ml. Propagations in Albimi broth had not lost their activity when tested after 15 months storage at 5 C. Phage filtrates which had been propagated in Trypticase soy broth were lyophilized in 0.1-ml amounts; no loss in titer was observed when the material was reconstituted.

Electron microscopy. Electron micrographs showed that the phage head is polygonal and approximately 65 mμ in diameter and the tail is about 16 mμ long (Fig. 3). The inset picture suggests fiber and end-plate components of the tail.

DISCUSSION

Bacteriophages are generally stable in their own lysates, provided these are free from specific inactivating substances derived from the lysed bacteria and contain suitable electrolytes (Adams, 1959). Parnas (1960) reported difficulty in maintaining active brucellaphage preparations in liver broth. We have not detected loss in titer of our preparations (in buffered beef-extract broth, Albimi broth, and Trypticase soy broth) during the 20-month period of study. We have, however, had considerable difficulty in keeping the host strain in a smooth-intermediate colonial phase susceptible to phage. Strain R 19 has a high rate of spontaneous dissociation to intermediate or rough forms, even when subcultured on solid medium free of phage. It is advisable to lyophilize R 19 and to open lyophilized stocks at frequent intervals.

Van Drimmelen (1959) heated phage preparations to 60 C for 1 hr to destroy bacteria. We observed an 18% loss in activity with this treatment, whereas use of Millipore filters to remove bacteria resulted in no loss in phage content.

Chloroform has been used extensively as a means of obtaining cell-free phage preparations and has also been used with brucellaphage (Kessel, Aronson, and Braun, 1960). We have found, however, that brucellaphage is inactivated by chloroform.

The difference in plaque size (also reported by Brinley-Morgan et al., 1960) can be attributed to the slow adsorption rate of brucellaphage to the host cell (3.6×10^{-11} ml/min). It was demonstrated by Wahl and Blum-Emerique (1952) that slowly adsorbing phage-bacterium systems produce a great diversity of plaque sizes on a single plate. The phages adsorbing early produced large plaques, and those adsorbing late produced small plaques.

As has been reported for other phage-bacterium systems (Adams, 1959), the long latent period (100 min) observed during the single-step growth experiments may be correlated with the long generation time of *Brucella* (approximately 4 hr in broth). Our data also show that the latent period lengthens with the age of the bacterial culture and that a 24-hr culture was capable of adsorbing phage without replication.

Although the brucellaphage which we studied was from a different source than the phage studied by Brinley-Morgan et al. (1960), the observations by electron microscopy were very similar. Both studies showed a polygonal phage head of about 65 mμ in diameter and a short wedge-shaped tail. The phage used by Brinley-Morgan and his coworkers was obtained by them through the courtesy of J. Parnas in Poland and P. A. Vershilova in Russia. It was labelled "Tbilisi" phage. The phage used in our studies and those of Jones (1960) was obtained from Dr. Stinebring, who had obtained it from Dr. Drozhevkina of Rostov on the Don, via the World Health Organization. This phage was labelled "*Brucella* bacteriophage, type *abortus*, strain 3."

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