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Spontaneous Mutation and Recombination Among Brucellaphages

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ABSTRACT

MERZ, GEORGE S. (University of Wisconsin, Madison), AND JOE B. WILSON. Spontaneous mutation and recombination among brucellaphages. J. Bacteriol. **91**:2356–2361. 1966.—Two plaque morphology variants, as seen on *Brucella abortus* 544A, termed c (for clear plaque) and lc (for late-clearing plaque) were isolated from stocks of wild-type brucellaphage and from colonies of *B. abortus* 544A which had undergone an alteration in colonial morphology associated with the establishment of the phage carrier state. Single-burst experiments showed that the phage variants arise by spontaneous mutation of the wild-type phage during its replication on *B. abortus* strain R19. Two-factor crosses of independently occurring c mutant phages showed the presence of wild-type recombinants among the progeny. Control experiments showed that there are no strong selective forces against either wild-type or c mutant phage inherent in the cross-procedure. Other control experiments ruled out the possibility that wild-type phage in the cross-progeny resulted from either back mutation of the c mutants or the presence of wild-type phage among the input c mutants.

Although the knowledge of bacteriophage genetics is extensive, primarily through the study of coliphage, relatively little is known about the genetics of brucellaphage. Jablonski (5) described brucellaphage variants which he designated as r (rapid lysis), rm (rapid lysis, minute) tu (turbid), and wild type, after the coliphage nomenclature. However, it is not known whether these plaque types result from genetically distinct phages or from variations in the experimental environment. Jones, McDuff, and Wilson (7) reported the existence of a mutant brucella phage. It was detected as an occasional clear plaque in turbid white areas formed by parent phage spotted on lawns of *Brucella abortus* 544A, a strain of intermediate colonial morphology. Phage isolated from these clear plaques gave rise to stocks of phage that likewise formed clear plaques on these strains. It was the purpose of the work reported here to try to determine the origin of these mutant brucellaphages and to determine whether they could be used to demonstrate recombination among brucellaphages.

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MATERIALS AND METHODS

Bacterial culture. B. abortus strain R19, a CO_2-independent strain of smooth-intermediate colonial morphology, and *B. abortus* 544A, a CO_2-independent strain of intermediate colonial morphology derived originally from the FAO/WHO reference strain 544, were grown on Trypticase Soy Broth (BBL) or on broth with added agar (Difco). A standard culture was prepared by harvesting the growth from slants (24 to 48 hr) and adding it to broth to give a final concentration of about 2×10^7 bacteria per milliliter. The broth cultures were shaken at 37 C for 15 hr, at which time the bacterial concentration was 10^9 to 2×10^9 per milliliter. Such standard cultures were used in all experiments involving controlled adsorption of phage.

Phages. The wild-type brucellaphage has been previously described by McDuff, Jones, and Wilson (10). Phage stocks having titers of 10^{10} to 10^{11} PFU (plaque-forming units)/ml were obtained by adding phage to a final concentration of about 10^9 PFU/ml to a young broth culture of strain R19 containing 10^8 to 5×10^8 bacteria per milliliter and incubating the mixture for 24 hr at 37 C with shaking. The lysates, which seldom cleared completely, were centrifuged (7,000 $\times$ *g*, 20 min), filtered (Millipore membrane filters, 0.45-μ pore size), and stored at 4 C. The mutant phages were propagated similarly on strain 544A.

Phage assays. All of the phage assays were done by

the agar layer method (1). Mixtures containing 2.5 ml of broth with 0.75% agar at 45 C, 0.1 ml of phage to be assayed, and a drop of standard broth culture were poured over the surface of plates containing about 30 ml of agar medium. The plates were incubated at 37 C.

RESULTS

Plaque morphology of wild-type and mutant brucellaphages. When several thousand isolated wild-type plaques were scored for plaque morphology variants on *B. abortus* 544A, three types of plaques were observed (Fig. 1). These photographs were taken after 24, 48, and 72 hr of incubation of an agar layer plate bearing a mixture of the three plaque types on a lawn of strain 544A. The c (for clear) mutant plaques were completely clear by 24 hr and underwent no further change except to increase somewhat in diameter. This plaque type corresponds to the clear plaque type called SW2 by Jones, McDuff, and Wilson (7). The lc (for late clearing) mutant plaques first appeared as turbid, white plaques after 24 hr, which became almost clear by 48 hr. This plaque type was not observed by Jones et al. (7). The wild-type plaques first appeared after 48 hr, as turbid, white plaques which never become clear, even after 72 hr of incubation. On *B. abortus* R19, the host propagating strain for wild-type phage, the wild type, c, and lc plaques were clear after 24 hr of incubation.

Origin of the c and lc mutant brucellaphages. In addition to being isolated occasionally from stocks of wild-type phage, the c and lc phages can be isolated readily from strain 544A colonies that have an alteration in colonial morphology associated with the establishment of the phage carrier state with wild-type phage (Merz, Jones, and Wilson, *in preparation*). The most likely origin for the c and lc phages was spontaneous mutation of the wild-type phage during replication. To test this possibility, single-burst experiments (8) were done. If the mutant phages did arise by spontaneous mutation, then the distribution of the mutant clone sizes should form a clonal distribution characterized by the presence of few, but statistically significant, large mutant clone sizes.

The experiments were conducted in the following way. Strain R19 cells were infected with wild-type phage by adding phage to a standard culture of strain R19 to a final concentration of 10^8 phage per milliliter and incubating at 37 C for 30 min to permit adsorption of phage. A sample of the mixture was diluted 10^{-1} in anti-phage serum (previously diluted 1:50 in broth) and incubated for 15 min at 37 C to neutralize free phage (10). After diluting a sample of the antiserum mixture 10^{-3} in broth, 0.1-ml samples were distributed into a series of tubes and incubated for 3 hr at 37 C to allow lysis of the infected cells. Then the contents of each tube was mixed with soft agar containing strain 544A cells, and was poured over the surface of an agar plate. At appropriate intervals, the plates were scored for the numbers and kinds of plaques present.

The results of one of several single-burst experiments are shown in Tables 1 and 2. Table 1 shows the distribution of number of c phages (clone size) in each tube. The observed distribution is quite different from the calculated Poisson distribution. Comparison of the two distributions by the chi-square test yields a value of 102 ($P < 0.0001$). Clearly, the observed distribution is not random, but rather resembles a clonal distribution with its long tail of a few, but statistically significant, large clone sizes. It is concluded, therefore, that the c phages can and do arise by spontaneous mutation of wild-type phage during its replication on strain R19. Mutation of wild-type phage during replication on strain 544A also occurs (Merz et al., *in preparation*). From the single-burst experiment data, it is possible to estimate the mutation frequency. There were 50 c mutations among approximately 5×10^6 progeny, giving a mutation frequency of about 10^{-5} per phage reduplication. Table 2 shows similar results for the distribution of lc phage clone sizes. The distribution appears to be clonal, and thus the lc phages likewise probably occur by spontaneous mutation of wild-type phage during replication on strain R19, with a frequency of about 10^{-5} per phage reduplication.

When the c and lc mutant plaques were picked and retested on strain 544A, their phenotypes were maintained through several successive transfers. If similar phage isolates were first tested on strain R19 and then retested on strain 544A, the respective phenotypes were likewise maintained.

Recombination between c mutant brucellaphages. Because of their independent origin, it was possible to obtain stocks of independently occurring c mutant phages and to test them for their ability to recombine. Two-factor crosses were done in the following way. Mixtures containing equal amounts of two independently occurring c mutants (2×10^{10} of each phage per milliliter) were added to an equal volume of a standard broth culture of strain R19, and were incubated for 30 min at 37 C to allow adsorption of the phage. A sample of the adsorption mixture was then diluted 10^{-1} in anti-phage serum (previously diluted 1:50 in broth), and was incubated for 30

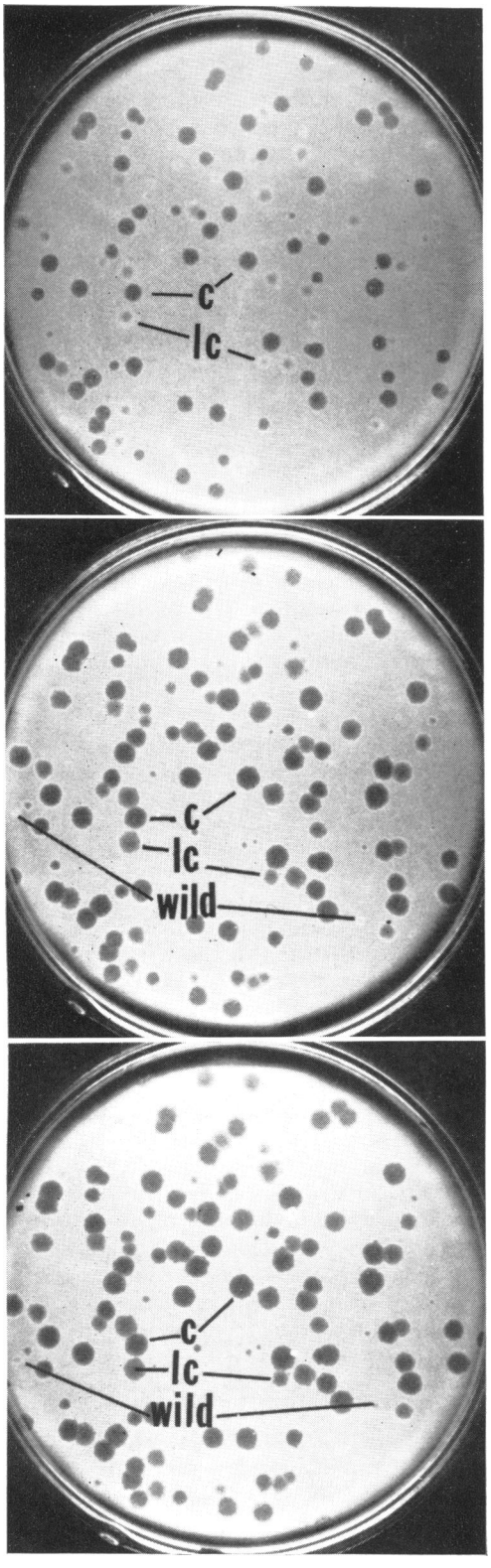

TABLE 1. *Distribution of c mutants among the plates of a single-burst experiment**

Clone size	Observed distribution	Calculated Poisson distribution
0	97	44
1	19	53
2	14	31
3	5	12
4	4	4
5	1	1
6	1	
7	1	
8		
9	2	
10		
11	1	
12		
13		
14		
15		
16	1	
17		
18		
19		
20	1	

* Average number of c mutants per plate, 1.2; number of tubes assayed, 147; number of c mutant plaques, 173.

min at 37 C to neutralize unadsorbed phage. A sample of the antiserum mixture was diluted 10^{-4} in broth, and was incubated at 37 C for 3 hr to allow lysis of the infected cells. Several thousand progeny phages were distributed over a convenient number of agar plates by the agar layer method, with a standard culture of strain 544A as a lawn. After 2 to 3 days of incubation, the plates were scored for the numbers and kinds of plaques present. Internal controls on the effectiveness of the cross procedure consisted of the following determinations: (i) a sample of the adsorption mixture was centrifuged (7,000 × *g*, 20 min), and the supernatant fluid was assayed for the amount of unadsorbed phage; (ii) a sample of the diluted antiserum mixture was assayed at the beginning of the 3-hr incubation period (which was well before the end of the 90-min latent period), to determine the number of infected cells; and (iii) a sample of the progeny was assayed on strain R19 to determine the relative efficiency of plating, which is defined in these experiments as the ratio of the phage titer on strain 544A to the phage titer on strain R19.

FIG. 1. *Plaques formed by wild-type, c, and lc phages on a lawn of Brucella abortus 544A after 24 hr (top), 48 hr (middle), and 72 hr (bottom) of incubation. The same plate is shown in all three photographs.*

TABLE 2. *Distribution of lc mutants among the plates of a single-burst experiment**

Clone size	Observed distribution	Calculated Poisson distribution
0	89	34
1	22	50
2	6	36
3	7	18
4	5	6
5	3	2
6	5	
7	3	
8	1	
9	2	
10		
11	2	
12		
13	1	
14		
15		
16	1	

* Average number of lc mutants per plate, 1.48; number of tubes assayed, 147; number of lc mutant plaques, 218.

TABLE 3. *Results of a typical two-factor cross*

Determination	No.
Input viable count*	6.4×10^8
Input cl PFU count*	1.2×10^{10}
Input c6 PFU count*	1.8×10^{10}
Unadsorbed PFU count (at 30 min)*	1.3×10^{10}
Adsorption rate constant, *k*	3.5×10^{-11}
Infective centers*	3.8×10^8
PFU adsorbed/viable cell	28
Progeny PFU*	4.9×10^{10}
Burst size (progeny/infective center)	128
Progeny efficiency of plating	0.84
No. of wild-type phage among progeny	20/1,037 (1.8%)

* Per milliliter of adsorption mixture.

The results of a typical cross are shown in Table 3. The adsorption rate constant and the burst size were quite reproducible, and the values shown are typical. The number of infected cells was about half of the number of input viable cells. This was a consistent finding and can be attributed to the decreased viability of brucellae that are incorporated into soft agar layers (Merz et al., *in preparation*). It can also be seen that the efficiency of plating of the progeny was less than 1.0. This too was a consistent finding, although the values obtained for different crosses were variable (0.45 to 0.9).

To determine whether these two observations had an effect on the outcome of a cross, the following experiment was performed. Mixtures containing different proportions of wild-type and c mutant phage were used as input mixtures in a control cross procedure. Three different mixtures, containing approximately 50% wild type and 50% c mutant, 10% wild type and 90% c mutant, and 90% wild type and 10% c mutant, were used. The fraction of each type in a given mixture showed no appreciable change after being subjected to the cross procedure (Table 4). The efficiency of plating for the input and progeny mixtures was about 0.4 and 0.5, respectively. These results indicate that there is no strong selection against either the wild type or the c mutant that is inherent in the cross procedure, and that the wild-type and c mutant phages are equally sensitive to whatever is responsible for the lowered efficiency of plating on strain 544A.

To show that any wild-type phage in the progeny of a cross was not the result of back mutation of the c mutant phages, another series of control experiments was performed. This consisted of employing the cross procedure with only one kind of c mutant as input phage. Each of the c mutants was tested in this way. In no case was any wild-type phage found among several thousand progeny plaques that were examined. As a further precaution, several thousand plaques from each c mutant stock were plated directly onto strain 544A to obtain an estimate of the frequency of wild-type phage in a c mutant stock. No wild-type plaques were ever observed. Thus, the frequency of back mutation to wild type, and the frequency of wild-type phage in a c mutant stock, must be less than about 2×10^{-4}.

The results of several two-factor crosses are summarized in Table 5. When a strain R19 cell was infected with two independently occurring c mutants, recombinant wild-type phage was found among the progeny. In each cross, the wild-type plaques, when picked and retested on strain 544A, always gave rise to wild-type plaques. When the same cross was repeated, the wild-type recombinant frequencies were usually reproducible. The recombinant frequencies shown in Table 5 were used to construct the linkage map

TABLE 4. *Control experiments showing the absence of strong selection against c mutant of wild-type phage in the cross procedure*

Mixture	Input assay		Progeny assay	
	C mutant	Wild type	C mutant	Wild type
	%	%	%	%
1	56	44	51	49
2	8	92	10	90
3	92	8	95	5

TABLE 5. *Results of two-factor crosses with c mutant brucellaphage*

Cross	Total progeny	No. of wild type	Per cent wild type
$c_1 \times c_6$	2,424	44	1.8
$c_1 \times c_6$	1,037	20	1.8
$c_1 \times c_4$	8,794	73	0.83
$c_1 \times c_4$	2,575	18	0.71
$c_6 \times c_4$	3,330	12	0.36
$c_6 \times c_4$	8,233	35	0.42
$c_6 \times c_4$	7,945	23	0.30
$c_6 \times c_{13}$	4,222	7	0.17
$c_6 \times c_{13}$	1,462	3	0.20
$c_6 \times c_{13}$	3,047	6	0.20
$c_1 \times c_{13}$	2,605	22	0.85
$c_1 \times c_{13}$	2,903	21	0.73
$c_4 \times c_{13}$	2,767	5	0.18

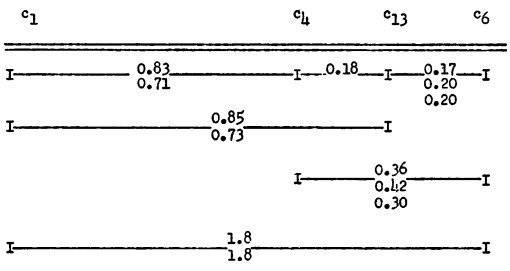

FIG. 2. *Brucellaphage linkage map (not drawn to scale).*

shown in Fig. 2. The positions of the c mutant loci were determined by the relative recombinant frequencies. The distances are given in terms of wild-type recombinant frequencies. More precise positioning by three-factor crosses was not possible because of the difficulty in isolating the double c mutant and the absence of outside markers.

DISCUSSION

Spontaneous mutation and recombination among bacteriophages have been known for some years (4, 8), and have been extensively studied (9). The work reported here, while leaving many questions unanswered, shows that brucellaphages are subject to these two mechanisms of genetic variation.

Demonstration of the origin of c and lc mutants by spontaneous mutation verifies the findings of Jones, McDuff, and Wilson (7). In their study of the alteration of the colonial morphology of strain 544A by the establishment of the phage carrier state, they isolated a phage, called SW2, which, unlike the parent phage, caused clear plaques on strain 544A. They believed this phage to be a mutant that pre-existed in the parent phage stock. The difference between SW2 and c mutant is only one of terminology. There is evidence (Merz et al., *in preparation*) that c and lc mutants also arise as spontaneous mutations of wild-type phage during its limited replication on strain 544A, and it is quite likely that the occurrence of these mutations is the initial event in the conversion of the strain 544A phage carrier colonies to sticky white colonies.

The demonstration of recombination and spontaneous mutation among brucellaphages might be of some practical importance. The focus of interest in brucellaphages has so far been centered around their possible use as epidemiological and taxonomic tools (2, 3, 6, 11). The major obstacle to the widespread use of brucellaphages for such purposes has been the lack of phages sufficiently varied in their host range. For example, Morgan (11) found that five phages, differing only in their place of isolation, showed the same host range when tested on 3,919 cultures of brucellae. A knowledge of the genetic mechanisms operating in brucellaphage could conceivably provide a means of developing strains of phage with species-specific or even strain-specific host ranges.

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