PHENOTYPIC ALTERATIONS IN THE COLONIAL MORPHOLOGY OF *BRUCELLA ABORTUS* DUE TO A BACTERIOPHAGE CARRIER STATE

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ABSTRACT

JONES, LOIS M. (University of Wisconsin, Madison), C. R. McDUFF, AND J. B. WILSON. Phenotypic alterations in the colonial morphology of *Brucella abortus* due to a bacteriophage carrier state. J. Bacteriol. **83**:860-866. 1962.—In the course of examining a number of *Brucella* cultures with a brucellaphage, it was observed that *B. abortus* cultures of intermediate colonial morphology, which had a blue-gray colonial appearance, were not lysed within 24 hr; in 48 hr they had developed sticky white growth in the area of the phage drop. When this growth was streaked on agar plates, both white and blue-gray colonies developed. White colonies which were sticky always carried phage and upon restreaking always gave rise to both white and blue-gray colonies. White colonies which were not sticky were rough and phage resistant. Blue-gray colonies produced only blue-gray colonies, did not carry phage, and were similar to the parent in their response to phage. When sticky white colonies were incubated for 6 hr or more in phage antiserum, all phage was eliminated and only blue-gray colonies developed. It was believed that the sticky white colonies were carrier clones in which lysis was delayed until after cell division, thus resulting in the establishment of a colony containing some phage-free progeny. With the accumulation of phage, the colony became sticky. This effect may be caused by the action of bacteriophage enzymes on the cell walls.

Brucellaphage had an extremely slow rate of adsorption on a culture of intermediate colonial morphology. A phage mutant which was more strongly lytic for cultures of intermediate colonial morphology was selected from the original phage. The adsorption rate of this phage was more rapid and the latent period shorter. A serological difference between phages could not be demonstrated.

In the course of examining a number of *Brucella* cultures with a brucellaphage, we observed partial lysis and an effect on the colonial morphology of some cultures. A phage mutant, which was more strongly lytic for cultures of intermediate colonial morphology, was obtained. Detailed examinations of these cultures and their interactions with phage will be reported here.

MATERIALS AND METHODS

The brucellaphage which we received from W. R. Stinebring, University of Pittsburgh, had originally been obtained from Rostov on the Don and was labelled "*Brucella* bacteriophage, type *abortus*, strain 3". The phage preparations used in this study are described in Table 1.

B. abortus strain R19 is a non-CO_2-requiring culture of smooth-intermediate colonial morphology which accompanied the brucellaphage from W. R. Stinebring. *B. abortus* strain 544A is a non-CO_2-requiring derivative of the FAO/WHO reference strain 544. It had been in our laboratory for many years and was of intermediate colonial morphology, producing large, blue-gray, granular colonies which gave an extremely fine agglutination with acriflavine. Of the remaining 132 cultures examined, representing four *Brucella* species, some were recently isolated, and some had been transferred as stock cultures for an unknown period of time.

Trypticase soy agar and Trypticase soy broth (Baltimore Biological Laboratory) were used throughout this study. Broth cultures, incubated for 12 hr at 37 C on a shaker, were used as lawn for all phage counts by the agar-layer technique and as sources of cells for all adsorption rate, single step growth, and serum neutralization experiments. The methods, as adapted from Adams (1959), have been described previously (McDuff, Jones, and Wilson, 1962).

In the routine examination of *Brucella* cultures for sensitivity to phage, agar plates were streaked with a cotton swab soaked in a broth suspension from an agar slant of 24 to 48 hr. Decimal dilutions of the phage were dropped on the inoculated plate with a single pipette, beginning with the highest dilution and proceeding to the lowest.

TABLE 1. *Efficiency of plating of different phage preparations on bacterial culture R19 and 544A by the agar-layer technique*

Phage	History of phage preparation	Bacterial lawn	Titer and morphology*	EOP†
P19	Single plaque (Russian phage on R19) picked and propagated in broth on R19	R19 544A	8.6×10^9 C 2.6×10^9 T 1.0×10^6 H	0.30
SW1	Sticky white colonies (from P19 on 544A) suspended in broth and filtered	R19 544A	1.6×10^{10} C 5.2×10^9 T 3.0×10^8 H	0.33
SW2	Hazy plaque (from SW1 on 544A) picked and propagated in broth on 544A	R19 544A	3.8×10^9 C 1.2×10^9 H	0.31
SW2/R19	SW2 propagated in broth on R19	R19 544A	4.4×10^9 C 1.3×10^9 H	0.29

* C = clear plaques with sharp and even margins; H = hazy plaques with irregular margins; T = very turbid plaques with indistinct margins.

† EOP = titer on 544A/titer on R19.

The pipette was standardized to deliver 0.02 ml per drop. Plates were incubated at 37 C for 48 hr in air or in an atmosphere of 10% CO_2.

RESULTS

All cultures, which had been recently isolated from cattle, and laboratory stock cultures of *B. abortus*, which had been maintained in the smooth or smooth-intermediate colonial form, gave confluent lysis from the drop of the 10^{-4} dilution of phage, and isolated clear plaques were seen in the areas of the drops of the 10^{-5} to 10^{-7} dilutions. Figure 1 shows typical results after 24 hr incubation.

With some *B. abortus* cultures of intermediate colonial morphology, only a few isolated plaques were seen at the lower phage dilutions after 24 hr incubation. After 48 hr the area in the position of the lower dilutions of phage was yellow-white in contrast to the blue-gray appearance of the surrounding lawn (Fig. 2). Isolated white areas were apparent at the higher dilutions to approximately the same end point as seen with smooth cultures. A few clear plaques were seen in the white areas at the lower dilutions. The growth in the white area did not appear to be diminished in quantity but was extremely sticky when touched with a needle. This effect was seen with strain 544A, with a culture strain 19 which had been transferred for many years, and with an intermediate colonial variant selected from a recently isolated *B. abortus* culture.

The white areas were picked and streaked on agar plates for observation of colonial morphology. In the area of the plate where the inoculum had been heavy, the growth was white and sticky with numerous plaques of almost complete lysis. Isolated colonies varied from white, to blue-gray, to small gray translucent colonies. The various types were picked, restreaked, examined for presence of phage as well as for susceptibility to phage. This information is given as follows:—1) White sticky colonies continued to produce the same variety colonies described above. They always carried phage capable of lysing R19. They were not lysed by the original phage but were lysed by the more strongly lytic mutant, SW2, whose origin will be described later.

2) Some white colonies showed homogenous white growth and did not carry phage. These were not sticky. They were agglutinated in large clumps by acriflavine and were, therefore, rough and no longer susceptible to phage. They had great selective advantage in the presence of phage within the colony.

3) The small gray translucent colonies were often nonviable and contained phage. When these were streaked, the few colonies which did appear were white, rough, and phage resistant.

4) Blue-gray colonies produced homogenous blue-gray colonies. They did not carry phage. If phage P19 was dropped, white sticky growth resulted, and if phage SW2 was dropped, lysis

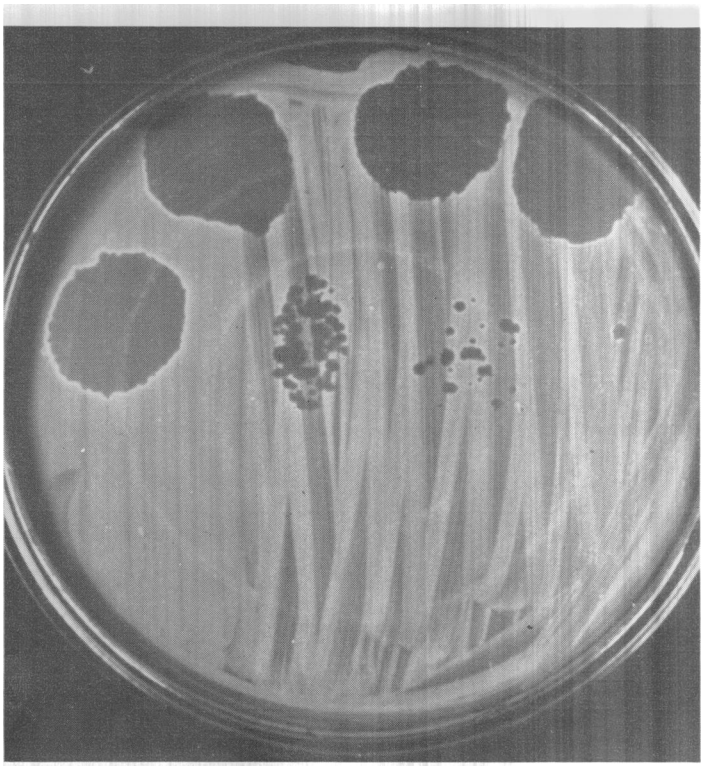

FIG. 1. *Decimal dilutions of Phage P19 dropped on a lawn of B. abortus R19 after 24 hr of incubation*

was complete. This colony type resembled that of the parent 544A.

In summary, phage could always be recovered from a sticky white colony but never from a blue-gray colony. Sticky white colonies always segregated into white and blue-gray colonies upon restreaking.

The alteration in the colonial morphology was obviously related to the presence of the phage. The possibility that a lysogenic conversion had occurred was eliminated when sticky white colonies were incubated in broth containing phage antiserum. After 6 hr the phage was all neutralized, and the cells produced only blue-gray colonies resembling 544A. It was apparent that the bacteria had not acquired the hereditary property of producing bacteriophage (or sticky white colonies), as infection of the progeny did not occur in the presence of phage antiserum. This suggested a carrier state in which both phage and cells existed.

The plaque morphology of brucellaphage was different on intermediate cultures from that on smooth ones. By the agar-layer technique only about one-third as many plaques were produced on the 544A lawn as on the R19 lawn. These plaques on the 544A lawn were very turbid, with hazy and irregular edges; they did not appear for 48 hr or more. In contrast, plaques on R19 were clear, with sharp even margins and were visible within 24 hr. The sticky white colonies, described above, were picked, suspended in broth, and filtered. This preparation was called SW1. When it was titrated by the agar-layer method, two types of plaques were seen on a lawn of 544A, turbid plaques and clear plaques which appeared earlier. The clear plaque was picked and propagated in a young broth culture of 544A, and in this way a more strongly lytic phage was obtained. This preparation, called SW2, produced complete lysis when dropped on a lawn of 544A or R19, but in the agar-layer method the plaques were a little hazy on 544A, though completely clear on R19. This strongly lytic phage was present in the original phage P19 in the frequency of about 1 in 10^3 particles. Propagation of SW2 on R19, the original host, did not result in a reappearance of the weakly lytic phage. It is believed that the more strongly lytic phage SW2 was the result of selection of a mutant which

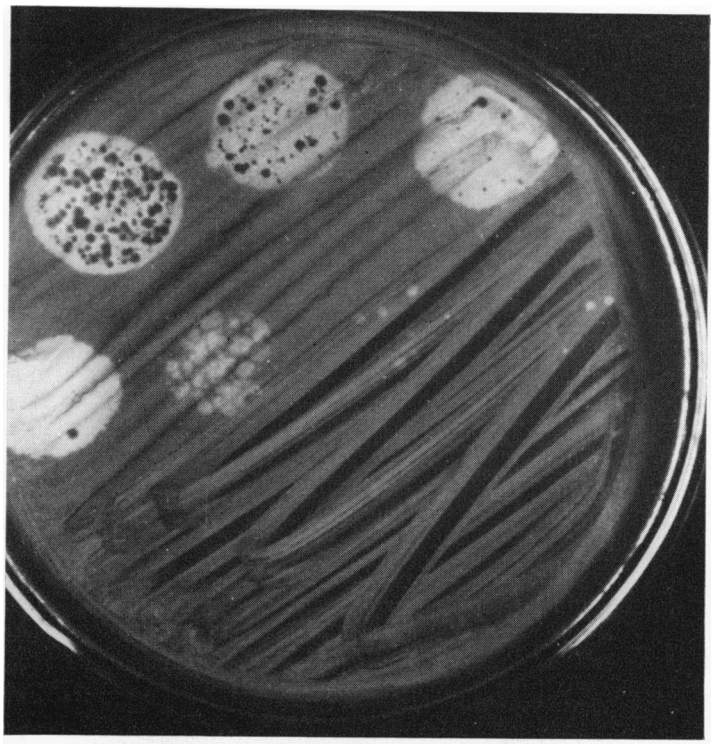

FIG. 2. *Decimal dilutions of Phage P19 dropped on a lawn of B. abortus 544A after a 48-hr incubation period.*

pre-existed in the P19 stock and was not due to a host-induced modification on the part of 544A. Phage SW2 was able to lyse the intermediate culture more effectively and, therefore, accumulated in the presence of 544A.

The rate of adsorption of P19 to 544A cells was determined by mixing phage and a 12-hr broth culture of cells, placing on a shaker at 37 C for the appropriate time period (Table 2), treating a portion with phage antiserum for 5 min to neutralize unadsorbed phage, diluting, and assaying on a lawn of R19. Plaques represented infected bacteria. The adsorption rate was extremely slow. The K values (Adams, 1959) obtained in different experiments varied from 2×10^{-12} to 2.5×10^{-13} ml per min (Table 2). In contrast the rate obtained on strain R19 and on a smooth culture of 544 (544 S in Table 2, experiment 3) was about 3×10^{-11} ml per min.

By passing a portion of the contents of the adsorption flask through a Millipore filter and assaying the filtrate for free phage, it could be demonstrated that there was no loss in free phage which could not be accounted for on the basis of infected bacteria. It was therefore apparent that adsorption did not occur without resulting in an infected bacterium and the release of phage.

A single-step growth experiment was performed with P19 and 544A. The minimal latent period was between 90 and 120 min, but phage continued to be released from infected cells for an additional 60 min. The burst size was about 100 particles. On 544A lawn these particles produced very turbid plaques.

The adsorption rate for the more strongly lytic phage SW2 (Table 2, experiment 5) was somewhat faster on 544A, the latent period was shorter (between 60 and 90 min) and the burst size was about the same.

Antisera were prepared in rabbits against P19 and SW2. A serological difference could not be demonstrated between these preparations (Table 3).

Dilutions prepared in the adsorption experiment were simultaneously spread on agar plates for colonial examination of surviving cells. Most of the colonies were blue gray and typical of 544A but a few sticky white colonies were seen.

TABLE 2. *Determination of rates of adsorption for several phage-cell systems*

Experiment number	Input ratio		Time	Number of infected bacteria*	Number of sticky white colonies†	Per cent of phage adsorbed	Rate of adsorption (K value)
	Phage	Cells					
			min				
1	P19	R19	15	2.4×10^5	0	26.0	2.6×10^{-11}
			30	5.1×10^5	0	55.0	3.6×10^{-11}
	9.2×10^5	7.5×10^8	45	6.9×10^5	0	75.0	4.1×10^{-11}
			60	7.9×10^5	0	86.0	4.3×10^{-11}
2	P19	544A					
	2.0×10^8	3.6×10^8	15	1.2×10^6	1.5×10^6	0.6	1.1×10^{-12}
			45	3.0×10^6	2.6×10^6	1.5	9.3×10^{-13}
	2.0×10^6	3.6×10^8	15	1.4×10^4	1.0×10^4	0.6	1.7×10^{-12}
			45	3.9×10^4	2.7×10^4	1.9	1.2×10^{-12}
	3.8×10^6	2.2×10^9	15	9.0×10^4	8.1×10^4	0.2	7.3×10^{-13}
			45	1.4×10^5	9.6×10^4	3.6	3.7×10^{-13}
3	P19	R19					
	1.5×10^6	8.0×10^8	15	8.0×10^5	0	53.3	6.3×10^{-11}
		544A					
	1.5×10^6	2.2×10^9	15	1.0×10^5	1.0×10^5	6.7	2.0×10^{-12}
		544S					
	1.5×10^6	3.0×10^9	15	1.2×10^6	0	79.3	3.5×10^{-11}
4	P19	544A					
	2.2×10^6	2.2×10^9	15	1.8×10^4	1.0×10^4	0.8	2.5×10^{-13}
5	SW2	544A					
	3.2×10^6	2.0×10^9	30	5.0×10^5	0	15.6	2.8×10^{-12}
			45	8.7×10^5	0	27.2	3.4×10^{-12}
			60	1.1×10^6	0	35.0	3.6×10^{-12}

* Determined by plating on a lawn of R19 by the agar-layer technique.
† Determined by spreading on an agar plate.

The number of sticky white colonies was the same as the number of plaques obtained by the agar-layer method (Table 2, experiment 2). Thus, only a single phage particle is necessary to initiate the eventual production of a sticky white colony. From this it would appear that an infected cell was not lysed but was able to initiate colonial growth. This was determined as follows. Dilutions of 544A cells were spread on agar plates, previously spread with undiluted phage, and on agar plates without phage. The colony counts were exactly the same on the two sets of plates. After 2 days incubation all colonies were small and blue gray, but after 4 days, the colonies on the phage plates were all sticky and white.

Preliminary observations were made on the cell morphology of 544A in the presence of phage. Thin agar plates were spread with undiluted phage P19 and a dilution of a 12-hr broth culture of 544A. Blocks of agar were cut out, mounted on slides, and observed under the microcope. Many cells had divided within 2 hr despite the presence of phage. In 4 hr some pleomorphism was observed within the microcolonies of short coccobacilli. Club-shaped, dumbbell-shaped, and fork-shaped bodies were seen, as well as large globular masses of varying granularity and long snakelike forms without cell-wall divisions but with dark round bodies at various points. By the third day the agar plate from which the slide had been prepared contained sticky white colonies. In control agar slides containing cells without phage, there was occasional variation in granularity or size of cells but the bizarre forms described above were not seen.

DISCUSSION

Burnet (1929) first observed that the change from a smooth to rough colonial form was accompanied by loss in phage sensitivity. He

TABLE 3. *Serological relationship of phages P19 and SW2*

Serum (1:100 dilution)	Phage	Time	Plaque count $\times 10^5$	Survival	K value
		min		%	
—	P19	0	650	100	
Anti-P19	P19	5	215	33	22.1
Anti-P19	P19	10	103	16	18.4
Anti-P19	P19	15	31	4.7	20.2
Anti-P19	P19	20	22	3.4	16.9
—	SW2	0	400	100	
Anti-P19	SW2	5	124	31	23.4
Anti-P19	SW2	10	32	8	25.2
Anti-P19	SW2	15	11	2.75	23.9
Anti-P19	SW2	20	2.8	0.7	24.8
—	P19	0	620	100	
Anti-SW2	P19	5	36	5.8	56.9
Anti-SW2	P19	10	13	2.1	38.6
Anti-SW2	P19	15	5.6	0.9	31.3
Anti-SW2	P19	20	5.2	0.8	23.9
—	SW2	0	300	100	
Anti-SW2	SW2	5	9	3.0	70.1
Anti-SW2	SW2	10	5	1.6	40.9
Anti-SW2	SW2	15	0	0	
Anti-SW2	SW2	20	0	0	

showed that the rough phage-resistant cultures resulted from the selection, under the action of the phage, of previously existing rough cells.

Wahl and Blum-Emerique (1952) characterized a third type of culture called "semi-resistant" whose behavior toward phage was intermediate between that of the resistant and sensitive cultures. Only a small fraction of cells in such a culture became infected and plaques were small, cloudy, and irregular in size and contour. *B. abortus* 544A would appear to fit the description of a semi-resistant culture.

Changes in the cell and colonial morphology of *Brucella* cultures in the presence of phage have been described by Nelson and Pickett (1951), Drozhevkina (1957), Drozhevkina and Kharitonova (1958), Dubrovskaya and Ostrovskaya (1960), Parnas, Feltynowski, and Bulikowski (1958), and Parnas (1961). Although lysogenization has been thought to occur by some workers, the basis for this belief has not been documented.

In the system described above, the alteration in colonial morphology was shown to be due to a carrier state. The question of how a cell infected with a lytic phage can establish a carrier colony has been dealt with by Fraser (1957). In studying host range mutants of phage T3 on *Escherichia coli* B, Fraser observed "lasting complexes" of cells and phage in which lysis was long delayed. Micromanipulation (by a modified de Fonbrune technique) of cells infected with phage revealed that a large proportion of infected cells can grow and divide. Some of their progeny give rise to phage-sensitive clones, some lyse and release phage, and some grow in a slow and abnormal way with increased cell length without cell division. The snakelike structures described by Fraser were also observed in phage-infected microcolonies of *Brucella*. It is conceivable that the intermediate strain 544A, which is semi-resistant to phage P19, is able to delay phage development until some time after cell division.

Li, Barksdale, and Garmise (1961) have described a bacteriophage carrier state in *Shigella dysenteriae* which resulted in a phenotypic alteration in lactose fermentation. Their description of segregating colonies is very similar to that described here. They attribute the alteration in lactose fermentation to endolysin produced by phage T7, which acts on the cell wall in such a way as to release a previously masked enzyme for lactose fermentation.

The change in the appearance and consistency of carrier colonies of 544A is a gradual one, which can be correlated with the increase of phage as well as the increase in the proportion of white rough forms in the colony. Bacteriophages are known to produce lysozymelike enzymes which act on cell walls (McQuillen, 1960). Gram-negative cell walls are made up, in part, of mucopeptides (Salton, 1960), and the release of large amounts of such materials might be expected to produce a sticky consistency in the colony.

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