SOME MUTANT PHAGES PRODUCED DIRECTLY BY BACILLUS MEGATHERIUM 899A WITH THEIR RATE OF OCCURRENCE

By JAMES S. MURPHY, M.D.

With the Technical Assistance of Robert L. Gosney, Jr.

(From the Laboratory of The Rockefeller Institute for Medical Research, Department of Bacteriology, University of California, Berkeley)

Plate 63

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The purpose of this study is to find out if bacteriophage mutants are produced directly by the lysogenic bacterial strain *Bacillus megatherium* 899a (1) and, if so, what types and frequencies are to be found. The results indicate that in all probability phage mutants are produced directly from lysogenic *B. megatherium* 899a and occur with a frequency of about one recognizable plaque mutant per 2000 normal individuals.

The experiment was designed to show whether passage through a sensitive cell was a major factor in the apparent production of mutants. The possibility of passage through a sensitive cell in the lysogenic culture was controlled by contrasting *B. megatherium* 899a growing in two media, one in which infection was virtually certain (peptone) and one in which it was highly unlikely (asparagine) (2). If the number of mutants was approximately the same in the two cultures, it would show that the infection of a sensitive cell occurring in the lysogenic culture did not play an important role in the production of mutants. If there was a significant variation in the two cultures and it could be demonstrated that the two mutation rates were not correlated, the plating suspension could be ruled out in the occurrence of mutants.

The results indicate that sensitive cells in the lysogenic culture do not play a significant role. The plating suspension test did not reveal as clear cut a result but gives every reason to believe that mutants are produced directly by *B. megatherium* 899a (lysogenic).

An attempt has been made to isolate and classify all the mutant phages recognized during this study and data are included which give some indication of the frequency of occurrence of these mutants. Several new mutants have been found, increasing the number described to 19 (3, 4).

Materials and Methods

Bacterial strains.—B. megatherium 899a (lysogenic) isolated by den Dooren de Jong (1) was sent to this laboratory by Gratia in 1949. It has been transferred weekly on 2 per cent

peptone agar (Difco) slants since that time. *B. megatherium* KM (sensitive) was used for all plating suspensions. This strain has been transferred daily on 2 per cent peptone agar slants for 4 years.

Media.—A modified asparagine medium (2) was used to prevent phage reinfection of any sensitive cells which might appear in the culture.

Asparagine medium.

	mg./ml.
Asparagine	10
$MgSO_4$	0.0012
$Fe(NH_4)_2(SO_4)_2$	0.00014

Dissolve in 0.02 M (pH 7.6) PO_4 buffer, autoclave, adjust to pH 7.5.

Dilute peptone (Difco) 0.35 per cent was used to provide a growth rate and phage production approximately equal to those in the asparagine medium. Stocks were made of sufficient volume to complete the entire experiment.

B. megatherium 899a growing in asparagine broth and dilute peptone broth was subcultured 1:5 daily for 7 weeks. Five times a week supernates of each culture were plated on seven per cent peptone agar by the method of Gratia (5) at such a dilution as to produce a total of about 10,000 plaques on 20 plates. The plates were carefully searched for plaque mutants and all those found were isolated and classified by plating together with known stocks. Extremely careful sterile technique was used throughout.

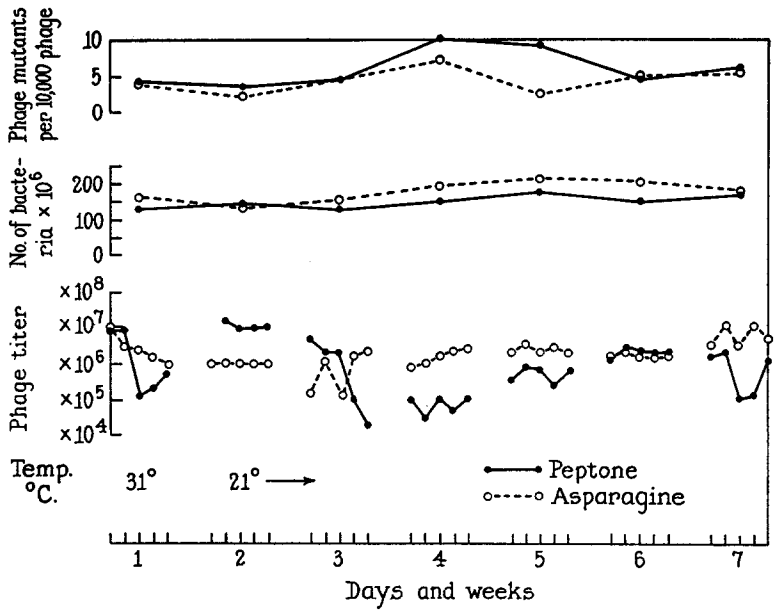
Controls.—Beside transferring *B. megatherium* 899a in asparagine and peptone, *B. megatherium* KM (sensitive) was also transferred in asparagine and peptone to check for accidental phage contamination. Furthermore, two tubes containing only medium were transferred to check against bacterial contamination. Since none of the control tubes was contaminated during the entire course of the experiment, there is a relatively good possibility that no contamination occurred in the experimental tubes.

To demonstrate that sensitive *B. megatherium* will not become infected with phage in asparagine medium, various concentrations of phage were added to a sensitive strain derived from *B. megatherium* 899a. The virus was freed from peptone by precipitating three times with ½ saturated $MgSO_4$. The conditions of the experiment were identical to those used throughout this study. Virus added up to 10^7 phage/ml. to 10^7 bacteria/ml. fell at a rate of about two logs per day to extinction. Above 10^7 phage/ml., occasional cultures were rendered lysogenic. No lysis was ever observed.

RESULTS

During the course of the experiment 396 mutant plaques were isolated and identified. Text-fig. 1 gives the over-all results with data averaged by weeks. It should be noted that after the 1st week the temperature was lowered. This was done in an effort to stabilize the cultures by lowering their growth rates and keeping them in log phase throughout the 24 hours. In spite of this the virus production continued to vary widely. No explanation of this is offered. The rate of production of phage mutants does not appear to have been altered by the change in temperature.

Table I shows the average number of phage mutants of all types found per week over the 7 weeks of the experiment. Mutants found on plates containing over 600 plaques have been omitted from this calculation, since marked errors may appear in counts of crowded plates. There is, very definitely, more varia-



TEXT-FIG. 1. Comparison of the data obtained from the two cultures, (peptone and asparagine) with respect to percentage of mutants/10,000, bacterial counts at time of subculture, final phage titer, and temperature. All data are in the form of weekly averages except bacteriophage titers in which daily averages are given.

TABLE I

*Average Total Mutants/10,000 Wild Type**

	Peptone		Asparagine	
	Total count	Mutants / 10,000	Total count	Mutants / 10,000
wks.				
1	39,900	4.3	24,700	4.1
2	16,600	3.6	35,800	2.2
3	13,300	4.5	No significant value	
4	31,000	10.0	45,500	7.2
5	28,000	9.1	31,500	2.5
6	32,300	4.6	37,300	4.8
7	19,600	6.1	35,500	5.6
Grand average.....	180,700	6.2	210,300	4.6

* Values derived from plate counts over 600/plate omitted.

tion from week-to-week in the mutation rate of both cultures than is allowed by the Poisson theory, indicating that the mutation rates are varying some what throughout the experiment. Therefore, the average mutation rate is only a relatively significant figure illustrating order of magnitude.

There is not very much difference between the two cultures. However, when compared on a week-to-week basis, using a chi square test, they are most assuredly not the same as if taken from the same tube. The 5th week alone is

TABLE II

Percentage of $\frac{\textit{Individual Mutants}}{\textit{Total Mutants}}$ *Found per Week*

		Peptone							Asparagine						
Weeks		1	2	3	4	5	6	7	1	2	3	4	5	6	7
Phage mutants	c	12		14	3	4	7	19	14			15	25	17	10
	c variant							5							
	d	6	5		6	4		5	14	9				11	
	vbr	6	5	14	6	3	4	5	7	9			13	11	24
	vbr variant														3
	br	6	36	14	9	1	9	5	14	9	15	18		6	
	lbr	18	16	14					35	9	12	15	13		
	w	6	10			14	18	19		9	12	6	13		7
	s		5		3	10	2		7		15			6	
	ss				3						4	3			
	s variant				6	1									
	bs	35	10	35	25	10	18	14	7	27	27	12	13	17	14
	bs variant	6								9					
	a				28	37	13	5	7	9	4	12	13	22	14
	ba					6								6	
	m									9		3			3
	u				6							3			
	u variant														3
	Not identified or lost	6	10	14	3	7	29	24			8	9			20

sufficient to rule out equality. Even if this value is excluded as erratic, the rest of the data rejects the equality hypothesis at the 5 per cent level.

This lack of equality indicates that the plating suspension is not producing the mutants. It may well be influencing the rates somewhat since there is a similarity between the two sets of values. However, there is no more reason to suspect the plating suspension than any other common factor between the two cultures such as temperature, pressure, or variation in laboratory procedure.

Table II is an analysis of the weekly occurrence of individual mutants in both cultures. For simplicity the values are given in per cent of the total

weekly "catch." It should be pointed out that br, lbr, and w are similar and belong to a group, while s, bs, bs var, a, and ba form another group. It was plaintly apparent to us that there was a smooth gradation between these groups and our classification was arbitrary. However, the task of completely differentiating these phages is beyond the scope of this project. In spite of these difficul-

TABLE III

Description of Mutants and Their Frequency of Occurrence

Mutant	Center	Intermediate zone	Halo	Size	Occurrence rate
c*	Absent	Clear	Present	N+‡	1/13,000
c variant	Absent	Clear	Present but thicker	N+	<1/100,000
d	Ring only	Clear	Present	N	1/30,000
vbr*	Present	3–4° lighter	Present	N	1/18,000
vbr variant	Present	3–4° lighter	Present	Smaller	<1/100,000
br*	Present	2° lighter	Present	N+	1/12,000
lbr	Present	1° lighter	Present	N	1/18,000
w	Partial	Turbid	Present	N	1/11,000
s*	Absent	Turbid	Present	N	1/20,000
ss	Absent	Turbid	Present	Smaller	<1/100,000
s variant	Absent and ragged	Turbid	Present	N	<1/100,000
bs	Absent	1° lighter	Present	N	1/7,000
bs variant	Absent (hole smaller)	1° lighter	Present	N	<1/100,000
a	Absent	2° lighter	Present	N	1/7,000
ba	Absent	3° lighter	Present	N	1/80,000
m*	Present	Turbid	Absent	Smaller	<1/100,000
vm*	Present	Turbid	Absent	Much smaller	Not found
u	Present	Very turbid	Present	N	<1/100,000
u variant	Present	Very turbid	Present	Smaller	<1/100,000
wild type	Present	Turbid	Present	Defined norm	

* These mutants have been previously described (3, 4) and are included here merely for comparative purposes.

‡ N = normal size. The presence or absence of a minute central hole in the middle of the colony is of no significance.

ties it can be seen that there was essentially no difference between the mutant populations of the two cultures.

One of the possibilities which was looked for in the daily results, was the persistence of any one mutant in day-to-day samples. It is obvious from inspecting the raw data that this did not occur. The occurrence of individual mutants seems entirely random, marked variations in the day-to-day population being the rule rather than the exception.

Table III gives a description of the various mutants found and illustrates

them. Mutants already described have been starred (3, 4). It will be seen that no radically different types have been found except u which is very turbid. All other newly found mutants appear as slight variations of those reported earlier.

Values are included in Table III showing the approximate frequency of occurrence of different mutants.

The possibility that the rarer mutants are more unstable has been explored. It can be said that all those mutants listed, with the exception of m, are stable in 5 per cent peptone at 4°C. over periods of time measured in months. The m mutant is only slightly less stable.

DISCUSSION

The relatively simple reasons enabling us to decide that phage mutants are produced directly by *B. megatherium* 899a (lysogenic) do not include any implications as to the possibility that more than one mechanism may be acting. We can only conclude that *some* mutants are being produced by *B. megatherium* 899a; not necessarily all.

Since some mutants are found in both peptone and asparagine cultures, the sensitive cells in the lysogenic culture can not be solely responsible for the mutations. As long as the two cultures are not identical in the way they vary then the plating suspension cannot be solely responsible for the mutations. Therefore, we conclude that some, if not all of the mutants were produced by the lysogenic cell directly.

The study of the different mutations proved to be quite difficult. Because of the fact that the method of plaque identification by direct visual comparison proved too crude, and recombination studies of each mutant would be too time-consuming to be practical at present we have had to be satisfied with groupings which might best be termed s-like or c-like, for example. There may be anywhere from 18 to 396 individual mutant "loci" represented in this collection and as far as we know, any value between these two is equally probable.

The mutation rates given may be markedly affected by the fact that reduplication of the mutant once formed may be taking place before it is liberated by the cell. This would give falsely high values for mutation rate. That this possibility must be seriously considered is indicated by Luria's demonstration of the clonal distribution of mutants in a coli-phage–sensitive bacterium system (6). This, of course, is so far removed from our lysogenic *B. megatherium* system that it makes serious comparisons impossible.

SUMMARY

The rate at which cultures of *B. megatherium* 899a produce certain mutants of its phage T (wild type) has been investigated in two media; one

(peptone) in which reinfection of sensitive cells, if present, is virtually certain, and one (asparagine) in which reinfection is extremely unlikely. Little difference either in the number or types of mutants produced has been detected.

It is concluded that phage mutants are produced directly by *B. megatherium* 899a and that no intermediate passage through a sensitive cell is necessary for the mutation.

396 individual mutant plaques have been isolated and classified into at least 18 types of plaque-forming mutants of *B. megatherium* 899a.

The over-all mutant ratio found was in the order of 1:2000 while individual mutants appeared with rates between 1:7000 and less than 1:100,000.

I wish to express my sincere appreciation to Dr. Joseph L. Hodges, Jr., for his most valuable help with the statistical analysis.

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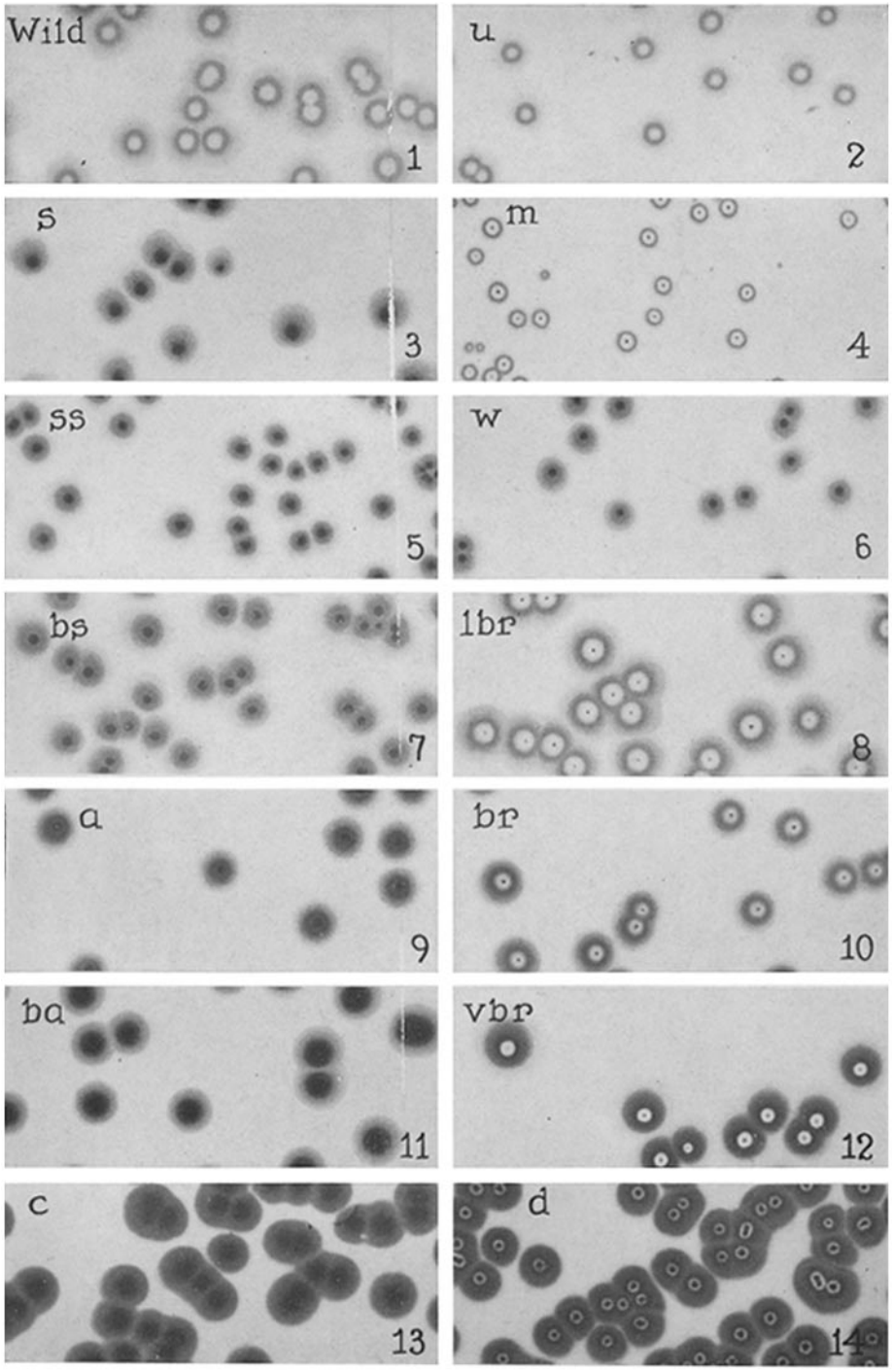
EXPLANATION OF PLATE 63

FIGS. 1 to 14. Photographs of the plaques formed by mutant phages of *B. megatherium* 899a on *B. megatherium* KM (sensitive). The mutants termed variants are so much like their prototypes that they can only be differentiated in mixtures and they therefore have been omitted from these illustrations. The variant u has a very faint halo which barely appears in this reproduction. × 2.



(Murphy: Mutant phages of *B. megatherium* 899a)