RECOMBINATIONS OF MUTANT PHAGES OF BACILLUS MEGATHERIUM 899A

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Plates 57 and 58

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In a previous report, the recombination phenomenon was shown to occur in the plaque mutant phages of *B. megatherium* 899a (1). The present study is an amplification of this work. Data are included on the percentages of recombination of the mutants isolated to date, and on the results of an analysis of these percentages. The *B. megatherium* system is unique in that all the mutants studied and most of the recombinants found can be readily recognized without recourse to special techniques. It is possible, in at least one case, to recognize plaques of phages with three mutations since each mutant produces its own characteristic effect on the plaque morphology.

Phage recombination has been extensively studied by Hershey and coworkers (2–5) who have shown that the patterns of recombination of T2 *coli*-phage mutants are essentially the same as those patterns found in the genetics of higher animals and are apparently genetic in nature. A further refinement of the work has been made by Doermann and Hill (6) using *coli*-phage T4, a virus closely related to T2. Their findings were used to test a theory advanced by Visconti and Delbrück (7) which indicates that the *coli*-phage T2 recombination can be explained on exactly the same basis as gene recombination in higher animals, with the major hypothesis that several rounds of mating occur during each infectious cycle. Doermann and Hill's results were in close agreement with those predicted by the hypothesis for five rounds of mating.

Burnet (8) and Hirst and Gotlieb (14) have been able to demonstrate recombination with influenza virus and Burnet has suggested the possibility that the phenomenon, if it occurs naturally, might play a role in the sudden changes in virulence that are found.

Materials and Methods

Phage from *B. megatherium* 899a (lysogenic) was used throughout all the studies, using methods previously reported (1, 9). The recombination experiments were done in a highly standardized way. The two phages under study were added simultaneously to a tube con-

taining 10 ml. of 5 per cent peptone with 5×10^7 bacteria per ml. The phage to bacterium ratio was as close as possible to 10 to 1 for each phage. After 20 minutes' shaking at 32°C. for adsorption, the tube was chilled, centrifuged, drained thoroughly, and the sedimented bacteria were transferred and resuspended in the original volume of fresh peptone. This reduces the remaining free phage to a concentration which will not influence later results. An aliquot was diluted 10^{-4} times in peptone and placed in the shaker at 32°C. Samples were plated at time zero, 1, 2, 3, and sometimes 4 hours. Controls included the concentrated bacteria-phage mixture and a tube of uninfected bacteria which were followed turbidimetrically. A titration was made of the supernate to insure that adsorption had been adequate. Table I

TABLE I

Theoretical Version of the Results of Crosses between m and s
Percentage of Recombinants = 5.4

Cross	Total plaque count	Individual plaque count
m × *s*	1000	473 *m* 473 *s* 27 *ms* 27 ++
ms × ++	1000	473 *ms* 473 ++ 27 *m* 27 *s*

TABLE II

Accuracy of Counts

Actual percentage	Majority count	Minority count	
		found	expected
per cent			
1	2511	21	25
2	2294	45	47
4	2212	91	92

gives a theoretical example of the behavior of *m* and *s* in crosses. The percentage of recombinants in all cases refers to the percentage of total recombinants with respect to the total plaque count.

Unknown mixtures were made up with great accuracy and coded in such a way that the observer did not know either the phages present or their percentages. Table II shows the checks obtained and indicates that the counting method is accurate to well within other experimental variations.

It will be seen that the recombination count is linear between 100 and 600 plaques per plate and only plates falling within those limits were used for calculation. From time to time a rough variant of *B. megatherium* appeared in the plating suspension and produced colonies with a granular surface which made accurate differential counts impossible. It was not found necessary to discard experiments in which the yields of the two "parental" types were differ-

ent since no lowering of the recombination percentage was found within the range of variation encountered.

Preparations for the electron microscope were made from lysates concentrated by ultracentrifugation in a Servall SS2 centrifuge for 1 hour at 12,500 R.P.M. The sediment was suspended in a smaller volume of 0.01 M phosphate buffer with 0.001 M $MgSO_4$ and filtered through supercel (10) to remove bacterial debris. Collodion supporting films were used and all material was fixed in formalin vapor at room temperature for 3 hours, followed by 2 per cent OsO_4 vapor for 20 minutes. After gentle washing in distilled water the preparations were dried. Photographs were made with an R.C.A. model E.M.C. electron microscope.

RESULTS

Origin and Terminology of Strains of Bacteriophage.—At this writing six mutants have been isolated which differ from each other sufficiently to be readily identifiable. One of these (*vm*) is not particularly stable, and, since it is so small as to be barely perceptible, it has not been included. All mutant strains used have been isolated from cultures of the bacteriophage of *B. megatherium* 899a (lysogenic) grown in cultures of *B. megatherium* KM (sensitive). However, four types of morphologically identical plaque-forming mutants have appeared in cultures of *B. megatherium* 899a in this laboratory. The two not found were the minute types (*m* and *vm*), the least likely to be recognized.

At the time of our previous report on some of the recombinations of these phages (1), it proved unnecessary to change the terminology of Gratia (11), who called the original phage "T" (turbid) and the clear mutant which he isolated "C." We merely added the "minor" mutant characters *br* (bright), *m* (minute), and *vm* (very minute) after the letters T or C termed "major" characters. However, since more information has become available on the recombinations, it has been decided to use only lower case letters to describe the phages, and to term T virus "wild type" (++) in order to conform with the *coli*-phage terminology which is based on common genetic practice. This means only slight changes, as is shown in Table III. It will be noted that the list includes a new mutant *vbr* (very bright) which is almost like *c* (clear) except for a turbid ring in the center. It is an excellent subject for differential counts (Figs. 1–12).

The double mutants on the list can be recognized by the presence of characters from each "parent;" *i.e.*, in *br m*, the size is like that of *m* (minute) and the intermediate zone is bright as in *br*. Two double characters have not been recognized (*s c* and *c vbr*). Also, *s vbr* looks very much like *c* and *br s vbr* like *br c*. Triple characters are more difficult, but can be recognized as such in *m br s*, a minute plaque (*m*) with a bright (*br*) intermediate zone and a clear center (*s*). *m br s* also may be slightly larger than *m* because of the property of *br* which gives a slightly increased plaque size.

Results of Recombination.—It has not been possible to demonstrate any significant difference between the percentages of the two recombinants produced by any given pair of mutants; nor has any significant difference been shown

between the recombination percentages of the reverse reaction. In other words, in the cross *br* × *s* the same percentage occurrence of recombinants will be found as in the reverse cross *br s* × wild type. Furthermore, in recombinations of phages differing by three factors the same percentages occur in crosses involving *br* and *s*. (See pair 1 in table V.)

In Table IV, the percentages found in ten recombination experiments on five phages are tabulated along with their probable errors and the total number of plaques counted in each case. At the top, the primary values are arranged to show the linear order of the five mutants, according to their recombination percentages. How well these "loci" fit the data can be seen from the "calculated" and "found" columns. The calculated values are the sum of the

TABLE III

Classification of Plaques

Former term	Present term	Meaning of symbol	Plaque description			
			Center	Intermediate zone	Halo	Size
T	Wild type (++)	Turbid	Turbid	Turbid	Stippled	Defined normal
C	*c*	Clear	Clear	Clear	Stippled	Slightly larger
S	*s*	Semiturbid	Clear	Turbid	Stippled	Normal
T(br)	*br*	Bright	Turbid	Bright	Smooth	Larger
T(vbr)	*vbr*	Very bright	Turbid	Clear	Stippled	Normal
T(m)	*m*	Minute	Turbid	Turbid and thin	Absent	¼ normal
T(vm)	*vm*	Very minute	Turbid	?	Absent	⅛ normal

individual "distances" between the loci indicated. The found column contains the experimental values. The two values in each case are well within the probable error except for *s vbr*. It can be seen that none of the values is high; in other words, the mutants are closely linked. It should be mentioned at this point that the low value for *s vbr* indicates a possibility that *c* and *vbr* may be reversed in their order. The arrangement shown seems to be the most likely, however.

Table V contains the results of a cross between the triple mutant phage *m br s* and wild type (++). There are three pairs of possible recombinants, all of which have actually been found, and all could be counted successfully except *m s*. The third pair ("double crossovers") were found in higher numbers than the number predicted by multiplying the two percentages of single recombination together (*i.e.*, 0.0056 per cent). A possible explanation of this difference will be considered in the Discussion.

Because of the fact that the *m s* recombinant was very difficult to count,

TABLE IV

Results of Recombination Experiments

m	*br*	*s*	*c*	*vbr*
1.4	4.0	1.5	0.8	

Cross	Total No. plaques	Recombination	Probable error
		per cent	
m × *br*	6,957	1.4	±0.19
br × *s*	1,853	4.0	±0.23
s × *c**	7,322	1.5	±0.44
c × *vbr**	2,506	0.8	±0.23

Cross	Total No. plaques	Recombination		
		Calculated from above	Found	Probable error
		per cent	*per cent*	*per cent*
m × *s*	3,650	5.4	5.5	±0.36
m × *c*	3,315	6.9	7.4	±0.60
m × *vbr*	5,000	7.7	7.6	±0.08
br × *c*	5,232	5.5	5.4	±0.52
br × *vbr*	11,131	6.3	6.5	±0.42
s × *vbr*	1,764	2.3	1.5	±0.08

* Only one recombinant could be counted.

TABLE V

The Results of a Cross between m br s and Wild Type (+++); Two Phages Differing by Three Factors

	Pair 1	Pair 2	Pair 3
Recombinants found	*m br* + ++ *s*	+ *br s* *m* ++	*m* + *s* + *br* +
Percentages found	4.0 ± 0.25	1.3 ± 0.16	0.4 ± 0.06*
Percentages expected	4.0	1.4	0.056

* Since the *m* + *s* count was impossible, this value was calculated by doubling the + *br* + value. Also a correction for mixed plaques has been applied.

although it was found occasionally on especially favorable plates, the value for the double crossover has been found by doubling the *br* value. Since this value was highly critical and there was a definite possibility that a mixed plaque (1) might be counted as a *br*, ten or one-half of the *br* plaques found were picked and replated. Three of these were found to be mixed. The other seven were pure, as judged by plates containing several hundred plaques each.

Statistical Treatment of the Data.—As already mentioned in the section on methods, several (3–8) plates were counted in each individual recombination experiment. On analyzing the data it was found that the counts from different plates within an individual experiment varied less than the values found in the separate experiments on the same cross. An example of this is shown in Table VI. In each case shown the value of recombination is based on three plates containing 250 to 400 plaques each.

TABLE VI

The Results of Two Experiments on the Same Cross

Experiment No.	Total count	No. of plates	Recombinants	
			Mean	Probable error
1	1100	3	4.8	±0.45
2	890	3	7.5	±0.85

TABLE VII

Size Measurements of Various B. megatherium Phages by Electron Microscopy

Strain	Head width	Tail length	Tail width
	mμ	mμ	mμ
899a	49 ± 10 per cent	330 ± 15 per cent	15 ± 30 per cent
No. 1 (Cowles)* (12)	100	260	40
B.M. (13)	60	150	15 (or less)

* The values actually given in this paper are in microns. It is assumed that this is a misprint.

Since the error within each experiment was less than the error between different experiments, the smaller was neglected and the mean and probable error were calculated from the following formula:

$$\frac{\Sigma A}{n} \pm \frac{0.84\Sigma(+d)}{n\sqrt{n-1}}$$

(in which n = number of experiments, A = average recombination value for each experiment, and $(+d)$ = sum of the differences without regard to sign between A and the mean.) An average of 4.5 experiments was used for each value.

Electron Microscopy.—In order to test whether some morphological difference might exist between various mutant phages, several were examined in the electron microscope using particles of Dow polystyrene latex (lot 10t 508G) of 280 mμ diameter as a reference for size determinations. No difference could be detected between wild type, *c*, *m c*, or *m br s*.

B. megatherium phages have been demonstrated in the electron microscope before. In Table VII our findings are tabulated along with two other reports. It is obvious that in each case an entirely different virus has been studied since both size and relative proportions vary considerably.

DISCUSSION

A linear linkage arrangement has been well established between mutant phages of both T2 and T4 *coli*-phage. This report shows that a similar system is present in *B. megatherium* 899a phage. Although all the mutants examined fall into an extremely close linkage group, the disadvantage of the small percentages of recombination is largely offset by the extreme differences between the mutants, which allows accurate counts to be made.

The accuracy of the method has been limited by some source of variation in the results of recombination experiments done on different days. It has been found that no correlation exists between this variation and any of the following values: (1) percentage adsorption, (2) number of bacteria, (3) burst size, (4) lysis time, (5) changes in passage numbers of stock virus, (6) lot numbers of media.

One three-factor cross was chosen after testing five possibilities, because of the ease with which this particular cross could be counted. Enough experiments were done to assure that the accuracy would be at least as high as the rest of the work. From this experiment a value for double crossovers was obtained which is far higher than had been expected, a finding which immediately suggests that recombination may be taking place more than once within the bacterial cell.

Visconti and Delbrück (7) have advanced a hypothesis according to which five rounds of mating will explain discrepancies in the percentages of recombination found in the *coli*-phage T2 system. This hypothesis also seems to explain the discrepancies in the T4 system (6). It is beyond the scope of the present paper to derive the parameters necessary for an analysis of this system along such lines. The important thing is to know whether multiple recombinations take place or not and, barring the two complications mentioned below, the evidence points to the conclusion that multiple recombinations occur in the *B. megatherium* system.

Two other possibilities suggest themselves. It is possible that a very high frequency of double crossovers does occur although this would be a departure from the experience of geneticists using other material. Also, the possibility must be considered that the data are influenced by an occurrence of mixed plaques. It is felt, however, that the actual picking and replating of the plaques have ruled this last possibility out.

The fact that the volume of the head of the T2 *coli*-phage is three times the volume of the head of *B. megatherium* 899a phage, and that three linkage

groups have been found in the *coli*-phage while only one has been found in the *B. megatherium* phage may be mentioned but no conclusions may be drawn until much more data are available. The finding that at least six mutants of *B. megatherium* phage exist demonstrates that the diameter of its "gene," if spherical, must be 14 mμ or less. This is assuming that the head of the phage contains nothing else. There is every reason to believe that the actual genetic unit is far smaller.

SUMMARY

A group of mutant phages stemming from the virus of *B. megatherium* 899a (lysogenic), growing on a sensitive *B. megatherium* strain (KM), have been studied with respect to their recombination reactions. All these mutants and many of their recombinations can be recognized by a characteristic plaque morphology. A similar group of phages have been isolated directly from a culture of *B. megatherium* 899a in this laboratory.

Previous work has shown that when two different plaque mutant phages both infect essentially all the bacteria in a culture, a characteristic per cent of recombinants is produced. This percentage depends on the two recombinants used, each pair having its own value. Hershey and coworkers (2–5) have demonstrated with *coli*-phage T2, that the percentages of recombination found can be handled mathematically and that they demonstrate the existence of a relationship between the mutations entirely comparable to crossover percentages as used in gene locus maps in genetics. This has been found to hold true for the phages studied in the present work.

Only one "linkage group" has been detected and all the mutants studied showed low percentages of recombination (0.8 to 7.6).

B. megatherium 899a phage and some of its mutants have been examined with an electron microscope and no differences have been detected between the different mutant strains.

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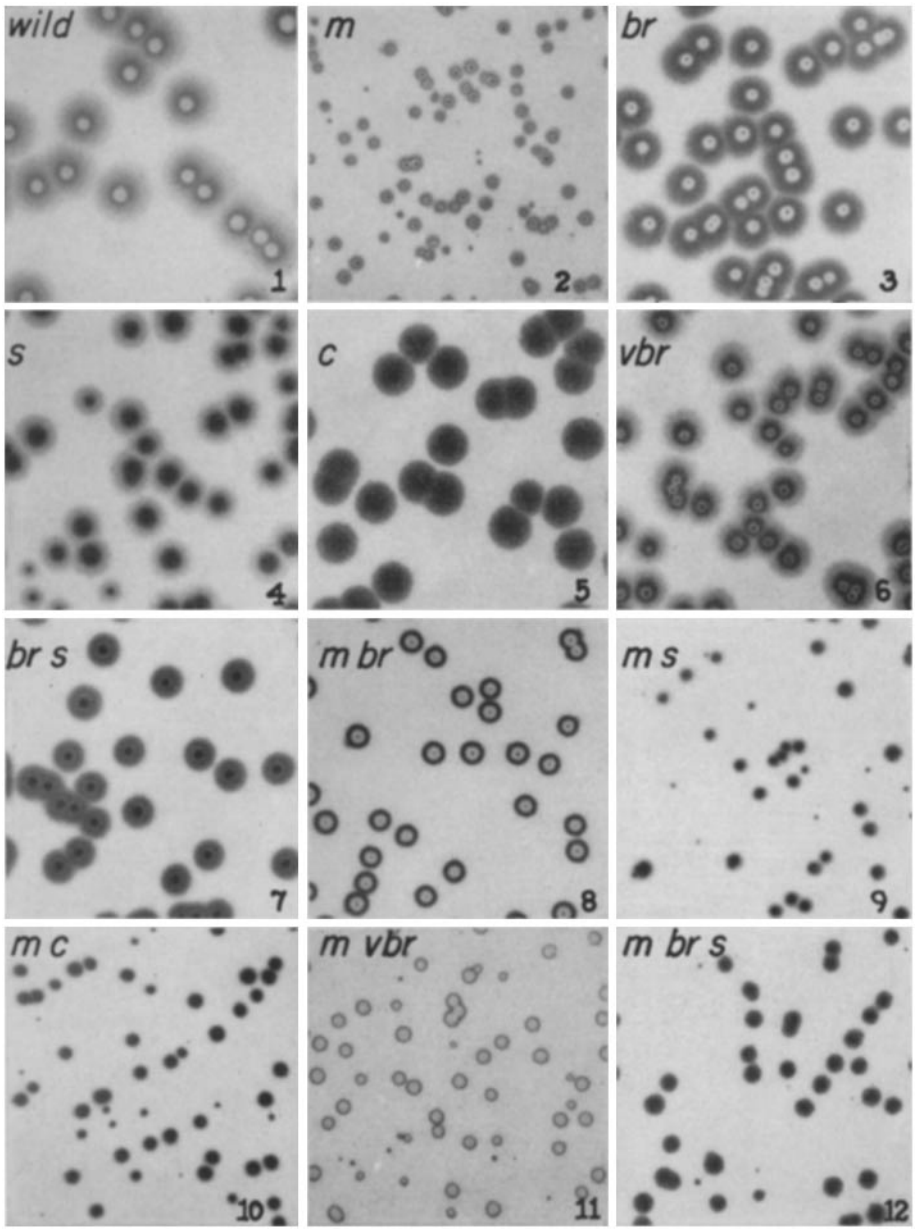
EXPLANATION OF PLATES

PLATE 57

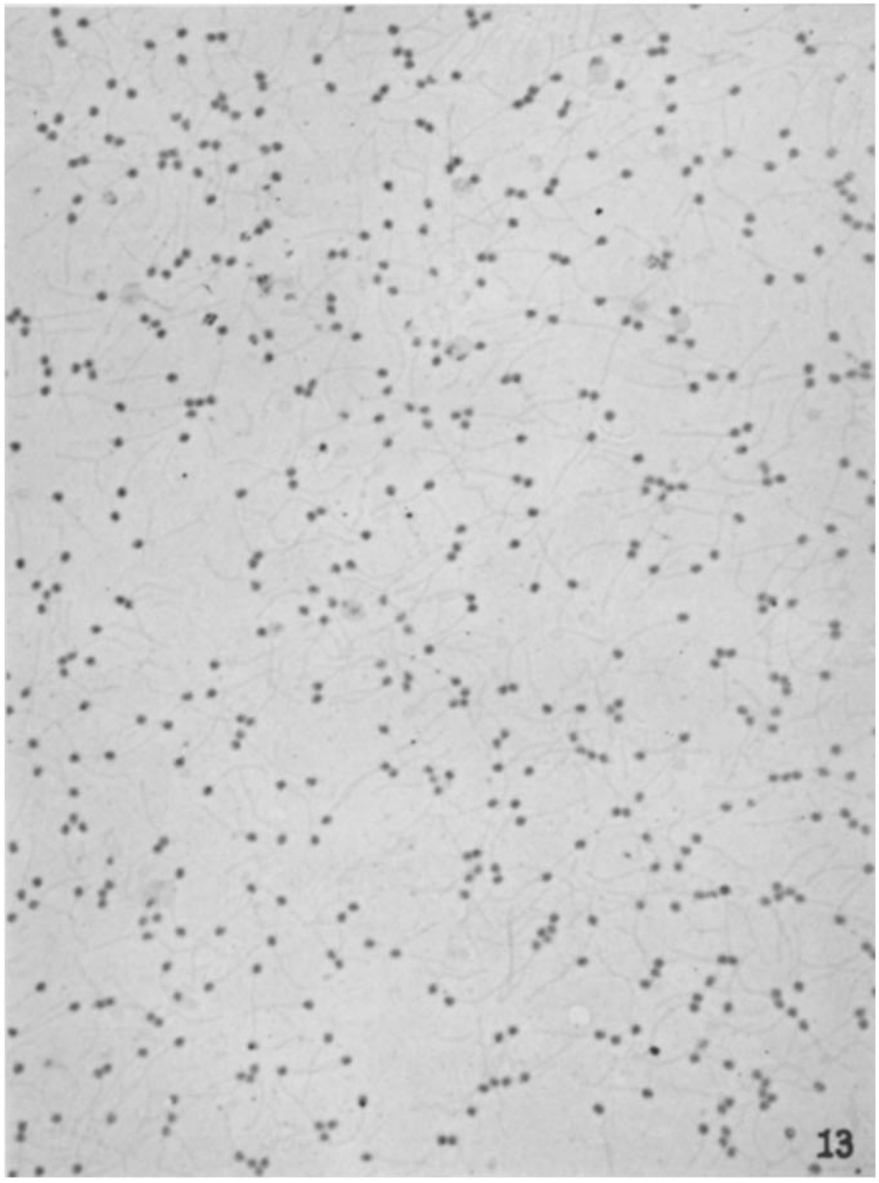
FIGS. 1 to 12. Photomicrographs of the plaques produced in a confluent sheet of *B. megatherium* KM (sensitive) by different mutants of the bacteriophage of *B. megatherium* 899a. (The pictures have been printed as photographic negatives.) × 2.



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

PLATE 58

FIG. 13. Electron micrograph of the phage of *B. megatherium* 899a. The phages appear as spheres with long thin tails. × 28,000.



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