BACTERIOPHAGE-RESISTANT MUTANTS IN ESCHERICHIA COLI

M. DEMEREC AND U. FANO
Carnegie Institution, Cold Spring Harbor, N. Y.

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BACTERIA are very promising as materials for studies of mutability, because of their great speed of reproduction and because of the opportunity they present for working with populations that comprise enormous numbers of individuals. Changes in bacterial cultures which make the bacteria resistant toward certain specific bacteriophage strains have been known for a long time and have been assumed by several workers to be due to mutations (GRATIA 1921; BURNET 1929; and others). This assumption has recently been confirmed in an important study by LURIA and DELBRÜCK (1943). The experimental method used by these investigators consists of determining how many resistant bacteria are present in small cultures of sensitive bacteria, especially when the size of cultures is kept so small that only a few resistants are found, on the average, in each culture. Under these conditions some cultures are still found to contain a comparatively large number of resistants. This indicates that such bacteria probably belong to a single clone stemming from an ancestor which mutated to bacteriophage resistance earlier in the growth of the culture.

Changes to bacteriophage resistance have been known to be fairly specific (BAIL 1923; BURNET 1929), inasmuch as each observed change usually involves resistance to only some of the bacteriophage strains capable of attacking the original strain of bacteria. The problem of resistance in bacteria therefore offers an unusual opportunity for a quantitative study of the occurrence of specific types of mutants, which outweighs the disadvantage of not being able to check the behavior of the mutants during a sexual process.

The purpose of the present study is to extend the investigation of LURIA and DELBRÜCK to cover resistance to several bacteriophage strains active on the same line of *Escherichia coli*. In particular, we wished to determine how bacteria attain simultaneous (or "multiple") resistance to various bacteriophage strains—that is, whether or not this resistance is due to separate, independent mutational steps.

MATERIALS AND METHODS

The materials used in our experiments consisted of the same bacterial strain —*E. coli* **B**—previously used by LURIA and DELBRÜCK (1943), of seven phage strains active on **B**, and of various strains of bacteria, derived by mutation from **B**, which were resistant to one or more of the phages. The phage strains were indicated as type 1 to type 7 (T1 to T7). T1 and T2, with which DR. LURIA supplied us, are the alpha and gamma strains of DELBRÜCK and LURIA (1942) and are identical with the P28 and PC strains of DR. J. BRONFENBRENNER (KALMANSON and BRONFENBRENNER 1939); T3, T4, T5, and T6 were isolated from a mixture of phages supplied by DR. TONY L. RAKIETEN; T7 was

isolated from the standard anti-coli-phage mixture prepared by DR. W. J. MACNEAL. Plaques produced by these phages were studied by plating a mixture consisting of phage and of bacteria obtained by washing a 24-hour slant with broth. For all our plates we used a nutrient agar, prepared by dissolving in the standard Difco nutrient broth 1.5 per cent of flaked agar and 0.5 per cent of NaCl. To minimize air contamination during manipulation of the plate, one drop of a saturated aqueous gentian-violet solution was added to each liter of the medium (BURNET 1929). We found it helpful against contamination, and particularly against condensation of the moisture within a Petri dish, to place filter paper on the inside of the Petri-dish cover. Cultures were incubated at 37°C.

In our experimental conditions T1 produces large plaques about 0.5 to 2 mm in diameter, visible after five hours of incubation, which after about 18 hours of incubation develop a large halo. Plaques of T2 are small, from 0.2 to 1.0 mm in diameter, without a halo and visible after about 18 hours of incubation. However, when T2 is grown on very soft agar, by the technique developed by HERSHEY, KALMANSON and BRONFENBRENNER (1943), an appreciable proportion of plaques develop small halos. T3 plaques are large, 1 to 2.5 mm in diameter; they become visible after five hours of incubation and later develop a large halo with a light ring around the central portion of the plaque. T4 plaques are small, 0.3 to 1.0 mm in diameter, and develop after about five hours of incubation; a small proportion of them show a small halo. Subsequently it was found that plaques without halos and those with halos breed true, and two lines of T4 were isolated—namely, T4a, which does not have a halo, and T4b, which has a halo. In all resistance tests made with these two strains their behavior was identical. T5 plaques are of medium size, about 0.5 to 1 mm in diameter; they show up after about five hours, and later develop a narrow halo. T6 has small plaques, similar to T2, but they develop early, after about five hours of incubation. T7 in appearance is very similar to T3—that is, it has large plaques with a large halo containing a ring. Plaques appear after five hours of incubation. Photographs of plaques of seven phages are reproduced in Plate 1, figures 1 to 7.

In this work we wanted to determine the number of mutants resistant to a particular phage that are present in a series of small, similar cultures of a bacterial strain sensitive to that phage. A suspension of sensitive bacteria was prepared in nutrient broth, containing about 1000 bacteria per cc. The desired amount, usually 0.3 cc, of this suspension was sucked up into the center of each of a large number of sterile 1-cc pipettes, after which the pipettes were stored in a sterile metal holder and incubated for about 24 hours. At the end of this period the concentration of bacteria in several sample pipettes was assayed for each experiment by the method of colony counts. This concentration was usually about 10^8 per cc—that is, the average number of bacteria per pipette was of the order of magnitude of 30 million. The contents of each remaining pipette were then spread on a nutrient-agar plate which had previously been coated with an excess of phage. At least 0.1 cc of phage suspension, of a titer of

about 10^9, was used on each plate. The plates were then incubated for 24 hours, and in later experiments for 48 hours, prior to observation and classification of the colonies of resistant bacteria that had developed.

To compare the numbers of mutants resistant to different phages and various combinations of phage mixtures, which were present in a particular culture of a sensitive bacterial strain, equal samples of the bacterial culture were mixed with excess amounts of one or more phages and spread on different plates.

Bacterial strains derived from colonies grown in the presence of excess phage were designated, following the earlier usage of BURNET and MCKIE (1936), by the symbol of the sensitive strain, a crossbar, and the number of the phage used in the experiment. Thus, resistant strains obtained as a secondary growth after plating the strain **B** on a Petri dish coated with phage T1 were designated as **B**/1. If **B**/1 was later plated on a Petri dish coated with a mixture of T_3 and T_4, the resulting colonies were designated as **B**/1/(3, 4).

In order to establish a representative set of mutant strains for purposes of further analysis, we had somehow to deal with the still unsolved problem of "lysogenicity." This term is used in connection with phenomena of apparently incomplete resistance, in which phage and bacteria multiply at the same time and in the same culture. The standard method of establishing a mutant strain was to inoculate broth with material from a bacterial colony that had grown on agar in the presence of excess phage. By this procedure the inoculum contained some phage as well as bacteria. The development of the culture appeared to be connected with the morphological appearance of the original colony as follows:

(a) Round colonies with the same structure as colonies of the parent strain give rise to normal growth in broth. The presence of some phage in successive cultures of the strains thus established does not make itself felt in tests of resistance to other phage types. The phage itself can be easily eliminated from the culture by repeated isolation of single colonies. This was done whenever a resistant strain was used in experiments other than routine cross-resistance tests.

(b) "Nibbled" colonies, with irregular edges and normal structure except on "chewed-up" sectors, which have a watery-translucent consistency, give rise to good growth in broth. The presence of phage in successive cultures of the strains thus established becomes evident, however, whenever a thick suspension of bacteria is plated onto agar: scattered plaques of the phage appear on the confluent bacterial growth. Phage assays indicate that the phage multiplies at the same time as the bacteria, and that only a small fraction of the phage particles present in the bacterial culture gives rise to plaques when the culture is plated on agar. Repeated isolation of single colonies from such cultures leads to eventual elimination of the phage, after which the bacterial strain does not appear to differ from the strains discussed under (a).

(c) "Thin" colonies, with watery-translucent structure throughout, do not give rise to good growth in broth. Attempts to culture them in broth yield instead a substantial concentration of phage. Repeated streaks on agar derived

from such colonies develop very slowly, but generally result in eventual elimination of the phage and in the establishment of a strain that does not appear to differ from the strains discussed under (a).

Tests of resistance of the bacterial strains to various phage strains were made by the cross-streaking method. On a nutrient agar plate, suspensions of bacteria and of phage were streaked with a large loop at right angles; and the growth, or lack of growth, of bacteria at the intersection of the streaks was observed. A bacterial strain was classified as sensitive to a phage if it showed lysis at the intersection of streaks. In a few instances, a variation in the degree of lysis was observed, but no attempt was made to record it.

RESULTS

Types of mutants.—Small broth cultures (0.3 cc) of the sensitive strain **B** regularly yielded mutants resistant to phages T1, T3, T4, T5, T6, and T7,

TABLE 1

Cross-resistance tests with various bacterial strains derived from B line.

TYPE	NUMBER OF STRAINS TESTED	RESISTANT TO PHAGE						
		T1	T2	T3	T4	T5	T6	T7
B/1	61	all	0	1	1	53	0	0
B/3	85	1+1	1	all	all	1+1	1	49+1
B/4	42	2	2	all	all	2	2	23+2
B/5	40	all	0	0	0	all	0	0
B/6	61	1	0	0	0	1	all	0
B/7	88	2+1	1	all	all	2+1	1	all

which were designated as **B**/1, **B**/3, **B**/4, **B**/5, **B**/6, and **B**/7, respectively. The frequency of occurrence of these mutants in the cultures varied between 10^{-8} and 10^{-5} approximately of the titer of normal bacteria, being in general lowest for **B**/1 and **B**/5.

No success was obtained in trying to isolate **B**/2 mutants by the standard method of plating **B** with an excess of phage T2, even though large amounts of high-titer cultures of **B** were plated in this way. Such platings generally gave rise to a thin, irregular growth of bacteria, which did not prove to be resistant. This growth is thought to be related to the peculiar slowness of action of T2 on **B** on agar, which is also apparent in the tardy appearance of T2 plaques. The observed growth may obscure the occurrence of a very small number of mutants truly resistant to T2.

Large numbers of strains of **B**/1, **B**/3, **B**/4, **B**/5, **B**/6, and **B**/7 were tested for resistance to all of our phage types. To make certain that the strains tested had stemmed from independent mutations, they were in the majority of instances derived from platings of separate cultures of **B**. Only occasionally were two strains derived from one plating, and that only when the plating of one

culture of **B** with excess phage showed two families of colonies clearly different in their morphology. In such cases one strain was established from each family. The numbers of strains that were tested and found to be resistant to the various phages are shown in table 1.

The strains entered in the table in italics proved to be resistant to all seven phages, including T2. Although the colony appearance is similar to that of the **B** strain, we do not feel certain that these colonies were not derived from contaminations, because they were resistant to T2, which is an exceptional condition, and because we did not make special identification tests. If these strains were not contaminants, they would seem to be mutants belonging to a different class than the other strains listed in the table.

In our case, as in others previously described (BURNET and MCKIE 1936), the phages may be grouped into cross-resistance groups. In the T1, T5 group all **B**/5 strains are also resistant to T1, while about 90 per cent of **B**/1 are also resistant to T5. This indicates the existence of two distinct types of mutants, one of which is resistant to T1 only, and the other to both T1 and T5. The first type is designated as **B**/1; the second should be designated either as **B**/1, 5 or **B**/5, 1, according to whether it was detected by plating **B** on T1 or on T5. Thus strains that are detected as **B**/1 are a mixture of **B**/1 and **B**/1, 5, while all strains thus far detected as **B**/5 are actually **B**/5, 1. No difference has been detected between strains designated as **B**/1, 5 and **B**/5, 1. The indication **B**/(1, 5) is used when the distinction is immaterial or when no distinction can be made.

As an additional check on the grouping of phages in resistance groups, a few experiments were done in which equal samples from one culture of **B** were plated on T1, on T5, and on a mixture of T1 and T5. It was expected that both types of mutants, **B**/1 and **B**/(1, 5), would form colonies on plates coated with T1 and that only **B**/(1, 5) mutants would form colonies on plates coated either with T5 or with the mixture of T1 and T5. Consequently, the number of colonies on the two sets of plates with T5 or with the mixture should be approximately equal and should as a rule be smaller than the number of colonies on the first set of plates. The colony counts in four experiments were:

Plates coated with:	T1	T5	T1+T5
Culture—1	135	62	29
—2	169	154	179
—3	241	43	51
—4	332	86	62

The colony counts in different experiments are unrelated, being derived from different cultures. In these cultures the total number of bacteria was high, and therefore it is to be expected that they would contain many mutants. The proportion of **B**/1 and **B**/(1, 5) mutants may differ from culture to culture, depending on which mutation happens to occur earlier. Culture 1 appears to contain **B**/1 and **B**/(1, 5) mutants in a ratio of about 2:1 (the difference between 62 and 29 in the count of colonies on T1 and on T1+T5 plates is as-

sumed to be due to a sampling accident). Culture 2 appears to contain B/(1, 5) but no appreciable amount of B/1 mutants. Cultures 3 and 4 appear to contain B/1 and B/(1, 5) in approximately a 4:1 ratio.

Resistance to T3, T4, and T7 also is due to a system of mutants which will be indicated as B/(3, 4) and B/(3, 4, 7). Mutants detected as B/3 or B/4 are either B(3, 4) or B/(3, 4, 7), while all mutants detected as B/7 seem to be B/(3, 4, 7). Experiments to support this interpretation, made by plating B cultures on Petri dishes coated with T3, T4, T7, and their mixtures, were carried out as for the T1, T5 group, with similar results.

Mutants detected as B/6 are generally resistant to T6 only.

Thus we may separate our seven strains of phage into four groups with regard to resistance reactions. T1 and T5 fall into one group; T3, T4, and T7 fall into a second group; while T2 and T6 form two other separate groups.

Table 1 shows occasional instances of mutants resistant to phages belonging to different resistance groups. For example, among 85 mutants detected as B/3 there was one B/3, 1, 4, 5; among 61 mutants detected as B/6, one was B/6, 1, 5; and among 88 detected as B/7, two were B/7, 1, 3, 4, 5. However, these multiple-resistant mutants occur with a much lower frequency than the mutants belonging to the regular resistance groups. Such multiple-resistant mutants can be most easily detected by plating a large number of bacteria on appropriate mixtures of phages.

In the experiments just discussed, the resistant mutants originated from the B strain, which is sensitive to all our phages. Table 2 summarizes the results of another set of experiments, in which the mutants were double or multiple mutants—that is, they were obtained from an already-resistant strain derived from B in previous experiments. Tests were made with a representative sample of resistant strains, and when uniform results were obtained no effort was made to complete the tests with all available resistant mutants. It is evident from the data in table 2 that there is no difference between the behavior of the resistant strains and the behavior of B strain in regard to the yield of new resistant mutants—that is, the pattern of resistance groups is the same irrespective of whether mutants were obtained from the B strain or from a resistant strain. For example, B/1 strain gives B/1/5, B/1/(3, 4), B/1/(3, 4, 7), and B/1/6 mutants; B/6 strain gives B/6/1, B/6/(1, 5), B/6/(3, 4), and B/6/(3, 4, 7) mutants, etc. New mutations are generally superimposed on the pattern of resistance which the strain had due to previous mutation; as a rule they are not affected by this pattern nor do they affect it. Table 2 shows the occurrence of one exceptional case, in which a B/6/1/7 strain was found not to be resistant to either T3 or T4.

Mutation rates.—LURIA and DELBRÜCK (1943) stressed the fact that considerable variation is observed in proportions of resistant mutants in parallel cultures of bacteria. This variation is the natural consequence of the mutational origin of resistance, because of the clonal grouping of mutants in each culture. Mutation rate is defined as the probability of occurrence of a mutation per bacterium per bacterial generation. Two alternate methods of estimating

the mutation rate have been proposed by LURIA and DELBRÜCK and used in our work.

(1) Determination based on the percentage (p_0) of cultures which do not contain any resistant mutant. The formula yielding the mutation rate (a) per bacterial generation is:

$$a = -\frac{1.6}{N}\log_{10} p_0 = -\frac{\ln 2}{N} \ln p_0$$

where N is the average number of bacteria per culture. The natural logarithm of 2 appears because the mutation rate refers to one bacterial generation—

TABLE 2

Cross-resistance tests with bacterial strains derived from various resistant lines.

PARENT STRAIN	NEW RESISTANCE	NO. OF TESTS	RESISTANT TO PHAGE						
			T1	T2	T3	T4	T5	T6	T7
B/1	/3	25	all	0	all	all	0	0	9
	/4	20	all	0	all	all	0	0	12
	/5	22	all	0	0	0	all	0	0
	/6	23	all	1	1	1	1	all	1
	/7	22	all	0	all	all	0	0	all
B/6	/1	37	all	2	2	2	34	all	2
	/3	39	0	0	all	all	0	all	21
	/4	21	0	0	all	all	0	all	12
	/5	12	all	0	0	0	all	all	0
B/4, 3	/1	13	all	0	all	all	all	0	0
	/5	6	all	1	all	all	all	1	1
B/4, 3, 7	/1	10	all	1	all	all	7	1	all
	/5	6	all	0	all	all	all	0	all
	/6	7	0	0	all	all	0	all	all
B/6/1	/3	20	all	2	all	all	1	all	10
	/5	25	all	0	0	0	all	all	0
	/7	30	all	3	29	29	3	all	all
B/6/3, 4	/1	10	all	0	all	all	7	all	2
	/5	11	all	1	all	all	all	all	1
	/7	5	0	0	all	all	0	all	all

that is, to the time during which the population doubles in number. This method is not very reliable, unless the conditions are so arranged that p_0 is neither too small nor too large; in fact, the method cannot be used if every culture contains mutants. Since we tried to use standardized experimental conditions, this prevented us from keeping p_0 steadily within the optimum range; and in a number of cases p_0 actually fell to zero. However, this method is inherently more accurate than the alternate method, inasmuch as it is affected to a much smaller degree by systematic errors.

(2) Determination based on the average number (r) of mutants per culture.

The formula correlating r with the mutation rate a per bacterial generation is:

$$r = 1.6\, a\, N \log_{10} \frac{CaN}{.693} = \frac{aN}{\ln 2} \ln \frac{CaN}{\ln 2}$$

where C is the number of cultures in the experiment. This method is applicable to a wider range of conditions than the previous one, but depends upon several assumptions that hold only very approximately. Furthermore, the incorrectness of these assumptions has the effect of making the calculated value of the frequency of mutations greater than the real value. The most important among such assumptions, as pointed out by LURIA and DELBRÜCK, is to disregard the chance of mutations early in the growth of the cultures.

The experiments on the detection of mutants resistant to phage T3 are listed in table 3. As was found in the case studied by LURIA and DELBRÜCK, the mutation rates computed by method (2) are generally larger than those computed by method (1). In three experiments all cultures carried mutants, and so method (1) could not be applied. The values obtained by method (1) range from 1.4×10^{-8} to 9.2×10^{-8}, a range that is not excessive in view of the limitations inherent in the method, besides those connected with accidental errors. The values obtained by method (2) range from 4.2×10^{-8} to 31.6×10^{-8}.

In view of the bias toward higher values inherent in method (2), it is probably fair to estimate that the rate of mutation to resistance to T3 is somewhere in the middle of the range 10^{-8} to 10^{-7}. This rate is actually the sum of the rates of a number of mutations leading to the same resistance, in this case to **B**/(3, 4) and **B**/(3, 4, 7), the first of which, according to the results shown in table 1, is probably somewhat (50 per cent) greater than the second.

An important conclusion to be drawn from table 3 is that strains of bacteria already resistant to T1 or T6, or both, appear to exhibit the same mutation rate to resistance to T3 as the original sensitive strain **B**. This indicates that mutations to different types of resistance are independent.

Series of experiments on the detection of mutants resistant to T1, T4, T5, T6, and T7, respectively, were carried out to an extent comparable to the series on resistance to T3. Table 4 is analogous to table 3 and includes all results. The rates of mutation to resistance to T1 and to T5 are probably lower than the rate of mutation to resistance to T3, inasmuch as they are probably smaller than 10^{-8}, which is in agreement with the determination of LURIA and DELBRÜCK for T1. On the basis of the data given in table 1, one must assume that the rate of mutation to resistance to T1, being the sum of rates of mutation to **B**/1 and to **B**/(1, 5), is actually somewhat greater than the rate of mutation to resistance to T5, which is the same as the rate of mutation to **B**/(1, 5) only.

The rate of mutation to resistance to T4, as expected, does not appear to be different from the rate of mutation to resistance to T3. The rate of mutation to resistance to T7, however, might appear from the data to be, on the whole, greater than the rate of mutation to resistance to T3 and T4. This would disagree with the results on the types of mutation to resistance to T3, T4, and T7

presented in an earlier section, which require that resistance to T_7, being due to mutation to **B**/(3, 4, 7) only, be somewhat less than half as frequent as resistance to T_3 and T_4, which is due to mutation to either **B**/(3, 4, 7) or **B**/(3, 4). In view of the greater reliability of the results on the types of mutation, we ascribe the apparent disagreement to inaccuracy in the determination of the rate of mutation to resistance to T_7.

TABLE 3

Frequency of mutants resistant to phage T3.
(Tabulation of all experiments.)

PARENT STRAIN	No. OF CULTURES	AVERAGE NO. OF BACT. PER CULTURE	PERCENTAGE OF CULTURES WITHOUT MUTANTS	NO. OF MUTANTS PER PLATE		MUTATION RATE	
				AVER.	RANGE	FROM f	FROM r
	C	N	f	r			
B	37	$.29\times10^8$ "	8.1	12	0–39	6.0×10^{-8} "	6.2×10^{-8} "
B	25	.67 "	4	47	0–254	3.3 "	9.3 "
B	23	.54 "	26	14	0–78	1.7 "	4.3 "
B	25	.38 "	8	110	0–988	4.6 "	31.6 "
B	41	.075 "	44	2.3	0–18	7.6 "	6.4 "
B	42	.16 "	12	8	0–36	9.2 "	8.1 "
B	46	.36 "	17	10	0–93	3.4 "	4.2 "
B/1	30	.25 "	—	51	9–144	—	25.2 "
B/6	20	.6 "	30	21	0–83	1.4 "	5.3 "
B/6	20	.16 "	15	6.9	0–55	8.3 "	8.1 "
B/6/1	29	.92 "	—	133.8	16–1000+	—	16.3 "
B/1/6	30	.4 "	—	46	3–157	— "	14.0 "

The rate of mutation to resistance to T6 is not very different from the rates of mutation to resistance to T_3, T_4, or T_7, and is probably somewhere between 10^{-8} and 10^{-7}.

Correlation between resistance and the appearance of the colony.—The strain **B** of *E. coli* used in these experiments forms on our medium, in about 24 hours at 37°C, isolated round colonies measuring 2 to 4 millimeters in diameter and having a semirough surface. The surface of the colonies has a characteristic appearance.

It has been observed that resistant colonies derived by plating a broth culture of **B** on a Petri dish that has been previously spread with excess phage frequently appear different from the colonies of the parent strain. In general, it is possible to distinguish the following types:

(1) Large colonies, similar to the parent type.

TABLE 4

Frequency of mutants resistant to various types of phage.
(Tabulation of all experiments.)

PHAGE TYPE	PARENT STRAIN	NO. OF CULTURES	AVERAGE NO. OF BACT. PER CULTURE	PERCENTAGE OF CULTURES WITHOUT MUTANTS	NO. OF MUTANTS PER PLATE		MUTATION RATE	
					AVER.	RANGE	FROM f	FROM r
		C	N	f	r			
T1	B	53	$.16\times10^8$	87	.94	0–23	$.63\times10^{-8}$	1.4×10^{-8}
T1	B	53	.24 "	89	3	0–114	.33 "	2.3 "
T1	B	17	.54 "	41	11.7	0–133	1.15 "	3.9 "
T1	B	25	.72 "	48	14	0–120	.71 "	3.1 "
T1	B	19	1.2 "	32	25	0–122	.66 "	3.2 "
T1	B	38	.23 "	66	8	0–107	1.26 "	6.1 "
T1	B	45	.68 "	80	2.6	0–57	.24 "	0.8 "
T1	B	44	.38 "	71	7	0–153	.63 "	3.2 "
T1	B	45	.36 "	85	4	0–72	.31 "	2.0 "
T1	B	45	.51 "	51	3.6	0–47	.92 "	1.3 "
T1	B/3,4	30	—	70	2.0	0–19	—	—
T1	B/4,3	30	.45 "	53	10.2	0–208	1.0 "	3.8 "
T1	B/4,3,7	30	.11 "	67	16.2	0–350	2.6 "	21.8 "
T1	B/6	30	.44 "	50	2.6	0–32	1.10 "	1.3 "
T4	B	53	.22 "	11	12.7	0–80	7.0 "	8.2 "
T4	B	19	1.2 "	—	≈750	90–4000	—	≈58
T4	B	25	.4 "	12	51	0–675	3.7 "	~16.3 "
T4	B	43	.14 "	30	14	0–272	6.0 "	14.3 "
T4	B/1	30	.25 "	—	~120	2–≈2000	—	≈52 "
T4	B/6	30	.44 "	—	~68	9–800	—	≈18 "
T4	B/1/6	26	.18 "	12	5.7	0–18	8.2 "	6.1 "
T5	B	54	≈.1 "	91	1	0–45	.66 "	2.4 "
T5	B	16	1.2 "	50	3.2	0–16	.40 "	0.7 "
T5	B	17	.72 "	65	7.6	0–65	.42 "	2.1 "
T5	B	30	1.2 "	83	3.3	0–89	.11 "	0.6 "
T5	B	48	.71 "	10	~102	0–20000	2.3 "	15.5 "
T5	B	47	.41 "	81	3.6	0–73	.37 "	1.7 "
T5	B/1	44	1.4 "	2.3	33	0–289	1.9 "	2.9 "
T5	B/3,4	30	—	63	5.5	0–117	—	—
T5	B/4,3	30	.23 "	80	37.8	0–890	0.7 "	20.9 "
T5	B/4,3,7	30	.11 "	80	8.7	0–180	1.4 "	12.7 "
T6	B	30	.23 "	—	32	1–150	—	≈18.3 "
T6	B	23	.11 "	—	93	3–331	—	≈100 "
T6	B	45	.25 "	33	10	0–58	3.1 "	6.0 "
T6	B	44	.46 "	7	108	0–2000	4.0 "	≈25.0 "
T6	B	43	.26 "	5	156	0–≈1500	8 "	≈61.5 "
T6	B/1	29	≈.56 "	—	150	2–500	—	≈28.6 "
T6	B/4,3,7	31	.11 "	—	77.2	5–300	—	80.0 "

TABLE 4—*Continued*

PHAGE TYPE	PARENT STRAIN	NO. OF CULTURES	AVERAGE NO. OF BACT. PER CULTURE	PERCENTAGE OF CULTURES WITHOUT MUTANTS	NO. OF MUTANTS PER PLATE		MUTATION RATE	
					AVER.	RANGE	FROM f	FROM r
		C	N	f	r			
T_7	B	29	.45 "	—	92	20–667	—	24.4 "
T_7	B	22	.54 "	—	49	4–197	—	11.7 "
T_7	B	25	.20 "	—	42	2–212	—	28.0 "
T_7	B	45	.21 "	9	26	0–151	8 "	16.2 "
T_7	B	46	.4 "	15	37	0–378	3.3 "	11.5 "
T_7	B	43	.45 "	14	32	0–224	3.0 "	8.9 "
T_7	B/1	25	.36 "	—	193	125–300	—	≈55.5 "
T_7	B/1	30	≈.56 "	—	228	29–432	—	≈41.1 "
T_7	B/6/1	29	.75 "	—	176	33–~1200	—	≈25.3 "

(2) Small colonies, about one-half the diameter of the large ones but otherwise similar to them in shape, appearance, and rate of growth.

(3) Tiny colonies, less than one millimeter in diameter, having the markings and color of the large colonies but requiring a longer period to attain full size.

(4) "Nibbled" colonies, already referred to on page 121 as type (b), varying in size from large to tiny, with irregular edges that appear as though chewed up, and having a normal semi-rough appearance except on the chewed-up sectors, which have a watery-translucent consistency.

(5) Thin colonies, already referred to as type (c), which have a watery-translucent appearance throughout, are variable in regard to shape and size, and take longer than the large colonies to develop.

Types (1), (2), and (3) can readily be grown, either on agar or in broth, and reproduce true to form. Type (4) and type (5), as mentioned previously, after repeated transfers eventually yield colonies of either type (1), type (2), or type (3).

Analysis of the distribution of bacterial mutants (LURIA and DELBRÜCK 1943) has shown that the mutants resistant to a phage within a population of sensitive bacteria are not all of independent origin, but have been derived by multiplication from a smaller number of mutations that occurred independently during the development of that population. Therefore we expect that if a few mutants are derived from one population, plated on a single Petri dish with excess phage, they may belong to one single colony type, or to two types, or—more rarely—to three, if each colony type is characteristic of a certain clone of mutants. This was actually found and is illustrated in table 5, which is a copy of the record of one experiment, and in figures 8 and 9 on Plate 1, which are photographs of two cultures derived from two populations of bacteria plated on Petri dishes previously coated with T_4. In each figure, two types of colonies are evident; figure 8 shows large and small, and figure 9 shows large and tiny colonies.

It has been observed that certain colony types are more frequent among the

mutants resistant to one phage, while other colony types are more frequent among the mutants resistant to another phage. This shows a correlation between colonial morphology and resistance to certain phages. As a rule, among colonies of **B**/1, **B**/(1, 5), and **B**/(3, 4) a high proportion are large in size, while among **B**/6 a high proportion are small, thin, and nibbled, and among **B**/(3, 4, 7) a high proportion are tiny and thin.

The correlation between the appearance of a colony and resistance to a particular phage is especially evident in experiments where **B**/(3, 4) and **B**/(3, 4, 7) types are isolated. As has been mentioned earlier, approximately 60 per cent

TABLE 5

Distribution of various types among resistant colonies, obtained by plating cultures containing about 6×10^7 **B**/6 *bacteria on Petri dishes previously coated with T3 (Experiment 12–51).*

CULTURE	NUMBER OF RESISTANT COLONIES				
	LARGE	SMALL	TINY	NIBBLED	THIN
1	56				
2	4	43			
3	1		3		
4	12				
5	19				
6	39				
7	19				
8	6	18			
9	37	17			29
10	29	5			3
11	2				
12	5				7
13	26				
14	44				

of **B**/(3, 4) are **B**/(3, 4, 7). When the resistance of mutants obtained by plating **B** cultures on plates phaged with T3 or T4 is tested, and the colonies are classified according to their appearance, then it is seen that among the large colonies a majority (about 65 per cent) are **B**/(3, 4) and among the small colonies a majority (about 80 per cent) are **B**/(3, 4, 7) (table 6).

Comparative growth of different strains.—The determination of mutation rates from the observed frequencies of mutants rests upon the assumption that the growth rates of mutant strains do not differ appreciably from the growth rate of the original strain. LURIA and DELBRÜCK (1943) determined and compared the growth curves of the original strain **B** and of two mutant strains resistant to T1. On account of the large number of mutant strains under investigation, we resorted to the simplified but strict test of growing mutant strains in competition with **B**.

A broth culture was prepared containing a mixture of two strains—for ex-

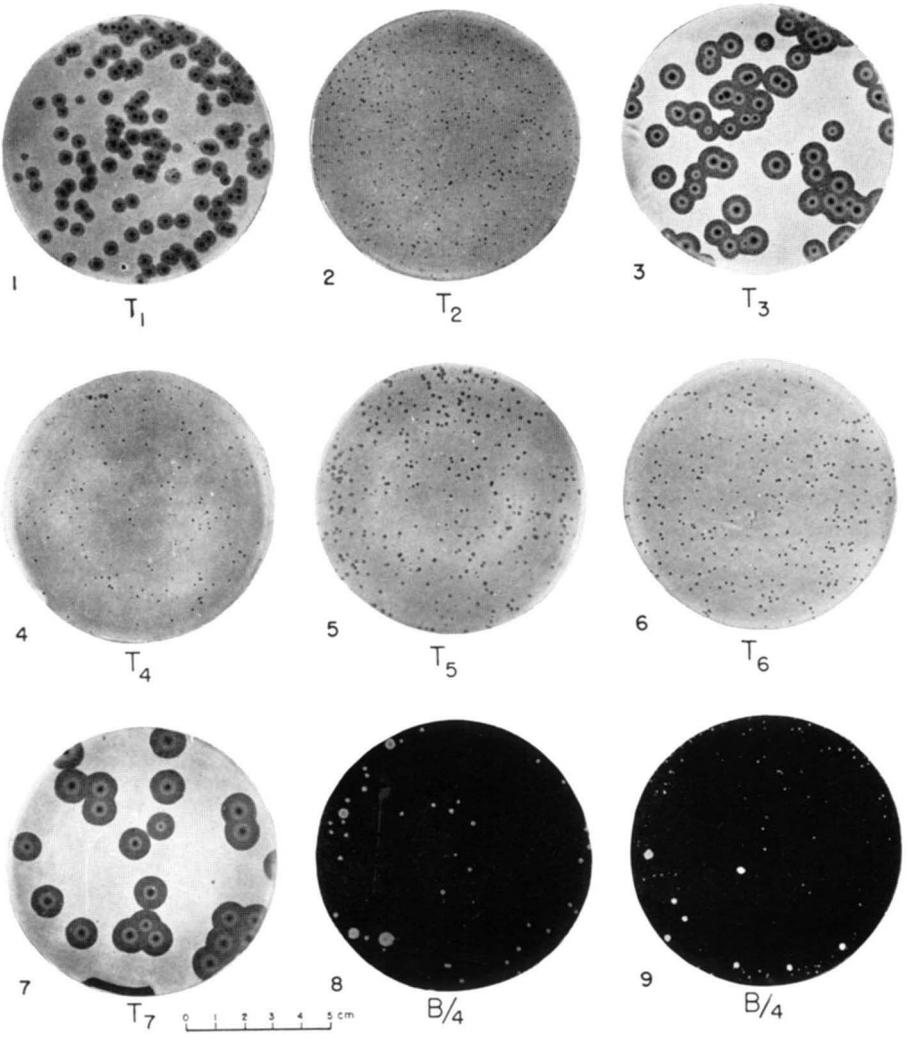

PLATE 1

FIGURES 1–7.—Photographs of plaques of phages T1 to T7 respectively. Appropriate dilutions of each phage were mixed with bacteria washed from 24-hour slant cultures; 0.05 cc of each mixture was added to 4 cc of melted agar (0.7 per cent) at 45°C and poured into Petri dishes containing about 20 cc of solid 1.0 per cent agar medium (HERSHEY et al. 1943). Photographs were taken after about 18 hours of incubation at 37°C.

FIGURES 8 and 9.—Colonies of **B**/4 bacteria obtained by plating about 3×10^7 **B**-bacteria on agar plates previously coated with about 10^8 particles of T4 phage. Figure 8 shows large and small colonies and figure 9 shows large and tiny colonies.

ample, **B** and **B**/6. These bacteria were derived by dilution from saturated cultures and were placed in the mixture in approximately equal numbers (a few thousand per cc). The mixture was assayed by placing small samples (a) on normal agar culture medium, and (b) on agar culture medium previously spread with excess phage (in our example T6). Colony counts on (b) plates determined the concentration of **B**/6 in the culture; the difference in count of (a) and (b) determined the concentration of **B**. The ratio **B**/(a−b) is the relative concentration of the two strains. The culture was then grown to saturation (approximately 10^8 per cc) and a new determination of the relative concentration of **B** and **B**/6 was made.

TABLE 6

Resistant strains derived by using T3 and T4 phage classified according to size of the colony and resistance to T7, indicating correlation between small size and resistance to T7.

COLONY SIZE		**B**/(3, 4)	**B**/(3, 4, 7)	TOTAL
Large:	observed	86	46	132
	expected	62	70	
Small:	observed	18	72	90
	expected	42	48	
Total		104	118	222

Table 7 lists the experiments carried out and their results, including the ratio of the final and initial values of the relative concentration of the pairs of strains. This ratio is 1 when the two strains grow at the same rate, greater or smaller than 1 if the mutant grows faster or slower, respectively. Under the experimental conditions, a small difference in the rate of growth of the strains, or any accident giving a small initial advantage to one strain, may strongly affect the final value of the relative concentration. Thus a 20 per cent difference in the duration of the division cycle would change the relative concentration during the experiment by a factor as large as 10. Also a delay of one hour in the onset of multiplication of one of the strains would have by itself an effect of similar magnitude. The importance of accidental variations can be appreciated by comparing the results of repeated experiments where the same strains were used. Repeated use of the same strain is indicated by ditto marks in the first two columns in the table.

In view of this, we feel that the results shown in the table indicate a considerable uniformity in the growth rates. On the whole, growth rates for mutants are lower than for the **B** strain; but no large competitive disadvantage seems to be systematically associated with the phenomenon of phage resistance. This seems to hold even when a multiple mutant strain is examined, and even when the strain's colonies on agar are much smaller than the colonies of the original strain.

TABLE 7

Results of experiments on competitive growth.

MUTANT STRAIN		SELECTING PHAGE	INITIAL ASSAY			FINAL ASSAY			b/a
RESISTANCE	COLONY		B	MUTANT	RELATIVE CONCENTRATION (a)	B	MUTANT	RELATIVE CONCENTRATION (b)	
B/1	large	T1	371	290	.78	132	303	2.3	3.0
B/1	nibbled large	T1	493	412	.84	0	646	∞	∞
"	"	T1	99	204	2.1	48	70	1.5	.71
"	"	T1	865	357	.41	107	232	2.2	5.3
B/6	large	T6	849	109	.13	1188	48	.04	.32
B/6	nibbled large	T6	132	354	2.7	58	172	3.0	1.1
B/1, 5	large	T1	218	121	.56	0	243	∞	∞
"	"	T1	81	110	1.4	90	22	.24	.18
"	"	T1	899	298	.33	87	126	1.4	4.4
B/1, 5	large	T1	485	189	.39	28	149	5.3	14.
B/1, 5	nibbled large	T1	110	122	1.1	38	58	1.5	1.4
B/5, 1	large	T1	124	91	.73	109	40	.37	.51
B/4, 3	large	T3	153	144	.94	262	18	.07	.07
"	"	T3	218	158	.77	220	25	.11	.14
"	"	T3	157	182	1.2	228	24	.10	.09
B/3, 4	large	T3	65	69	1.1	153	19	.12	.12
B/3, 4	small	T3	487	41	.08	141	2	.01	.17
B/3, 4, 7	small	T3	32	65	2.0	18	44	2.4	1.2
"	"	T3	100	16	.16	6	3	.50	3.1
B/4, 3, 7	small	T3	676	91	.13	240	4	.02	.12
"	"	T3	101	25	.25	156	0	0	0
B/7, 3, 4	tiny	T3	58	64	1.1	136	11	.08	.07
B/6/1	large	T6	123	798	6.5	297	193	.65	.10
B/1/3, 4	large	T3	45	117	2.6	155	33	.21	.08
B/1/3, 4	medium	T3	63	70	1.1	165	2	.01	.01
B/6/1, 5	large	T1	131	226	1.7	34	84	2.5	1.4
B/6/1, 5	large	T6	168	221	1.3	99	92	.93	.71
B/6/1, 5	nibbled large	T1	121	135	1.1	64	48	.75	.67
B/5, 1/6	large	T6	500	180	.36	612	89	.15	.40
B/6/3, 4	large	T3	866	157	.18	507	12	.02	.13
B/4, 3/1, 5	large	T3	778	90	.12	431	11	.03	.22
B/4, 3/1, 5	large	T1	104	48	.46	89	5	.06	.12
B/3, 4/1, 5	medium	T1	116	86	.74	68	5	.07	.10
B/3, 4/1, 5	medium	T1	84	41	.49	115	10	.09	.18
B/4, 3/1, 5	small	T3	545	122	.22	371	9	.02	.11
"	"	T1	388	211	.54	122	6	.05	.09
B/4, 3/1,5	small	T3	602	62	.10	477	0	0	0
"	"	T1	84	70	.83	113	0	0	0
"	"	T1	116	86	.69	141	2	.01	.02
B/4, 3/1, 5	small	T3	538	78	.15	317	7	.02	.15
"	"	T1	110	65	.59	170	0	0	0
"	"	T1	149	52	.35	191	4	.02	.06
B/7, 3, 4/1, 5	small	T1	429	256	.60	487	16	.03	.06
"	"	T1	72	218	3.0	137	9	.07	.02
B/7, 3, 4/1, 5	small	T1	103	114	1.1	165	0	0	0
B/6/1/7	thin large	T1	149	345	2.3	85	121	1.4	.61
B/6/1/7, 3, 4	thin medium	T1	181	239	1.3	89	103	1.2	.92
B/6/1/7, 3, 4	thin medium	T1	226	106	.47	141	163	.87	1.9
"	"	T1	93	143	1.5	214	28	.13	.08
B/6/1/7, 3, 4	thin medium	T1	110	48	.44	177	148	.84	1.9

DISCUSSION

The evidence presented in this paper shows that we isolated eight different mutants by exposing the **B** strain of *E. coli* to the seven bacteriophage strains

(T1–T7) used in our experiments. Two of these mutants (**B**/1 and **B**/6) are resistant to only a single phage strain each; two others (**B**/1, 5 and **B**/3, 4) are resistant to two strains each; and the remainder (**B**/3, 4, 7, **B**/1, 3, 4, 5, **B**/6, 1, 5 and **B**/7, 1, 3, 4, 5) possess multiple resistance. Of these mutants, **B**/1, **B**/1, 5, **B**/3, 4, **B**/3, 4, 7, and **B**/6 arise more frequently than the others. The fact that some of the mutations confer on the bacteria a resistance not to a single phage strain but to several strains suggests the existence of certain relationships among the latter (BURNET and MCKIE 1936). The phages in our collection may accordingly be classified into four resistance groups. T1 and T5 form one group; T3, T4, and T7 form another; while T2 and T6 are the single known representatives of two further groups. Mutants that confer on the bacteria a resistance to phages belonging to different groups are rare (**B**/1, 3, 4, 5, **B**/6, 1, 5, and **B**/7, 1, 3, 4, 5).

Theoretically, the origin of bacterial lines with multiple resistances may be accounted for in two different ways. Thus a **B**/1, 3, 4, 5 strain may arise from **B** owing to a single mutational step that confers resistance to these four types of phage at once, or else mutations to **B**/1, 5 and **B**/3, 4 (which are known to arise separately) may by a coincidence occur in the same cell or cell line. Taking into account the frequencies of the mutations (see below), the probability of such coincidences is extremely low, and a great majority of mutants with multiple resistance derived directly from a sensitive strain must be regarded as representing particular one-step mutational types.

Our technique of detection of mutants has been based on obtaining colonies of bacteria resistant to certain lines of phages, on agar plates on which all the nonresistant bacteria are destroyed by phage action. It was soon noticed that, at least among the five most frequent mutant types as detected by phage resistance, there is a clear variation in the morphology of the colonies. Although this variation might be continuous, the colonies can be classified into three fairly discrete groups: large, small, and tiny. Special experiments showed that the bacteria breed true as to which colony type they form. Taking into account colony type as well as resistance, we can say that about 20 distinct mutant types were present in our experiments. Evidence exists that *E. coli* is capable of producing a still greater variety of mutant types. BURNET and MCKIE (1936) worked with 37 different phage lines lysing one strain of *E. coli*, and there is very little doubt that this number could be increased. If there are mutants resistant to all types of phages, the number of different mutants that arise in *E. coli* must be considerable, probably running into hundreds.

As already stated, the different mutant types differ in frequency of occurrence. In the strain of *E. coli* that we used, the rate of mutation to **B**/2 is so low that we did not succeed in measuring it. The rates of mutation to **B**/(3, 4), **B**/(3, 4, 7), and **B**/6 range between 10^{-8} and 10^{-7}, while the rate of mutation to **B**/1 and **B**/(1, 5) is below 10^{-8}. This difference is significant. It is interesting to note that strains of bacteria which have by mutation become resistant to a certain phage still show rates of mutation to resistance to other phages similar to that of the original strain **B**. Thus, a line which had acquired resistance to

T6 (B/6) gives mutations leading to resistance to T_3, and thus is transformed into B/6/(3, 4) or B/6/(3, 4, 7), at a rate equal to that of the transformation of B into B/(3, 4) or B/(3, 4, 7). Furthermore, the multiple-resistant strains obtained in two or three steps from the original B line show a pattern of group resistance identical with that of the mutants obtained separately. It seems probable, therefore, that different mutants are independent of one another in origin and that they are caused by changes comparable to mutations in sexually reproducing organisms.

From the results obtained by us, it is evident that similar phenotypes may be obtained in different ways and may or may not have similar genotypes. For example, B/(1, 5) either may be obtained from B directly, by means of a single mutational step, or else may arise through summation of two independent changes: from B by mutation to B/1, and from B/1 by an independent mutation to B/1/5. Similarly, B/1, 3, 4, 5, 7 is occasionally obtained from B in one mutational step, but it can also arise in four steps: B to B/1, B/1 to B/1/5, B/1/5 to B/1/5/3, 4, and finally B/1/5/3, 4/7. On the whole, the observations suggest that two strains, B/1/5/3, 4/7 and B/1, 3, 4, 5, 7, although not distinguishable in their reactions to the phage lines at our disposal, actually possess different genotypes.

It may be questioned whether we are justified in referring to changes in the resistance of bacteria to the action of bacteriophages as "mutations," and particularly as "gene mutations." Since these changes are retained by the entire offspring of the modified colonies, and the properties of the changed strains are as stable as those of the original one, we are dealing with hereditary changes. Since these changes arise, as far as we can tell, in a single discrete step, it seems legitimate and proper to call them mutations. The justification for calling them gene mutations is much less convincing. The existence of genes in higher organisms is inferred from the Mendelian segregations in crosses between unlike strains or races; the unitary nature of a gene is defined by its behavior as a discrete unit in heredity, crossing over, mutation, and finally in chromosome breakage. Strictly speaking, it is the concurrence of all these criteria that permits us to retain the idea of discrete genes notwithstanding the evidence of their partial mutual dependence (position effects). Since bacterial reproduction, as far as is known, is exclusively asexual, inheritance in bacteria cannot be studied with the aid of the methods employed in work on higher organisms, nor can the criteria characterizing gene mutations be applied directly. We have given evidence, however, showing that several distinct types of mutational change arise in *E. coli*, each with its characteristic frequency and—what is perhaps most important in this connection—independently from one another. This is at least indicative of the existence in these bacteria of several independent self-reproducing and separately changeable entities, which to that extent remind one of genes as the latter are known in sexually reproducing organisms.

SUMMARY

In a single strain, B, of *Escherichia coli*, changes to resistance to seven strains

of bacteriophage (T1 to T7) were studied. A total of 377 mutants was investigated (table 1), and eight different groups were detected. Five of these—**B**/1, **B**/1, 5, **B**/3, 4, **B**/3, 4, 7, and **B**/6—occur with higher frequency than the other three—**B**/1, 3, 4, 5, **B**/6, 1, 5, and **B**/7, 1, 3, 4, 5. (The numbers following the bar indicate the phages to which the mutant is resistant.)

In addition, 364 mutants were derived from lines already resistant to one or more phages. The cross-resistance pattern in these tests does not significantly differ from the pattern obtained in the previous experiments, where mutants were derived from the sensitive **B** strain.

The rate of mutation to **B**/(1, 5) was approximately 10^{-8}, to **B**/(3, 4) between 10^{-8} and 10^{-7}, and to **B**/6 slightly higher.

Extensive tests made with 34 different mutants representative of the material used in this work indicate that the growth rates of mutants do not differ systematically from that of the original **B** strain, when they are grown together with it.

The rate of mutation to resistance to a given phage is similar for the **B** strain and for strains already resistant to some other phage or phages.

These results indicate that mutations to different resistance types are independent of one another and are probably produced by changes comparable to gene mutations.

Evidence indicates that complex mutants resistant to two or more phages may be obtained from **B**, either by means of a single mutational step or through summation of two or more changes.

It was found that mutants frequently show morphological differences in colony size, which breed true. At least three colony sizes can be distinguished for each of the eight types of mutants—namely, large, small, and tiny colonies. Altogether about 20 distinguishable mutant types showing some degree of resistance to our seven phages appeared in our experiments. Since many more phages affecting the **B** strain could be isolated, it is evident that the actual number of mutants affecting resistance is considerably larger.

A correlation between colony size and resistance type was noted. The frequency of small colony types is higher among **B**/(3, 4, 7) than among **B**/(3, 4) mutants.

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Before the experiments were completed, the junior author took leave of absence to join a war research project.

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