SPONTANEOUS MUTATION TO STREPTOMYCIN RESISTANCE AND DEPENDENCE IN ESCHERICHIA COLI

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Bacteria give promise of yielding quantitative information on the process of gene mutation, which could not be obtained from the classical genetic materials (Luria, 1947). However, detailed studies have so far been confined almost entirely to a particular group of mutations, namely, those producing phage resistance (Demerec, 1946; Witkin, 1947b; Bryson, 1948; Beale, 1948; Newcombe, 1948; Newcombe and Scott, 1949). For this reason, the present investigation has been directed toward obtaining accurate data for a different kind of mutational change, namely, the development of drug resistance.

The genetic origin of resistance to three of the most common antibacterial drugs has already been studied. These are penicillin (Demerec, 1945, using *Staphylococcus aureus*), sodium sulfathiazole (Oakberg and Luria, 1947, using *S. aureus*), and streptomycin (Klein and Kimmelman, 1946; Alexander and Leidy, 1947; Demerec, 1947; Scott, 1949; using, respectively, *Shigella* spp., *Hemophilus influenzae*, *S. aureus*, and *Escherichia coli*). Of these, streptomycin was chosen since it appeared to be the only one to which a high degree of resistance could be developed in a single mutational step.

MATERIALS

Escherichia coli strain B/r (Witkin, 1946, 1947a) and streptomycin (calcium chloride complex, Merck) have been used throughout. Liquid cultures were grown in Difco nutrient broth plus 0.5 per cent sodium chloride, and platings were made with Difco nutrient again having the same salt concentration. One unit of streptomycin is the equivalent of one microgram of streptomycin base.

EXPERIMENTAL RESULTS

Degrees of streptomycin resistance. Survival in concentrations of streptomycin ranging from 0 to 4,096 units per ml was measured by plating known numbers of bacteria with melted agar containing streptomycin and counting the colonies that developed on incubation.

Curves were first obtained for the parent strain and then for cultures derived from the colonies surviving 1, 2, 4, 8, ... 1,024 units per ml. These are shown in figure 1 and are numbered to indicate the concentration from which the colonies were isolated. Numbers 1 to 8 are from means of four tests each. Of seven colonies surviving 16 units per ml, three possessed slightly increased resistance (curves 16a, b, and c), and four were resistant to 4,096 units per ml. The two types will be termed "slightly " and "fully" resistant. All colonies surviving

32 units, or more, per ml were of the latter type (table 1). This absence of intermediate forms possessing higher degrees of resistance has also been found in *Hemophilus influenzae* (Alexander and Leidy, 1947), and in *Mycobacterium tuberculosis* and *Mycobacterium ranae* (Yegian and Vanderlinde, 1948). The *Meningococcus* data (Miller and Bohnhoff, 1947) appear at first sight to constitute an exception, but the apparent difference could be due to the use of surface platings, a condition that greatly reduces the selective action of the drug.
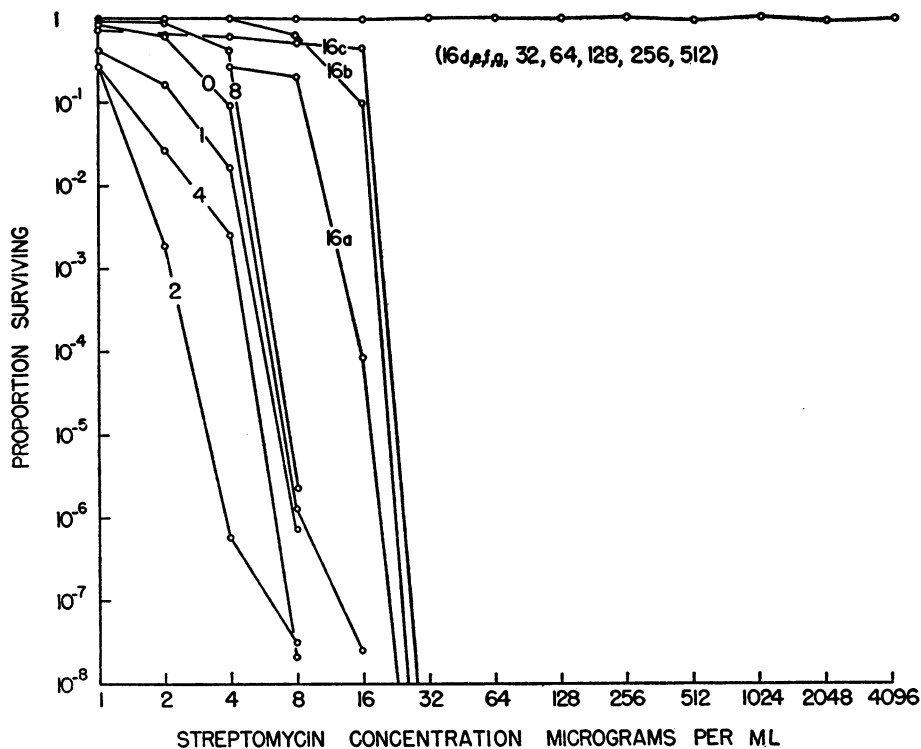

Figure 1. Survival curves for strains isolated from various concentrations of streptomycin. The number of each curve indicates the streptomycin concentration in units per ml from which the strain was isolated.

Two types of the fully resistant variant were observed: one capable of growing either in the absence or in the presence of streptomycin and the other only when streptomycin is present. These have been termed "resistant" and "dependent," and will be referred to as *sr* and *sd*, respectively. Similar resistant and dependent types have been described in *Meningococcus* by Miller and Bohnhoff (1947), in *Staphylococcus aureus*, *Proteus morganii*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Pseudomanas aeruginosa*, by Paine and Finland (1948), and in *Mycobacterium* by Hobby and Dougherty (1948).

Both the *sr* and the *sd* variants have been subcultured serially, the former in the absence of streptomycin and the latter in 128 units per ml. Single colonies from a dilution of the previous culture were used as the inoculum in each case.

Both variants were unchanged after ten such subculturings, this being the equivalent of a factor increase of 10^{105}, or of more than 300 cell generations.

Mutation rates. Series of similar cultures were grown from small inocula, the whole cultures (centrifuged where the volume was greater than 1 ml) plated with agar containing 64 units of streptomycin per ml, and colony counts made. Each colony was then tested to determine whether it was *sr* or *sd*. Mutation

TABLE 1

Proportions of fully resistant colonies obtained when sensitive populations are plated with increasing concentrations of streptomycin

CONCENTRATION OF STREP-TOMYCIN (UNITS/ML)	NO. OF COLONIES TESTED	NO. OF COLONIES RESISTANT TO 1,000 UNITS/ML	PERCENTAGE OF RESISTANT COLONIES
8	14	1	7
16	13	5	38
32	5	5	100
64	41	41	100

TABLE 2

Mutation rates from sensitivity to sr and sd

EXPERI-MENT	c	VOL. ML	$n_2 \times 10^8$	MUTATION TO *sr*				MUTATION TO *sd*			
				p_0	r	$a_1 \times 10^{-10}$	$a_2 \times 10^{-10}$	p_0	r	$a_1 \times 10^{-10}$	$a_2 \times 10^{-10}$
A	90	1	3.7	0.96	0.10	0.9	1.1	1.00	0.09	0.8	1.0
B	100	1	2.9	0.95	0.35	1.2	3.3	0.98	0.02	0.5	0.6
C	100	1	4.3	0.96	0.14	0.7	1.1	0.93	0.07	1.2	0.8
D	100	1	4.9	0.92	0.20	1.2	1.3	0.92	0.10	1.2	0.8
E	16	10	24	0.75	0.44	0.8	0.9	0.69	0.50	1.1	0.9
F	16	10	24	0.75	0.38	0.8	0.8	0.75	0.44	0.8	0.9
G	16	10	35	0.75	0.81	0.6	0.8	0.50	0.50	3.1	0.6
H	16	10	33	0.94	0.06	0.2	0.3	0.69	0.56	1.7	0.8
Averages of mutation rates................						0.8	1.2			1.3	0.8

C, number of cultures, n_1, bacteria in the inoculum (not listed above, is between 10^2 and 10^4), n_2, bacteria in the fully grown culture, p_0, proportion of cultures in which no mutant cells occurred, r, average mutant cells per culture, a_1 and a_2, estimates of mutation rate using formulas 1 and 2.

rates to *sr* and *sd* were estimated by the two standard methods (Luria and Delbrück, 1943; formulas 1 and 2 of Newcombe, 1948) and were found to be similar and equal to approximately 10^{-10} per bacterium per division cycle (table 2).

Evidence for the spontaneous mutational origin of the *sr* variants was obtained by comparing the numbers of mutant cells in individual test cultures with the numbers expected on the mutation hypothesis (table 3). A comparison similar to this has been made by Luria and Delbrück (1943) for phage resistance. The present case differs from theirs in that it has been possible to avoid correcting for the occurrence of two mutations in the same culture by using data solely from

the 1-ml cultures. Since only about 5 per cent of these contained *sr* mutants, the number of cultures in which two such mutations had taken place would be negligible. The table shows that there was good agreement.

TABLE 3

Numbers of mutations giving rise to various numbers of descendants

(Comparison of observed distributions with those calculated assuming a constant mutation rate and equal growth of mutant and parent strains)

DIVISION PRIOR TO END OF GROWTH	NO. OF DESCENDANTS FROM A MUTATION	NUMBERS OF MUTATIONS			
		sr		*sd*	
		Calc.	Obs.*	Calc.	Obs.*
0	1	10.5	11	11.0	18
1	2	5.8	6	5.5	4
2	3–6†	2.9	1	2.8	0
3	7–11	1.4	2	1.4	0
4	12–22	0.7	0	0.7	0
5	23–45	0.4	1	0.4	0

* Data from the 1-ml test cultures only. No correction is made for those cultures in which two mutations may have occurred, since the probable number of these is negligible.

† Where small whole numbers are involved the limits of the classes cannot be set with accuracy. As a result this class is somewhat larger than it should be and the preceding one somewhat smaller.

TABLE 4

Competitive growth of susceptible and sr strains in broth

No. of replicate cultures......................................	10
Vol. of cultures, ml..	10
Avg. *sr* bact. in inoc. (m_1)...................................	0.73×10^4
Avg. total bact. in inoc. (n_1).................................	1.94×10^4
After 18 hours' incubation	
Avg. *sr* bact. (m_2)...	0.96×10^9
Avg. total bact. (n_2).......................................	2.45×10^9
Factor increase in population.............................	1.3×10^5
No. of generations...	17.1
Avg. $m_2/n_2 \times n_1/m_1$....................................	0.91 ± 0.165
After 5 days' incubation	
Avg. *sr* bact. (m_3)...	0.63×10^9
Avg. total bact. (n_3).......................................	1.59×10^9
Avg. $m_3/n_3 \times n_1/m_1$....................................	1.10 ± 0.264

The *sd* variants are similarly treated in the table, but owing to the streptomycin dependence of this form no test culture would be expected to contain large numbers. That the two comparisons differ in the manner anticipated is evidence of the validity of the test.

It should be noted that *sr* and *sd* individuals did not occur together in the same culture more often than would be expected from the action of chance.

Possible sources of bias. Formula 2 assumes that the mutant bacteria multiply at the same rate as the parent strain. That there are no appreciable differentials of growth or of death in the case of the *sr* variant has been shown by growing cultures from mixed inocula (table 4). Bacteria of the *sd* strain similarly treated failed to undergo more than two divisions during 17 generations of the parent strain. It follows that formula 2 will not be biased in the case of mutation to *sr*, but will underestimate the rate of mutation to *sd* as a necessary consequence of the streptomycin dependence.

Both formulas would give biased estimates if the growth of some of the resistant cells were inhibited by the presence of large numbers of sensitive bacteria. That this does not occur in the case of *sr* cells has been shown by mixing known

TABLE 5

Recovery of sr mutants that have been added to large susceptible populations

No. of replicate tests..	14
Vol. of suspension used in each test, ml......................	10*
Avg. no. of bact. in test suspensions.........................	7.4 × 10⁹
Avg. resistant mutants in test suspensions...................	6
Avg. resistant mutants added.................................	264
Avg. recovered after centrifuging.............................	313
Avg. ratio observed/expected recovery.........................	1.19 ± 0.76

* Note: The 10-ml samples used in each of the 14 replicate tests were all from the same culture. One sample with, and one without, added mutants were centrifuged and plated in each test. To minimize death from metabolic products formed during and after centrifuging while the bacteria were crowded in the pellet, the suspensions were chilled and kept at approximately 5 C until plated.

numbers with approximately 10^{10} sensitive bacteria, plating with streptomycin agar, and making colony counts in the usual manner (table 5).

Single-step origin of the fully resistant variants. It was conceivable that the slightly resistant forms might mutate more readily to full resistance than did the parent strain, and that as a consequence an appreciable proportion of the changes to full resistance might take place via this intermediate step. Two of the slightly resistant forms described earlier were tested, and the mutation rates were found not to differ significantly from those for the parent strain. These rates, with formula 1, were 0.3 and 1.1×10^{-10} for mutation to *sr*, and 1.8 and 1.4×10^{-10} for mutation to *sd* as obtained from series of 24 ten-ml cultures of each strain.

DISCUSSION

The possible effects of dominance and phenotypic delay. Approximately equal estimates of the rate of mutation to the *sr* form have been obtained using formulas 1 and 2. If the bacteria contained only a single nucleus, this would indicate that gene mutation alters the phenotype immediately, or within a negli-

gible fraction of a division cycle. However, there is cytological evidence of two, and sometimes four, linearly arranged nuclei in the cells during logarithmic growth (Robinow, 1945; Boivin, 1947). Where four nuclei are present, cleavage appears to separate sister pairs. Although observation is more difficult during the period immediately prior to saturation, it is possible that more than one nuleus is present throughout the whole of the growth cycle.

If the bacteria contain more than one nucleus, it is necessary to take account of dominance, since a mutant gene when it first appears would be present in only one nucleus. In this heteronucleate state it might be either dominant or recessive. If recessive, expression could not occur until the segregation of a homonucleate mutant. Also, after the development of the appropriate genotype (heteronucleate dominant or homonucleate recessive) phenotypic expression could follow either immediately or after a delay of one or more generations. There are four possible combinations of these conditions: (a) dominant mutation, no phenotypic delay; (b) dominant mutation, phenotypic delay; (c) recessive mutation, no phenotypic delay; and (d) recessive mutation, phenotypic delay. It is inherent in the formulas used for the calculation of the mutation rates that the relative values of the two estimates obtained by formulas 1 and 2 (Newcombe, 1948) would depend upon which of these conditions applies. Thus, in case (a) estimate 1 would be greater than estimate 2; in case (b) estimate 1 would equal estimate 2 if the number of generations required for phenotypic expression was equal to the number required for a homonucleate individual to develop (one generation if there are two nuclei, and two generations if there are four) but, failing this, it could be either greater or less; in case (c) the two estimates must be equal; and in case (d) estimate 1 would be less than estimate 2. Thus, the equal estimates obtained for mutation to the *sr* form could be interpreted in terms either of dominance of the mutant gene or of absence of phenotypic delay.

It should be pointed out that formulas 1 and 2 do not yield equal estimates of the rate of mutation to phage resistance (Luria and Delbruck, 1943; Demerec and Fano, 1945), and it is clear that phenotypic change must occur two or three generations after the formation of a homonucleate mutant cell (Newcombe, 1948). Whether streptomycin resistance differs in being controlled by a dominant gene or in having no phenotypic delay cannot be decided at the present time.

The possibility of related genes in other organisms. It is of interest that mutation to streptomycin resistance in *Hemophilus influenzae* occurs at a rate, 0.4×10^{-10} (Alexander and Leidy, 1947) that is not appreciably different from that for *E. coli*. This, together with the fact that the development of resistance and of dependence both appear to be widespread phenomena, suggests that the gene, or genes, responsible may be related by descent and relatively unchanged with respect to one another.

SUMMARY

The estimated rates of mutation of *Escherichia coli* strain B/r to streptomycin resistance and to streptomycin dependence are both approximately 1×10^{-10} per bacterium per division cycle.

Mutation to streptomycin resistance differs from mutation to phage resistance in that equal estimates are obtained by the two standard formulas. The difference could be due either to dominance of the gene for streptomycin resistance or to absence of phenotypic delay.

E. coli and *Hemophilus influenzae* have similar rates of mutation to streptomycin resistance, and it is suggested that the genes responsible may be related.

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