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Fluctuation Analysis of Mutations to Nalidixic Acid Resistance in *Escherichia coli*

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Mutations of *Escherichia coli* from sensitivity to nalidixic acid resistance were studied by fluctuation analysis. The mutant distributions in replicate cultures were not significantly affected either by the age of the carbon-starved preculture used for inocula or by the inoculum size. The data from 23 fluctuation tests (48 cultures each) were pooled. The mean number of mutations per culture was estimated to be 0.71 from the fraction of cultures without mutants or 0.74 and 0.77 by maximum-likelihood estimation based on the two models under consideration. When the pooled data were compared with the theoretical expectations, the fits were unsatisfactory ($P < 0.005$). The lack of fit was caused mainly by too high a frequency of cultures with between 17 and 32 mutants and too high a frequency of cultures with more than 128 mutants. Possible reasons for the lack of fit and its implications with respect to estimation of mutation rates from fluctuation tests are discussed.

The statistical analysis of bacterial mutations came into prominence with the publication of Luria and Delbrück (16); the system they studied was that of resistance to bacteriophage T1 in *Escherichia coli*. When a culture of sensitive *E. coli* is plated in the presence of an excess of the phage, although virtually all the bacteria are killed, a small number survive and give rise to colonies on the plate. The problem analyzed by Luria and Delbrück was whether the survivors were the result of mutations that occurred prior to the plating, i.e., during the growth of the culture, or whether they occurred after plating, i.e., as a direct consequence of the bacteria being exposed to the phage. These two principal conceptions may be labelled the pre- and postadaptive theories, respectively (7). Luria and Delbrück studied the problem using mathematical models. If the mutation occurred postadaptively, the distribution of mutants in a number of replicate (parallel) cultures should be a Poisson distribution, provided that all bacteria have a small, finite probability of mutating to become resistant after the exposure to the phage. The preadaptive mutation theory leads to a quite different prediction. Although the replicate cultures are the same with respect to the total size of population, some may have experienced a mutation at an early generation and thereby yield a large number of mutants, while with others, a mutation may have occurred just prior to the plating so that only one or a few mutants are present in these cultures. The difference in the predictions of the pre- and postadaptive mutation theories could therefore be reduced to a prediction of the variance of the number of mutants in replicate cultures: the postadaptive theory predicted that the variance would equal the mean, whereas the preadaptive mutation theory predicted that the variance would be much larger, owing to a few cultures yielding a large number of mutants.

Luria and Delbrück grew a number of replicate cultures that were plated in the presence of excess of phage T1 and, after incubation, counted the number of mutant colonies obtained from each culture. They did not correct for sampling error, nor did they deduce the theoretical distribution expected from the preadaptive mutation theory; they did not need to do so, as the empirical variance was very much larger than the empirical mean. They correctly concluded that mutations to T1 resistance occur preadaptively (as pointed out by Cairns et al. [6], this mutation cannot occur postadaptively, as it has a phenotypic delay and the selection is lethal). Luria and Delbrück also provided two methods for the estimation of mutation rates. In method 1, the mutation rate is estimated as the complementary probability to not mutating, i.e., from the fraction of cultures without mutants (derived from Luria and Delbrück's equation 5): $a = -(N_t - N_0)^{-1}\ln P_0$, where a is the mutation rate measured per generation, N_t is the number of cells at the time of plating, N_0 is the number of cells used as inoculum, and P_0 is the fraction of cultures without mutants. In method 2, the mutation rate is estimated by iterative trials from the following equation (Luria and Delbrück's equation 8): $r = aN_t\ln(aCN_t)$, where r is the mean number of mutants per culture and C is the number of cultures.

As mentioned, Luria and Delbrück did not deduce the theoretical distribution of mutants, but it is evident that assumption or knowledge of the theoretical distribution allows alternative and more efficient estimates of the mutation rate. Lea and Coulson (14) provided a numerical method for calculating the theoretical distribution based on the assumptions that all parental cells have the same probability of mutating, all mutant cells have identical probabilities of dividing within a short time interval, and the growth rates of wild-type and mutant cells are identical. Many later studies have modified this solution both to optimize the calculations and to account for the effects of factors such as phenotypical delay, different growth rates of mutants and the wild type, sampling error, etc. (for examples, see references 2, 3, 10, 12, 15, 17–19, 27, 28). The most extensive study is probably that of Stewart et al. (28); for a review on fluctuation analysis, see reference 11. Considering the many recondite theoretical studies, it may seem surprising that few authors have found it

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TABLE 1. Distribution of Nal^r mutants as a function of the age of the preculture (carbon starvation at 37°C)[a]

No. of mutants	No. of cultures with mutants in expt no. (incubation time):					
	14 (24 h)	15 (48 h)	16 (76 h)	17 (24 h)	18 (48 h)	19 (72 h)
0	29	24	28	28	22	23
1	7	8	6	6	6	5
2	2	1	1	3	6	3
3 or 4	2	5	3	1	4	8
5 to 8	3	7	3	3	0	4
9 to 16	1	3	2	2	5	2
17 to 32	3	0	2	2	3	2
33 to 64	1	0	1	1	1	0
65 to 128	0	0	2	0	1	0
129 to 256	0	0	0	2	0	1
>256	0	0	0	0	0	0

[a] In the contingency tests the values are grouped in the following classes: experiments 14 to 16, cultures with one and two, cultures with two to eight, and cultures with more than eight mutants; experiments 17 to 19, cultures with two to four and cultures with more than four mutants.

worthwhile to test whether the theory of Lea and Coulson (14) fits with experimental observations.

Armitage (2) tested the data of Luria and Delbrück (16) and of Newcombe (21); both cases were fluctuation analyses of mutations of *E. coli* to resistance to phage T1. Newcombe had, on the basis of the discrepancies between different estimates of the mutation rate, correctly realized the existence of phenotypic delay for this mutation, and consequently Armitage (2) introduced this as a variable in the model of Lea and Coulson, but even with this extra parameter the fit between theory and experimental data was unsatisfactory (level of significance, $P < 0.05$). This was, however, not the only surprise in the study by Armitage (2). Newcombe (21) had published the mutant distributions of eight series of 25 cultures each. Four series were inoculated with 10 cells, and the remaining four series were inoculated with 10^4 cells; each of these sizes is negligible in comparison with the final population size, and the distributions for all of the eight series would a priori be expected to be identical. Armitage (2) found, nevertheless, that the distribution of mutants differed significantly between the two halves. Armitage (2) offered no explanation for this, but an explanation could be that a small fraction of cells in the precultures used for inocula were predisposed to give rise to progeny with a mutation rate different from that for the average cell. In the inocula of 10 cells they were absent, whereas in the inocula of 10^4 cells they were present. Although it may be doubtful how much weight should be attached to conclusions based on such a limited set of data, the finding of Armitage (2) should certainly serve as a warning that, in contrast to the theory, the outcome of fluctuation tests may depend on the inoculum size.

Ryan (25) made similar tests comparing the theory of Lea and Coulson with the observed distributions of the number of lactose-utilizing (*lac*$^+$) mutants of two *lac*-negative strains of *E. coli*. One of the strains resulted in an unsatisfactory fit ($P < 0.01$), whereas the other resulted in some degree of fit ($P = 0.2$). However, in this fluctuation test the average number of mutations per culture was large, and, as Ryan grouped the cultures with more than 64 mutants, all data basically fall in four classes; therefore, this level of fit is not to be considered conclusive.

In retrospect, neither mutations to T1 resistance nor reversion of a *lac* mutation can be considered ideal as test mutations. The T1-resistant phenotype is usually the result of mutations disrupting a membrane protein that is used as a receptor by the phage. Thereby, many different mutational events such as point mutations, frameshift mutations, deletions, insertion element translocation, etc., may result in the resistant phenotype. Some of these mutations may be the result of replication errors and occur generation dependently, whereas others may not. Secondly, some of the mutant progeny will retain wild-type receptor protein and thereby also retain phage sensitivity. In principle, some of the mutant progeny will never become fully resistant. The *lac*$^+$ mutation used by Ryan

TABLE 2. Distribution of Nal^r mutants as a function of inoculum size[a]

No. of mutants	No. of cultures with mutants in expt no. (dilution):											
	6 (10^4)	7 (10^5)	8 (10^6)	9 (10^7)	10 (10^4)	11 (10^5)	12 (10^6)	13 (10^7)	20 (10^4)	21 (10^5)	22 (10^6)	23 (10^7)
0	22	34	22	16	23	19	22	25	22	21	23	21
1	7	5	5	10	6	13	10	4	6	8	13	7
2	3	1	9	6	6	2	5	8	6	5	2	6
3 or 4	3	2	1	8	7	5	7	5	4	6	3	6
5 to 8	3	1	6	3	4	2	0	2	1	4	4	3
9 to 16	4	2	4	2	0	3	2	1	4	3	0	3
17 to 32	4	2	0	1	0	0	1	3	3	1	2	2
33 to 64	0	1	0	1	0	1	0	0	1	0	1	0
65 to 128	1	0	0	1	0	0	1	0	0	0	0	0
129 to 256	0	0	0	0	1	0	0	0	1	0	0	0
257 to 512	1	0	0	0	0	2	0	0	0	0	0	0
>512	0	0	1	0	1	1	0	0	0	0	0	0

[a] In the contingency tests, the values are grouped in the following classes: experiments 6, 10, and 20, cultures with three to eight and cultures with more than eight mutants; experiments 9, 13, and 23, cultures with three or four and with more than four mutants. With the pooled data, cultures with more than 16 mutants are grouped.

TABLE 3. Goodness of fit between the theoretical distributions and the experimental distribution of Nal^r mutants at 37°C

No. of mutants	No. of cultures with mutants				
	Observed	Lea-Coulson distribution		Haldane distribution	
		$\hat{m}$	m_0	$\hat{m}$	m_0
0	543	528.5	543.0	511.2	543.0
1	169	194.7	192.7	196.8	192.7
2	92	100.7	98.4	136.2	130.5
3 or 4	99	100.9	97.8	109.2	101.3
5 to 8	72	78.1	75.2	71.2	64.8
9 to 16	51	48.5	46.6	39.1	35.3
17 to 32	41	26.2	25.1	20.0	18.1
33 to 64	13	13.3	12.7	10.1	9.2
65 to 128	9	6.6	6.3	5.1	4.6
129 to 256	7	3.3	3.1	2.5	2.3
256 to 512	4	1.6	1.6	1.3	1.1
>512	4	1.6	1.5	1.3	1.1
Avg no. of mutations		0.737	0.710	0.770	0.710
χ^{2a}		25.5	27.6	69.9	81.3
No. of degrees of freedom		8	8	8	8
P		<0.005	<0.001	≪0.0005	≪0.0005

[a] Cultures with >128 mutants were grouped.

(25) is also doubtful as a test mutation since at least some *lac*$^+$ reversions seem to occur postadaptively (6).

The wide use of fluctuation tests in the study of mutagenesis in bacteria and the limited attempts to compare the theory with experimental observations make it worthwhile to revive the question of whether the theory describes the facts. Furthermore, it has recently been revealed that Haldane devised, but did not publish, a theory alternative to that of Lea and Coulson (14) (see reference 26 for Haldane's solution). The existence of two alternative models for the distribution of mutants in replicate cultures makes it almost a necessity to compare the alternative theories with experimental observations. To simplify, we have used only the unmodified theories.

MATERIALS AND METHODS

Bacterial strain. A derivative of the *E. coli* K-12 strain MG1655 was used throughout this study.

Media and growth conditions. AB minimal medium (8) supplemented with 0.05% glucose as the limiting growth factor was used as liquid medium. Luria-Bertani agar (4) containing nalidixic acid (100 mg/liter) was used as solid medium selective for nalidixic acid-resistant mutants. All incubations were performed at 37°C for 24 h unless stated otherwise.

Fluctuation test. The fluctuation tests were performed essentially as described by Luria and Delbrück (16). An outgrown culture (containing approximately 4.0×10^8 CFU/ml) was diluted in minimal medium (10^5 times unless indicated otherwise), and 3 ml was distributed to each of 48 tubes. The cultures were incubated until stationariness was ensured (24 to 36 h, depending on inoculum size), and the cells were harvested by centrifugation, resuspended in 0.1 ml of medium, and plated on medium selective for mutants. After overnight incubation the number of mutant colonies originating from each culture was counted.

Statistical methods. For a general description of the statistical methods, see reference 9.

Calculation of the theoretical mutant distributions. The Lea-Coulson distribution was calculated according to equation 4 in reference 27; the Haldane distribution was calculated from an unpublished computer program compiled by N. B. Jensen and N. Tholstrup. An outline of the solution used in the maximum-likelihood estimation is presented in Appendix.

Maximum-likelihood estimates of the mean number of mutations per culture. Consider a fluctuation test consisting of N cultures. Let the vector $\vec{n} = (n_0,n_1,n_2,n_{3-4},n_{5-8},n_{>8})$ denote the observed number of cultures with 0, 1, 2, 3 and 4, 5 to 8, and more than 8 mutants, respectively. These numbers are assumed to follow a multinomial distribution with fixed probabilities. The likelihood of a sample can therefore be written as $L(\vec{n},m) = L(n_0,n_1,n_2,n_{3-4},n_{5-8},n_{>8},m) = \binom{N}{n_0 n_1 n_2 n_{3-4} n_{5-8} n_{>8}} P_0(m)^{n_0} P_1(m)^{n_1} P_2(m)^{n_2} P_{3-4}(m)^{n_{3-4}} P_{5-8}(m)^{n_{5-8}} P_{>8}(m)^{n_{>8}}$, where m denotes the mean number of mutations per culture and $P_r(m)$ is the theoretically expected frequency of cultures with r mutants. $P_r(m)$ is calculated according to the model under consideration. The log likelihood is $\log L(\vec{n},m) = K + n_0 \log P_0(m) + n_1 \log P_1(m) + n_2 \log P_2(m) + n_{3-4} \log P_{3-4}(m) + n_{5-8} \log P_{5-8}(m) + n_{>8} \log P_{>8}(m)$, where K is a constant. The maximum likelihood estimate, $\hat{m}$, is determined as the value of m that maximizes the log likelihood, that is, $\hat{m} = arg \max \log L(\vec{n},m)$. Identification of $\hat{m}$ was carried out by iterative methods.

Goodness-of-fit test. The $\chi'^2 = \Sigma \frac{(\text{observed} - \text{expected})^2}{\text{expected}}$ value is calculated as the sum over classes, and the level of significance (P) is obtained by comparison with the proper $\chi^2(\phi)$ distribution, ϕ being the number of degrees of freedom (ϕ = number of classes − 2). The expected values are calculated according to the model considered.

Contingency test. The contingency test method was used to test if there are significant differences between the outcomes of a number of fluctuation tests. $\chi'^2 = \Sigma \frac{(\text{observed} - \text{expected})^2}{\text{expected}}$ is calculated as the sum over experiments and classes, and the level of significance (P) is obtained by comparison with the proper $\chi^2(\phi)$ distribution. "Observed" is in this case the number of cultures in each class in each separate experiment, while Expected is the average number of cultures in each class, calculated from the total number of experiments to be compared. Data were grouped in such a way that the expected number of cultures in each group is as little above 5 as possible.

RESULTS

Mutation to nalidixic acid resistance as a test mutation. Several considerations made us decide on mutation to high concentrations of nalidixic acid (Nal^r) as a test system. This phenotype is the result of well-defined point mutations, usually in the *gyrA* gene (30), though point mutations in the *gyrB* gene giving the Nal^r phenotype have also been reported (29). Nal^r mutants are easy to select, and the colonies formed on selective plates are usually uniform and about the same size as that of the wild type on nonselective medium. Phenotypic delay of Nal^r mutations was a priori assumed to be short, as treatment with nalidixic acid has been shown to increase the rate of synthesis of the DNA gyrase two- to threefold (20). However, in the present setup, the cells spend some hours in the stationary phase before they are plated on selective medium (see Materials and Methods), and the question at hand is therefore whether a Nal^r mutation occurring in the last generation of growth can be expressed during the last part of growth and the subsequent stationary phase. To test this, a Nal^r Hfr strain was mixed with a streptomycin-resistant Nal^s recipient, both in the stationary phase, and samples were plated with intervals on plates containing both streptomycin

and nalidixic acid. Transconjugants could be detected within an hour (the map distance between the origin of transfer and *gyrA* was approximately 15 min), indicating that Nal^r alleles can be expressed during stationary phase. It should, however, be stressed that we cannot exclude the possibility that some Nal^r mutants may have a reduced recovery on selective medium as the result of insufficient phenotypic expression time.

The generation times of four randomly chosen Nal^r isolates were determined in a standard growth experiment using the same medium as that used in the fluctuation tests; the mean value was 72 min (standard deviation, 5.0 min). A similar experiment with four wild-type isolates gave the mean generation time of 73 min (standard deviation, 5.9 min), indicating that, for the present purpose, the generation times of mutants and the wild type can be considered identical. Preliminary experiments showed that Nal^r mutations could be sib selected, demonstrating that the majority of mutations occurred prior to plating on selective medium (for details of sib selection, see reference 7).

Roles of the time of carbon starvation of the preculture and the inoculum size on mutant distribution. As mentioned, a convenient hypothesis to account for the observation made by Armitage (2), that the mutant distributions reported by Newcombe (21) were conditionally dependent on the inoculum size, could be that the progeny of a few of the cells in the preculture had an abnormal probability of mutating. It would not be so surprising if epigenetic mutator cells could arise in the preculture, for example, by translational errors (5, 22). Furthermore, quasi-genetic mutator cells could arise, for example, as the result of DNA amplifications and duplications which seem to appear quite frequently in bacteria (1). For these reasons we decided to investigate the roles of carbon starvation of the preculture and the inoculum size on the distribution of Nal^r mutants.

Table 1 shows two series of fluctuation tests, each consisting of three media inoculated at 24-h intervals with bacteria from the same preculture kept at 37°C, the inoculum being a 10^5-fold dilution of the preculture (approximately 1.2×10^4 cells). In experiments 14 to 16, media were inoculated with bacteria from the same preculture at 24-h intervals. The preculture was maintained at 37°C. Experiments 17 to 19 were performed in parallel but with another preculture. To test whether the age of the preculture affected the mutant distributions, we performed contingency tests on the two sets of data. This revealed that the homogeneity was relatively high both among experiments 14 to 16 ($P = 0.4$) and among experiments 17 to 19 ($P = 0.6$), and, although the data are not impressive, it seems safe to conclude that the age of the preculture has little effect on the mutant distribution.

Table 2 shows the results of three independent series of fluctuation tests, with each series consisting of four media inoculated with bacteria from the same preculture but with different inoculum sizes (one preculture was used for experiment 6 to 9, a second preculture was used for experiments 10 to 13, and a third preculture was used for experiments 20 to 23). If we focus on the two extreme dilutions, we find an acceptable degree of homogeneity of both the fluctuation tests with 10^4-fold dilutions (i.e., experiments 6, 10, and 20 [$P = 0.3$]) and those with the 10^7-fold dilutions (i.e., experiments 9, 13, and 23 [$P = 0.6$]). This allows us to compare the pooled data of these series, i.e., to perform a contingency test on the pooled data of experiments 6, 10, and 20 against the pooled data of experiments 9, 13, and 23. Doing this, we find that these series are very alike ($P = 0.8$), and consequently the data do not support the hypothesis that events in the stationary phase of the preculture have any significant influence on the mutant distribution obtained in the present fluctuation tests.

TABLE 4. Cultures with mutants in 23 fluctuation tests

No. of mutants	No. of cultures with mutants in expt no.:											
	1	2	3	4	5	6	7	8	9	10	11	12
0	25	16	31	26	21	22	34	22	16	23	19	22
1	5	9	5	7	11	7	5	5	10	6	13	10
2	1	4	4	3	5	3	1	9	6	6	2	5
3	1	2	1	1	2	1	2		6	4	3	4
4	4	3	1	2	2	2		1	2	3	2	3
5		3	2	1	1	2	1	2	1	1	1	
6	1		1	2	2	1		3			1	
7		1	1		1			1	1	3		
8	1	1	1						1			
10						1						
11						1	1		2		1	2
12		1	1		1	1		3				
13				1							1	
14	1					1						
15	1											
16		1			1		1	1			1	
17	1											
18		1		1					1			1
19		1		1								
24					1	1						
26				1								
27	1					1						
29	1					1						
30		1				1	1					
31							1					
36				1								
39	2											
40											1	
42		1										
49							1					
52									1			
66	1											
68				1								
73						1			1			
74	1											
107												1
132		1										
151										1		
152		1										
258											1	
265	1											
320											1	
482						1						
>512[a]		1						1		1	1	

[a] Plates with >512 mutants could not be counted with precision.

Comparison of the distribution of nalidixic acid-resistant mutants with the Lea-Coulson and the Haldane distributions. The pooled data of all fluctuation tests performed at 37°C, a total of 1,104 cultures, are shown in Table 3 (the raw data are shown in Table 4). Besides the data from Tables 1 and 2 we have included data from five analogous fluctuation tests performed at 37°C from an unpublished work on the effect of temperature on mutation. Cultures containing more than two mutants have been grouped. The rationale behind this is, besides reducing the number of data, that the recovery of mutants is not quite 100%. Reconstructing selection experiments have shown that the recovery of mutants in the present setup is approximately 95% (data not shown), and therefore some of the cultures harboring (say) eight mutants will be registered as harboring fewer. By grouping the data for cul-

TABLE 4—*Continued*

No. of mutants	No. of cultures with mutants in expt no.:										
	13	14	15	16	17	18	19	20	21	22	23
0	25	29	24	28	28	22	23	22	21	23	21
1	4	7	8	6	6	6	5	6	8	13	7
2	8	2	1	1	3	6	3	6	5	2	6
3	3	2	4	2	1	3	3	2	5	1	4
4	2		1	1		1	5	2	1	2	2
5		1	2	2			2		1		2
6	2		2	1	3			1	1	2	
7			2						1	2	
8		2	1				2		1		1
9		1				1	1	2			
10			1			2	1	1	1		1
11					1		1				1
12			1								1
13				1	1	1					
14			1					1	1		
16	1			1					1		
18	1									1	
19				1	1	1					1
20	1					1					
21		2			1		1				1
23						1					
25								1			
26		1		1			1	1			
27	1										
28									1		
29								1			
32										1	
34					1						
35										1	
37		1									
41				1							
57								1			
59						1					
69						1					
105				1							
116				1							
137								1			
140					1						
146					1						
192							1				

tures with high counts, the error caused by incomplete mutant recovery is diminished.

The average number of mutations per culture (m) was estimated both from the fraction of cultures without any mutants (m_0) and by maximum-likelihood estimates ($\hat{m}$) (see Materials and Methods) by using either the Lea-Coulson distribution or the Haldane distribution. The expected distributions were calculated on the basis of these estimates, and goodness of fit between the observed and the expected distributions was tested by a chi-square test. The distributions calculated from the maximum-likelihood estimates gave better fits than those based on m_0. The best fit was found to the Lea-Coulson distribution, but even in this case the goodness of fit was unsatisfactory ($P < 0.005$).

DISCUSSION

The major conclusion to draw from the present work is that the distribution of Nal^r mutants does not follow any of the two theoretical distributions. Deviations common to both the Lea-Coulson and the Haldane distributions are (when calculated from the present estimates of m) too many cultures with more than 128 mutants, too many cultures with 17 to 32 mutants, and, to a lesser extent, too few cultures with only 1 mutant. With respect to the Haldane distribution, another important deviation was that too few cultures with two and four mutants were observed (of the 99 cultures with three or four mutants shown in Table 3, 57 contained three and 42 contained four; the Haldane distribution predicts 43 and 66, respectively). There are many possible explanations for the lack of fit between theories and observations, and we shall here briefly discuss some of them. For convenience these may be grouped as (i) the possibility that some of the mutations were caused by mutagens, (ii) the question of mutant recovery, and (iii) the question of whether the assumptions behind the theories are fulfilled.

Question of mutagens. A situation we had to consider was whether some of the test tubes were contaminated with mutagenic substances. We tried experimentally to test for presence of a mutagen by mixing equal numbers of otherwise isogeneic *lac*$^+$ and *lac* strains and perform fluctuation tests on this mixture. Five experiments, each consisting of 48 cultures, were employed, and we found no significant correlation between Nal^r mutants of the *lac*$^+$ and the *lac* types in the individual cultures (data not shown). We therefore rule out the possibility that the presence of cultures with many mutants was the result of mutagen contamination.

Another possibility to be considered is whether nalidixic acid, an antibiotic that interferes with DNA replication, can act mutagenically, i.e., whether some of the mutant colonies were the result of mutational events that occurred after plating. An inspection of the data in Table 3 does not support this hypothesis. Postadaptive mutants would be expected to follow a Poisson distribution, and as the majority of cultures were without any mutants, this would result in an overrepresentation of cultures with exactly one mutant. This is not the case, and taken together with the fact that Nal^r mutants could be sib selected, it seems safe to rule out the possibility of postadaptive mutagenesis.

Question of mutant recovery. A source of error that has to be taken seriously is whether all mutants in the cultures form colonies upon plating on selective medium. Some of the mutants may die before plating, and some of the mutants may be lost during the plating procedure. From reconstructing selection experiments we have estimated that only a small fraction (approximately 5%) of the mutants are lost during the plating, and as the grouping of cultures with more than two mutants should reduce the effect of this, it seems unlikely to be a major cause of the lack of fit.

Another aspect of mutant recovery is phenotypic delay. The conjugation experiment mentioned previously indicated that Nal^r mutants in the present context have little phenotypic delay, but it must be stressed that this is only a qualitative observation which certainly does not rule out the possibility that some fraction of the mutations occurring in a fluctuation test fail to produce colonies owing to insufficient expression. Phenotypic delay will basically affect the detection of mutants that originated from mutations occurring in the last generation of growth, thereby causing too high an estimate of the proportion of cultures without mutants and too low an estimate of the proportion of cultures with exactly one mutant. This is to some extend in agreement with the data presented in Table 3, and it therefore seems likely that some of the lack of fit between theory and observation can be accounted for by assumption of phenotypic delay for Nal^r mutants. However, as we also observed an overrepresentation of cultures with 17 to 32 mutants, it seems unlikely that this is the sole explanation.

Assumptions behind the theories. In applying mathematical models to biological principles, it should be kept in mind that

the models are usually based on simplifying assumptions. In the present context, the assumptions may be divided into assumptions of growth and assumptions of mutation. Regarding growth, the assumption behind the Lea-Coulson model is that all cells have identical probabilities of dividing in the interval *dt*, whereas Haldane assumed synchronous growth. Common for both is, thus, the assumption that growth can be described as if all individual cells have identical division times. We know that this is not the case. The generation time in a real bacterial population is, in fact, a reflection of a rather broad distribution of individual division times (13, 23, 24). Although it is difficult to predict the effects of this, it is evident that, if mutations occur predominantly in, for example, fast-growing cells, this will affect the goodness of fit between the observed and the theoretical mutant distributions.

Both models considered are based on the assumption that all individual cells have the same probability of mutating during a cell cycle and that this probability remains constant throughout the different growth phases. As we at present have no knowledge of the distribution of individual mutation rates in bacterial populations, it is impossible to make any prediction of how this may affect the fit between the theoretical and the experimental mutant distributions. We can, however, make some qualitative predictions of the effects of a change in mutation rate during growth. If, for example, the mutation rate increases in the last generation, or if mutants can accumulate in the stationary phase prior to the plating, we have a situation that closely resembles postadaptive mutagenesis. We would in the present setup expect too few cultures without mutants and too many with exactly one, a situation which does not agree with our observations. If, on the other hand, the mutation rate decreases in the last generation, we will have the opposite situation, i.e., a situation analogous to phenotypic delay; this is in accordance with our observations.

Conclusion. When the many factors that can affect the fit between the theoretical model and the experimental observation are considered, it may not seem surprising that we did not observe close resemblance between the prediction of the models and the distribution of Nal^r mutants. It seems that a part, but probably not all, of this discrepancy can be explained by the assumption of a phenotypic delay or a reduced mutation rate in the last generations of growth, and incorporation of these concepts in the models could increase the fit. However, before doing so, it is our opinion that we will need an experimental foundation of these assumptions. The introduction of more free parameters in the models may be misleading, as the resulting improvement of the fit may be purely coincidental, i.e., owing to a counterbalancing of variables whose existence the theories do not take into account.

In the present study, as well as in former studies (2, 25), an unsatisfactory fit between the theoretical models and the experimental data was obtained. It should be apparent that, in such cases, calculation of the mutation rate by assumption of a theoretical model (e.g., estimates from the median or maximum-likelihood estimates) would be in error or, at least, very doubtful.

APPENDIX

On the generation of the Haldane distribution. The solution to the Haldane model used in Table 3 was obtained from a computer program made by N. B. Jensen and N. Tholstrup. This program (which is available upon request from L.B. or T.T.-N.) generated the distribution of mutants from a large matrix containing the outcomes of different combinations of mutations along with their respective probabilities. However, the maximum-likelihood estimation of the average number of mutations per culture (m) required a solution where the fraction of cultures with r mutants is expressed as a function of m. We shall here present an outline of this.

The deduction is based on the assumption of an infinitely large number of cultures, synchronous growth of both wild-type and mutant individuals, and all cell divisions having the same small probability of producing a mutant daughter cell; therefore, mutations are assumed to be distributed according to a Poisson distribution with the mean m.

Let $f(j)$ denote the fraction of cultures in which exactly j mutations occurred, $f(j)$ being the frequency distribution of the Poisson distribution. The proportion of cultures without mutants, $P(0)$, is identical to the proportion of cultures in which no mutation took place: $P(0) = f(0) = e^{-m}$. Now, consider the fraction of cultures where exactly one mutation occurred, $f(1)$. In half of these cultures the mutation has occurred in the last generation, as half of the total number of cell divisions in the cultures took place in the last generation. Therefore, the fraction of cultures with one mutant is $P(1) = f(1)/2 = (1/2)me^{-m}$. Likewise, one quarter of the cultures in which exactly one mutation occurred will contribute to the fraction of cultures with two mutants, one eighth will contribute to the fraction with four mutants, and so on.

In the fraction of cultures in which two mutations occurred the probability of both mutations having occurred in the last generation is $(1/2)^2$. Therefore, the proportion of cultures harboring two mutants is $P(2) = f(1)/4 + f(2)/4$. The fraction of cultures with exactly three mutants can be obtained by exactly three mutations in the last generation [probability, $f(3)/2^3$] or from the fraction of cultures in which two mutations occurred, provided that one occurred in the last generation [probability, $f(2)/2$] and the other in the preceding generations [probability, $f(2)/4$]. However, there are two combinations of this event [i.e., the binomial coefficient $B(2,1)$]: either the one mutation occurred in the last generation and the other in the preceding or vice versa. Thereby, the fraction of cultures harboring three mutants is given as $P(3) = 2f(2)/8 + f(3)/8$. Although the complexity increases with the number of mutants to be considered, it is not difficult to continue this deduction. The method is to write the number of mutants to be considered as a sum of 2^0, 2^1, 2^2, ... and then multiply the corresponding probabilities (i.e., $2^{-1} \times 2^{-2} \times 2^{-3} \times \ldots$) by the number of combinations for arranging the mutations. To give an example, five mutants can be obtained in four ways: $2^0 + 2^2$ [probability, $2^{-1} \times 2^{-3} \times f(2)$; combinations, $B(2,1) = 2$], $2^0 + 2^1 + 2^1$ [probability, $2^{-1} \times (2^{-2})^2 \times f(3)$; combinations, $B(3,1) = 3$], $2^0 + 2^0 + 2^0 + 2^1$ [probability, $(2^{-1})^3 \times 2^{-2} \times f(4)$; combinations, $B(4,1) = 4$], $2^0 + 2^0 + 2^0 + 2^0 + 2^0$ [probability, $(2^{-1})^5 \times f(5)$; and combinations, $B(5,0) = 1$]; thereby, we get $P(5) = 2f(2)/16 + 3f(3)/32 + 4f(4)/32 + f(5)/32$. By use of this approach, we could generate the Haldane distribution. This solution gave identical results to those obtained from Jensen and Tholstrup's solution, and both were verified by comparison with computer-generated fluctuation tests based on the assumption of synchronous growth and random mutation.

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