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Integration Efficiencies of Spontaneous Mutant Alleles of *amiA* Locus in Pneumococcal Transformation

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The distribution of integration efficiencies of independent mutations spontaneously occurring in the *amiA* locus was determined in two strains of pneumococcus. Strain Cl_s integrates genetic markers with different efficiencies during transformation, whereas strain 401, isogenic with strain Cl_s, does not discriminate between markers and integrates all markers with the same high efficiency. The discriminating strain Cl_s gives rise to spontaneous mutations in the locus *amiA*, which fall into four classes with respect to their individual integration efficiencies. Mutations with a low efficiency of integration are equal in number to mutations with a high efficiency. Mutations from the two other classes corresponding to intermediate and very high efficiencies are about five times less frequent. The same four classes were also found among *amiA* mutants spontaneously occurring in strain 401. However, the two distributions of integration efficiencies of *amiA* mutants arisen either in strain Cl_s or strain 401 are significantly different. The number of spontaneous *amiA* mutants, estimated by two methods, was found to be higher in strain 401 than in strain Cl_s. The increase of the mutation rate in strain 401 could be accounted for by the excess of mutations falling in the two less-efficient classes observed in this strain. The discriminating process which acts during transformation presumably also intervenes in the appearance of spontaneous mutations.

A striking feature emerging from genetic studies of recombination in *Diplococcus pneumoniae* is the difference in the efficiency with which single-site mutations are successfully integrated by transformation. Auxotrophic markers or those conferring resistance to antibiotics are generally divided into several classes according to the efficiency with which they are integrated into recipient cells. Four classes were found for mutations in the amylomaltase locus (7) and for mutations in the structural gene of dihydrofolate reductase (15). Mutations in the *amiA* locus, which determine resistance to aminopterin (3), or mutants requiring uracil (11) or thymidine (4) fall into only two distinct classes, a high-efficiency class and a low-efficiency class. The relative integration efficiencies of different mutations within the locus determining resistance to streptomycin (1) or erythromycin (6) vary over a wide range. Such a discrepancy in the classification of mutations on the basis of their individual integration efficiency may have several origins. The nature of the physiological function of the gene considered, the nature of the mutagens used to induce mutations, or the genomic background of the pneumococcus strains could possibly affect these classifications.

To determine the kind of structural differences that are associated with mutations manifesting different integration efficiencies, it was thought of interest to find out whether these mutations fall into well-defined classes. Mutants in locus *amiA* whose product is presumably not essential to the cells (Trombe and Sicard, to be published) were retained for this investigation. Since mutations occurring spontaneously in bacteria contain a multiplicity of different mutational lesions (2), integration efficiencies of only spontaneous *amiA* mutations were analyzed. Aminopterin-resistant mutants were isolated in two isogenic strains, one showing marker effects during transformation, strain Cl_s, and the other integrating all markers with the same high efficiency, strain 401.

Integration efficiencies of spontaneous *amiA*

mutants isolated in the two strains fall into the same four classes reported by Lacks (9) and Sirotnak and Hachtel (15). The manner in which these mutants fall in these four classes is different, however, in the discriminating Cl_3 strain and the non-discriminating 401 strain. The enhancement of the spontaneous mutation rates for *amiA* mutants in strain 401 is apparently related to the excess of mutants in the two less-efficient classes.

MATERIALS AND METHODS

Strains. Strain Cl_3, a capsule-deficient derivative of a type II strain of *D. pneumoniae* (pneumococcus), was used as the drug-sensitive recipient. Genetic markers are integrated with characteristic and distinct efficiencies during transformation of strain Cl_3. Strain 401, having lost this property by mutation, integrates all markers with the same high efficiency (Tiraby and Sicard, Genetics, in press). The new character displayed by strain 401, similar to that of the *hex*$^-$ strains of Lacks (8), was introduced by transformation into strain Cl_3. Strain Cl_3 is referred to as the discriminating strain, and strain 401 as the non-discriminating strain. Spontaneous *amiA* mutations were isolated in strains Cl_3 and 401, both carrying the streptomycin resistance marker *str-r41*.

Selection of transformants. Details of media and methods of transformation are described elsewhere (Tiraby, Claverys, and Sicard, Genetics, in press). Competent cells were added to deoxyribonucleic acid (DNA) extracted from strain Cl_3 or 401 carrying the same *str-41* marker and an independently arisen mutant marker, and were incubated for 20 min at 37 C. A portion of each transformed culture was frozen at −70 C after addition of 10% glycerol. The remaining portion was used the same day to estimate the frequency of transformation. Cells were embedded in nonselective nutrient agar and grown for 2 h, and transformants were scored by addition of a second layer containing streptomycin or aminopterin. The following day a more accurate number of Str-r and Ami A-r transformants was determined by the same procedure after appropriate dilutions of thawed cultures.

Isolation of spontaneous mutations in locus amiA. A thawed culture of strain Cl_3 *str-41* or of strain 401 *str-41* was diluted in pre-warmed neopeptone medium to a final density of about 10^8 bacteria per ml. The diluted culture was then distributed in several 1-ml samples in small tubes. When the cells reached a sufficient density after several hours of incubation at 37 C, growth was stopped by chilling the tube; a fraction of each individual culture was then plated in a small petri dish with neopeptone agar containing streptomycin (200 μg/ml) and aminopterin (10^{-5} mol/ml). Small cultures were initiated from a single, resistant colony picked in each plate. Cultures were then tested for sensitivity to an excess of isoleucine in minimal medium (14). Since *amiA* mutants are sensitive to an excess of isoleucine (14), clones resistant to aminopterin but showing the same level of resistance to isoleucine as the wild type were discarded; the number of such clones amounted to about 10% of the colonies picked.

Preparation of DNA. A thawed culture of an aminopterin-resistant mutant of either strain Cl_3 or 401 was diluted 20 times in 10 ml of growth medium and incubated for 5 to 6 h at 37 C. Cells were collected by centrifugation, and the pellet was suspended in 1 ml of 0.1 M tris(hydroxymethyl)aminomethane 0.01 M ethylenediaminetetraacetic acid, and 0.1% sodium deoxycholate. After lysis of the cells, the DNA was precipitated with 2 ml of alcohol. The nucleic acid fibers were withdrawn and dissolved in 0.2 M sodium chloride-0.02 M sodium citrate. These DNA preparations were stable for 1 month; after that period they gradually lost their transforming activity.

Measurement of spontaneous mutation rates. The rate of spontaneous Ami-r mutations in strains Cl_3 and 401 was determined by two different methods. The first method, devised by Luria and Delbruck (10), depends upon fluctuations in the number of mutants in different cultures grown to the same cell density. The average number of Ami-r mutants able to form colonies in selective medium (r) was calculated from 25 independent cultures (C) prepared for the isolation of spontaneous mutations in the *amiA* locus, as described above. The final number of colony-forming units (Nt) was determined by plating cells from five cultures in a nonselective medium. Cells from strain Cl_3 *str-41* and strain 401 *str-41* grew with the same average number of cocci per chain of 2.3, as judged by visual counting in a Petroff-Hauser chamber under a microscope.

The mutation rate (a) was estimated by interpolation from the equation: $r = a\,Nt \ln(C\,Nt\,a)$.

The second method, devised by Newcombe (12), is based on the determination of the number of new mutants appearing during one growth interval of a bacterial population. The mutation rate is given by: $a = [M_2 - M_1)/(N_2 - N_1)] \ln_2$. M_2 and M_1 are, respectively, the number of mutants at time t_2 and t_1; N_2 and N_1 are the number of cells at time t_2 and t_1, respectively.

A frozen culture of Cl_3 *str-41* or 401 *str-41* was diluted 1,000-fold in neopeptone medium and incubated for 30 min. Then 0.1 ml of the diluted culture was spread onto the surface of a petri dish containing 10 ml of nutrient agar and 0.5 ml of horse blood. Approximately 30 plates prepared in the same manner within about 10 min were incubated at 37 C. Aminopterin-resistant mutants were scored at 30-min intervals, starting at 150 min of incubation, by cautiously pouring a second layer of 10 ml of nutrient agar containing twice the selective concentration of aminopterin (2×10^{-5} M). The number of cells at the various time intervals was determined by resuspending cells in 5 ml of medium, scraping the surface of plates, and then plating in a nonselective medium after appropriate dilution.

RESULTS

Distribution of integration efficiencies of amiA mutants occurring in the discriminat-

ing strain. The integration efficiency of a given genetic marker has been defined similarly by several workers (1, 3, 6, 7, 9). It is determined as a ratio of the number of transformants for a given marker to the number of transformants for a reference marker. The reference marker (*str-41* in this report) serves to measure, essentially, the relative amount of DNA bearing the *str-41* mutation taken up by the cells that is integrated into recipient genomes.

Mutants in which the *amiA* locus is affected are characterized by their resistance to aminopterin and sensitivity to an imbalance in the isoleucine, leucine, and valine ratios. The majority of the mutants resistant to 10^{-5}M aminopterin fall into this group. The remainder are mutated in other different loci (4, 15). The distribution of the integration efficiencies of 105 independent *amiA* mutants spontaneously arisen in strain Cl_3, measured in the recipient strain Cl_3, is shown in Fig. 1. With our transformation procedure, these values are reproducible, and usually variability lies within the error of the plating technique (C 10%). On the basis of distribution frequency, individual integration efficiencies appear to be nonrandom and distribute into four classes: low (0.05 to 0.30), intermediate (0.3 to 0.7), high (0.7 to 1.3), and very high (1.3 to 1.75). However, the distinction between classes is not as sharp as reported by Lacks (7) for amylomaltase mutants, or by Sirotnak and Hachtel (15) for amethopterin-resistant mutants. Mutants falling into the high-efficiency class or the low-efficiency class are roughly equal in number, in fairly good agreement with results of Ephrussi-Taylor et al. (3), whereas mutants with an intermediate efficiency or a very high efficiency are far less numerous.

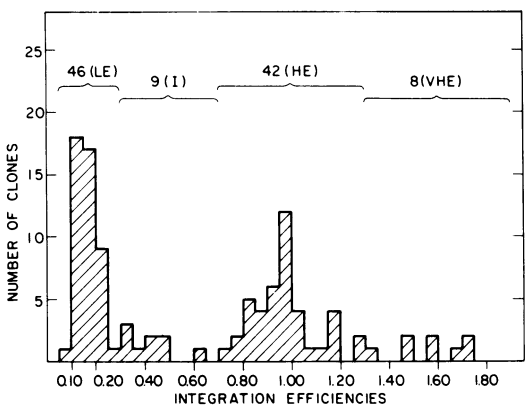

FIG. 1. *Distribution of amiA mutations occurring in Cl_3 with respect to integration efficiency. The integration efficiency of a given amiA marker is determined by the ratio of the number of transformants for this marker to the number of transformants for the reference marker conferring resistance to streptomycin (str-r41). The discriminating strain Cl_3 is used as recipient. Each bar represents the number of mutations with integration efficiencies within an interval of 0.01. Brackets indicate the classes: low efficiency (LE), intermediate efficiency (I), high efficiency (HE), and very high efficiency (VHE), and the number of distinct mutations in each.*

Distribution of integration efficiencies of amiA mutants arisen in the non-discriminating strain. When used as recipient, the non-discriminating strain 401 yields about the same number of transformants for various markers with different integration efficiencies as when measured in Cl_3. The relative integration efficiencies for 88 independent *amiA* mutants spontaneously occurring in strain 401, determined in the recipient strain Cl_3, are given in Fig. 2. Classes similar to those found with strain Cl_3 are discernible among these mutants. The striking feature emerging from these data is that the frequency of mutants among the four classes in strain 401 is significantly different from that in strain Cl_3, as shown by the result of the statistical test of the chi-square distribution (Table 1). For the same number of low-efficiency mutations in both strains, mutations with a high or very high efficiency of integration appear with a considerably lower frequency in strain Cl_3.

The distribution of the integration efficiencies of mutants isolated in the non-discriminating strain might be accounted for by a reduced frequency of initial production of the most-efficient mutations. A more plausible explana-

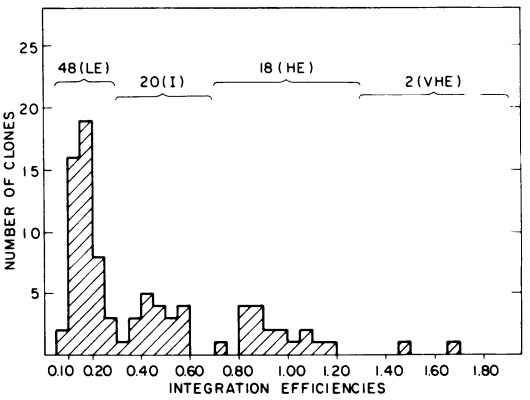

FIG. 2. *Distribution of amiA mutations occurring in 401 with respect to integration efficiency. Integration efficiencies of amiA mutations are measured in recipient strain Cl_3. Each bar represents the number of mutations with integration efficiencies within an interval of 0.01. Brackets indicate the classes and the number of distinct mutations in each.*

TABLE 1. *Calculation of χ^2 for the two distributions of integration efficiencies of amiA mutants isolated in Cl_3 and 401*[a]

Determination	O	E	O - E	(O - E)²	(O - E)²/E
Cl_3					
LE	46	51.14	-5.14	26.42	0.52
IE	9	15.78	-6.78	45.97	2.91
HE and VHE	50	38.08	11.92	142.09	3.73
401					
LE	48	42.86	5.14	26.42	0.62
IE	20	13.22	6.78	45.97	3.48
HE and VHE	20	31.92	-11.92	142.09	4.45
					(χ^2 = 15.71)

[a] Numbers reported here are taken from Fig. 1 and 2. Numbers from the two highest-efficiency classes were grouped together, the number of mutants from the VHE class in 401 being less than 5. O, observed; E, expected. With two degrees of freedom, the probability under these conditions of obtaining a χ^2 as large as or larger than 15.71 is less than 0.5%. Consequently, the two distributions are different.

tion would be that early events leading to a spontaneous mutation, such as formation of a heteroduplex region, might be identical to transient events appearing during the transformation process. Less-efficient mutations, whose structural singularity could be recognized and eliminated only in the discriminating strain, would, for that reason, be less numerous in strain Cl_3 than in strain 401. Consequently, the spontaneous mutation rate for Ami-r mutants should be higher in the non-discriminating strain. And indeed such an expectation proved to be correct, as shown by measurement of mutation rates for spontaneous aminopterin-resistant mutants in both strains.

Spontaneous mutation rates for Ami-r mutants. The number of aminopterin-resistant mutants present in each of several cultures of Cl_3 or 401 was determined in independent experiments differing from one another in respect to the time of incubation of cells. When values of the final number of cells are plotted against the corresponding number of mutants able to form colonies in solid medium containing 10^{-5}M aminopterin, a straight line joins the various points for each of the strains (Fig. 3). The two curves, although parallel, are quite distinct. Mutation rates estimated by the graphic method of Luria and Delbruck (10), from any points taken on the curves, are three times higher in strain 401 than in strain Cl_3. To be more confident about this result, we also measured mutation rates by the method of Newcombe (12). The two strains have the same pattern of growth on the surface of plates. However, strain 401 generates more Ami-r mutants able to form colonies after the aminopterin selection than does strain Cl_3 in the same interval of time (Fig. 4). Values of mutation rates estimated by the two methods are shown in Table 2. These values are consistent with an increase of about a factor three of the mutation rate to aminopterin resistance in strain 401.

DISCUSSION

On the basis of their integration efficiencies, spontaneous mutations occurring in the *amiA* locus fall into four classes. High-efficiency mutations are integrated with the same frequency as the standard reference markers, whereas low-efficiency mutations are integrated with about one-eighth that frequency. Mutations with intermediate efficiencies range between these two classes. The last class corresponds to mutations with very high efficiencies of integration. Mutants from the low-efficiency class and the high-efficiency class, originating from the discriminating strain, are equal in

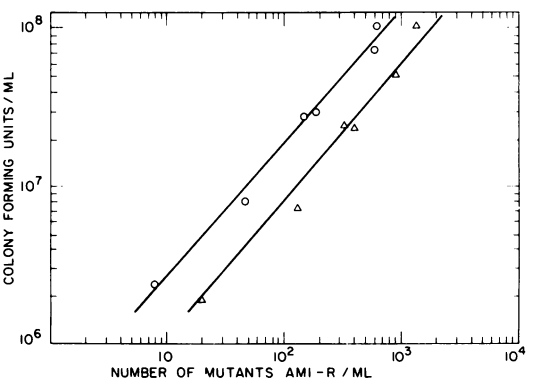

FIG. 3. *Average frequency of Ami-r mutants in cultures of Cl_3 and 401. Each point represents the average number of Ami-r mutants in 25 independent cultures grown to the same density of cells. The final number of cells in the various experiments was within an order of magnitude of about 2. Circles, Cl_3; triangles, 401.*

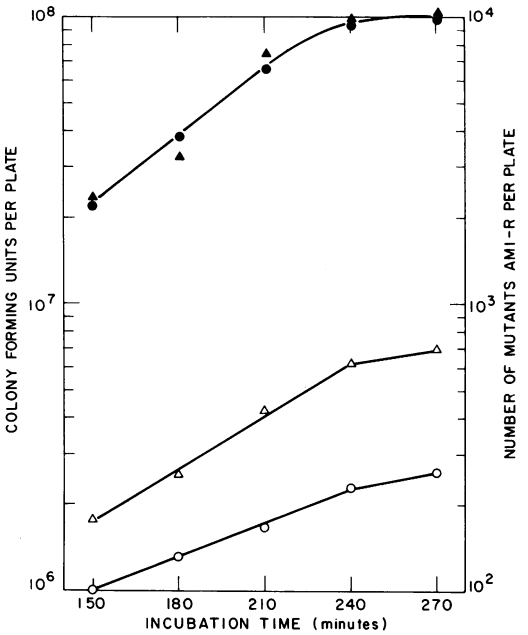

FIG. 4. *Kinetics of appearance of Ami-r mutations in strains Cl_3 and 401. The increase of new Ami-r colonies as a function of time is shown by open symbols; circle, Cl_3; triangle, 401. Filled symbols represent the corresponding increase of the number of colony-forming units.*

TABLE 2. *Spontaneous mutation rates of Ami-r mutants in strains Cl_3 and 401*

Methods	Mutation rates[a] of strains	
	Cl_3	401
Newcombe	10^{-6}	3×10^{-6}
Newcombe	1.4×10^{-6}	4.2×10^{-6}
Luria and Delbruck	10^{-6}	3.2×10^{-6}

[a] Values determined by the method of Luria and Delbruck (10) were estimated by the graphic method from any points taken on the two curves shown in Fig. 3.

number and are predominant over mutants from the other two classes. Ephrussi-Taylor et al. (3) and Gray and Ephrussi-Taylor (5) have found only high-efficiency and low-efficiency mutations with the same *amiA* mutants and the same recipient strain. The absence of the two additional classes can be explained by the fact that these authors did not examine enough mutants of spontaneous origin to be able to discern the four classes. The majority of their *amiA* mutants were induced by mutagens proven to be specific for only one class.

A distribution of mutations at the amylomaltase (*mal*$^-$) locus into four efficiency classes has been reported by Lacks (7). He expressed efficiencies in terms of the *sulf-d* marker, which itself shows an integration of 1.7 relative to *str-r41*. When efficiencies of *amiA* mutants are divided by this factor for comparison of results, the four classes appear to be roughly identical in both cases. However, classes reported here are not as distinct as those observed by Lacks (7). This difference could be related to the fact that *mal*$^-$ mutants were assumed to be single-site mutations, whereas *amiA* mutants included multisite and deletion mutations as well as single-site mutations. Indeed, it was observed that some mutants, especially from the intermediate class, segregate after transformation at least two separate mutations with different integration efficiencies (Gasc and Sicard, unpublished result). These multisite mutations whose efficiencies have been shown to vary over a wide range (7, 5) presumably tend to broaden the distribution.

Mutations conferring resistance to amethopterin were also separated into four distinct groups on the same criterion of differential efficiencies by Sirotnak and Hachtel (15). A direct comparison of their data and those obtained with *amiA* mutants is not possible since they used a different streptomycin resistance marker as reference. Nevertheless, on examination of the relative differences observed between their classes and those reported here, it appears that in both cases the classes might be similar.

The distribution of integration efficiencies of spontaneous mutations to erythromycin or streptomycin resistance was not found to be as characteristic as those of various markers described above (1, 6). In addition to the lack of discernible classes, the distribution of efficiencies of Str-r mutants, for instance, showed a variation as high as 500-fold between less-efficient and more-efficient mutations (1). Such a result could be accounted for by the unusually high frequency of multisite mutations among these mutants (13). However, the analysis of 20 independent Ery-r mutations (0.5 μg/ml) spontaneously occurring in our strain Cl_3 could provide an alternative explanation. According to their efficiencies relative to the *str-r41* reference marker, all these mutations ranged into classes defined for *amiA* mutants: high efficiency, 12; intermediate efficiency, 2; low efficiency, 6 (Tiraby, unpublished results). The discrepancy between this latter observation and results from Ravin and his associates may be due to differences in the pneumococcus strains that were used. The importance of the background of the strain used is emphasized by the

finding that two isogenic strains (Cl_s and 401) give rise to spontaneous *amiA* mutations whose distribution of efficiencies is significantly different from one strain to the other. As the result of presumably a single mutation, strain 401 is distinct from strain Cl_s in its behavior toward various markers during transformation. Thus, Ami-r mutants falling into the four efficiency classes on recipient strain Cl_s are integrated with about the same efficiency classes on recipient strain 401 (Tiraby and Sicard, Genetics, in press). The distribution of efficiencies of *amiA* mutations arising in 401 shows qualitatively the same four classes as found for Cl_s. However, frequencies of mutants falling into the two more-efficient and the two less-efficient classes are significantly different in the two strains. The number of mutants from the two lower classes is, in 401, much higher than that of mutants from the other two classes, otherwise equal in value in Cl_s. This observation appears to be related to the fact that the non-discriminating strain 401 exhibits a higher mutation rate for Ami-r mutants and optochin-resistant mutants (Tiraby, Ph.D. thesis, University of Toulouse, 1971) than the discriminating strain Cl_s. A plausible hypothesis is that the mechanism which eliminates certain classes of transformants in the discriminating strain also eliminates a fraction of spontaneous mutations. As a consequence of mutation, this mechanism acting during transformation and mutation would be inactivated in strain 401.

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