Marker Discrimination in Transformation and Mutation of Pneumococcus
(bacterial transformation/spontaneous mutation/mismatched base pairs)

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Communicated by Rollin D. Hotchkiss, July 30, 1973

ABSTRACT Earlier investigations of pneumococcal transformation revealed a function (hex*) responsible for severely reducing the transformation yield of certain markers. A mutational alteration (hex-) responsible for the loss of this function has been transferred into a hex+ strain to permit a comparison of the hex+ and hex- phenotypes in an isogenic background.

The loss of the hex* function results both in a loss of the capacity to eliminate the low efficiency markers in transformation and a substantial increase in the spontaneous mutation rate. These properties of the hex+ strain could result from the loss of a capacity to eliminate certain classes of mismatched base pairs that occur as intermediates in both transformation and mutagenesis.

Genetic transformation of pneumococcus proceeds by the insertion of a single-strand fragment of donor DNA into the DNA of the recipient bacterium. The product, a heteroduplex structure, is also heterozygous when a mutational difference distinguishes the donor and recipient strains occurs within the heteroduplex region (1-4). For several markers the yield of transformants begins to approach the number of donor genomes in the DNA taken up by the cells (5, 6). These markers are referred to as high efficiency (HE) markers.

For other markers the yield of viable transformants, when compared to the yield of HE transformants, is substantially reduced. A striking class of such markers, that include point mutations, show a 10- to 20-fold reduction in relative yield of transformants and are referred to as low efficiency (LE) markers (7, 8).

Pneumococcal strains which do not discriminate between high and low efficiency markers have been identified among existing stock cultures as well as after exhaustive mutagenesis (10, 11; Fox, M. S., unpublished results). In order to examine the phenotype of these mutants, the mutation responsible for the loss of capacity to discriminate [referred to as hex- by Lacks (10)] was introduced, by transformation, into our standard discriminating strain (hex*). The integration efficiencies of both HE and LE markers are the same in two of these hex- strains and are the same as the efficiency observed for HE markers in our otherwise isogenic hex+ strain.

The hex+ function appears to be responsible for the selective loss of transformants harboring certain base-pair mismatches. Since spontaneously occurring point mutations would be expected to exist transiently as heterozygotes, harboring base-pair mismatches, it was thought that the presence of the hex+ function might result in the loss of some of these mutants by the same mechanism that is responsible for the loss of the LE transformants.

The spontaneous mutation rates at several loci are indeed substantially lower in the discriminating strain than in the nondiscriminating strain. Examination, using the discriminating hex+ recipient, of the integration efficiencies of markers arising as spontaneous mutants in the hex+ strain and the hex- strain substantiate the proposal that the reduced mutation rate observed in the hex+ strain is due to a reduction in the observed frequency of mutations that fall into the low efficiency class.

MATERIALS AND METHODS

Bacterial Strains. Diplococcus pneumoniae strain R36A subline 6 (R6) served as recipient. DNA from the nondiscriminating strain Rx (12) and Hex-1 (10) was used to introduce, by transformation, the hex- mutations into the R6 strain. Strain 119, the source of donor DNA, was constructed by successive transformation of R6, and carried markers conferring resistance to: sulphanilamide, sulf-f (13); streptomycin, str-4l (14); fusidic acid, fus-A; novobiocin, nov-rl (15); optochin, opt-r2 (16); rifampin, rif-r23; streptolydigin, stg-rF', as well as the thymidine-requiring mutation, thy'-A2 (17).

Isolation of 3H-Labeled DNA. Strain 119 was grown to a density of 5 X 10^8 colony-forming units per ml in 10 ml of M medium (18) containing 250 μCi of [methyl-3H]thymidine. Cells were collected, washed twice at 4°C, and resuspended in 1 ml of 0.15 M NaCl-0.01 M EDTA (pH 8.0). After lysis of the cells by addition of 0.02% of sodium dodecyl sulfate and 0.01% of sodium deoxycholate, 200 μg of pancreatic ribonuclease ( Worthington) and 200 units of ribonuclease T1 ( Worthington) were added. After 1 hr of incubation at 37°C, 400 μg of Pronase (Calbiochem), previously heated at 60°C for 10 min, was added, and the lysate was incubated for 6 hr at 45°C. After alcohol precipitation, the DNA fibers were washed and dissolved in 20 mM NaCl-2 mM Na citrate.

Measurement of Integration Efficiency of Markers. [3H]-DNA from strain 119 was added to freshly prepared competent cells at 10^6 colony-forming units per ml (3) and samples, removed at various times, were mixed with an equal volume of warmed B medium (4) containing 50 μg/ml of deoxyribonuclease I ( Worthington) and 1 mM magnesium acetate. After 5 min of incubation at 37°C, a 4-ml portion was collected on membrane filters (Millipore), washed three times with 0.15 M NaCl-0.015 M Na citrate and three times with 5% trichloroacetic acid. Filters were then dried and radioactivity counted in Liquasol ( New England Nuclear Corp.) in a liquid scintillation spectrometer. The remaining portion was allowed to grow for 2 hr after a 1:5 dilution. The number of

Abbreviations: HE, high efficiency; LE, low efficiency.
Inactivation of \( \text{non-r}^{-1}(\text{LE}) \) and \( \text{str-r41}(\text{HE}) \) markers by exposure to ultraviolet light. Irradiation was carried out with a G.E. 15 W germicidal lamp at a distance of 33 cm. Transforming activity was tested on recipient strains \( \text{R6} \) and \( \text{R6x} \). The initial ratio of \( \text{non-r}^{-1} \) to \( \text{str-r} \) transformants was 1 with \( \text{Rx} \) and 0.1 with \( \text{R6} \). \( \bullet \), \( \text{non-r}^{-1} \) \( \text{R6} \); \( \bigcirc \), \( \text{str-r} \) \( \text{R6} \); \( \bigcirc \), \( \text{str-r} \) \( \text{Rx} \); \( \bigtriangleup \), \( \text{non-r}^{-1} \) \( \text{Rx} \).

Measurement of Spontaneous Mutation Rates. Mutation rates of strains \( \text{R6 str-r41 non-r}^{-1} \) and \( \text{R6x str-r41 non-r}^{-1} \) were measured. A growing culture was diluted in 100 ml of warm broth to a final density of about \( 10^9 \) cells per ml, distributed in 100 small tubes, and incubated at 37°. When the cells reached the appropriate density, the contents of each tube was poured into a small petri dish, and mixed with 5 ml of 1.5% agar-M medium containing one of a number of chosen selective agents. After 24 hr of incubation, the fraction of plates without any resistant colonies was determined. The average number of bacteria was estimated by microscope counts from six different tubes in a Petroff–Hauser chamber. The \( P(o) \) method of Luria and Delbrück (19) was used to calculate the mutation rates.

In order to determine the appropriate density of cells suitable for the \( P(o) \) method for a given mutation, the mutation rate was first estimated from 25 independent cultures grown to a high density. From the average number of resistant colonies per culture, and the number of bacteria plated, the mutation rate was estimated graphically (19).

Distribution of Integration Efficiency of Spontaneous Markers. Cultures were inoculated from single colonies picked from the plates prepared for the estimation of spontaneous mutation rates. In order to exclude mutations of common origin, only one colony from any plate, usually one of the largest, was selected. After several hours' growth, the cells in 10 ml of culture were collected by centrifugation and the pellet was resuspended in 1 ml of 0.1 M Tris·HCl-0.01 M EDTA-0.1% sodium desoxycholate. After 10 min at 37° to allow lysis of cells, the DNA was precipitated with 2 ml of alcohol. The fibers were removed and dissolved in 20 mM NaCl-2 mM Na citrate. The transforming activities of these DNA preparations were determined. The common marker \( \text{str-r41} \) was used as a basis for determination of the efficiency of the other antibiotic resistance marker present.

Antibiotics. Streptomycin sulfate USP was purchased from Eli Lilly and Co., Indianapolis. Aminopterin was purchased from Nutritional Biochemical Corp., Cleveland. Streptolydigin was a gift of the Upjohn Co., Mich. Fusidic acid was a gift of Leo Pharmaceutical Products, Ballerup, Denmark. Rifampin was a gift of the Ciba Pharmaceutical Co., N.J.

RESULTS

Isolation of \( \text{hex}^{-} \) Strains Isogenic with the \( \text{hex}^{+} \) Strain \( \text{R6} \). Although our standard discriminating strain (\( \text{hex}^{+} \)) \( \text{R6} \) and the nondonorserting strain (\( \text{hex}^{-} \)) \( \text{Rx} \) (12) originate from the same parent \( \text{R36A} \), they have had different histories. In order to make a direct comparison of the two phenotypes, we

![Graph 1](image1)

**Fig. 1.** Inactivation of \( \text{non-r}^{-1}(\text{LE}) \) and \( \text{str-r41}(\text{HE}) \) markers by exposure to ultraviolet light. Irradiation was carried out with a G.E. 15 W germicidal lamp at a distance of 33 cm. Transforming activity was tested on recipient strains \( \text{R6} \) and \( \text{R6x} \). The initial ratio of \( \text{non-r}^{-1} \) to \( \text{str-r} \) transformants was 1 with \( \text{Rx} \) and 0.1 with \( \text{R6} \). 

![Graph 2](image2)

**Fig. 2.** Transformation (transformants/ml) as a function of quantity of DNA taken up. Filled symbols, \( \text{R6} \); open symbols, \( \text{R6x} \). Triangles, \( \text{str-r41} \); squares, \( \text{str-r41} \); circles, \( \text{str-r4} \).
introduced, by transformation, the mutation responsible for the lack of discrimination from strain Rx into R6.

Exposure of transforming DNA to ultraviolet light results in a loss of the biological activity of the DNA. When tested on the discriminating strain R6, the transforming activity of LE markers is lost much more rapidly than that of HE markers (7, 11; Fox, M. S., unpublished result). When tested on the nondiscriminating strain, Rx, transforming activities of LE and HE markers decline at the same rate as the activity of HE markers on the hex+ strain R6 (Fig. 1) (9, 10). This distinguishing feature between hex* and hex− strains permitted us to recognize the transfer of the hex− property into strain R6.

Competent R6 bacteria were exposed to DNA isolated from strain Rx and allowed to grow for several generations. These bacteria (R6 transformed), as well as untreated R6 and Rx cultures, were made competent and exposed to UV-irradiated DNA bearing the markers str-r41 (HE) and nov-r1 (LE). Transformants were plated on selective medium after allowing 2 hr of incubation at 37°C. When tested on Rx, both markers yield equal numbers of transformants whereas with the untreated R6 culture, str-r41 transformants were 104 times more frequent than were nov-r1 transformants. The R6 bacteria that had been transformed with R6 DNA showed only a 30-fold excess of str-r41 transformants. 20 Colonies of nov-r1 transformants were picked, and tested by transformation with DNA bearing str-r41 (HE) and sty-gF (LE) markers for their ability to discriminate. Among the nov-r transformants originating from the R6 bacteria transformed with Rx DNA, nineteen no longer discriminated (hex−), while all of the nov-r1 transformants originating from the control R6 culture retained their hex+ property. The frequency of bacteria displaying the hex− phenotype in the Rx culture treated with Rx DNA was about 3%, which is the usual level of transformation for a single HE marker. It seems unlikely that more than one mutational alteration is responsible for the hex− property in view of the high efficiency with which the marker is introduced by transformation. Using another nondiscriminating strain, Louarn et al. (20) have also provided evidence for loss of capacity to discriminate as the consequence of a single mutational alteration. Using the same procedure, other R6 hex− transformants were isolated after transformation of R6 by DNA from an additional nondiscriminating strain Hex-1. One clone, designated R6H, isolated by transformation of R6 with Hex-1 DNA, and one clone, designated R6x, isolated by transformation of R6 with Rx DNA, were further examined.

### Integration Efficiencies of Various Markers in hex− and hex+ Strains

The hex− strains R6x and R6H were compared with the parent R6 hex+ strain with regard to yields of transformants for a variety of markers as well as the quantitative yield of transformants for a given amount of DNA taken up by the competent bacteria.

Transformation was carried out at a bacterial density of 10^8 colony-forming units per ml with DNA isolated from strain 119 carrying the LE markers, sty-rF, rif-s3, nov-r1, opt-r2, thy-A3, two HE markers, str-r41 and fuc-A, and a very HE marker sulf-d (Table 1). The hex− strains yield transformation frequencies varying over a range of a factor of 20, while the nondiscriminating strain yields nearly the same frequency of transformation for all of the markers tested.

<table>
<thead>
<tr>
<th>Antibiotic resistance markers</th>
<th>R6</th>
<th>R6x</th>
<th>R6H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin: str-r41</td>
<td>195</td>
<td>1.0</td>
<td>230</td>
</tr>
<tr>
<td>Sulfanilamide: sulf-d</td>
<td>350</td>
<td>1.8</td>
<td>290</td>
</tr>
<tr>
<td>Fusidic acid: fuc-A</td>
<td>275</td>
<td>1.4</td>
<td>270</td>
</tr>
<tr>
<td>Novobiocin: nov-r1</td>
<td>23.0</td>
<td>0.12</td>
<td>315</td>
</tr>
<tr>
<td>Optochin: opt-r2</td>
<td>37.0</td>
<td>0.19</td>
<td>280</td>
</tr>
<tr>
<td>Streptolydigin: sty-gF</td>
<td>27.0</td>
<td>0.14</td>
<td>285</td>
</tr>
<tr>
<td>Rifampin: rif-r23</td>
<td>41.0</td>
<td>0.21</td>
<td>250</td>
</tr>
<tr>
<td>Aminopterin: thy-A3</td>
<td>17.5</td>
<td>0.09</td>
<td>205</td>
</tr>
</tbody>
</table>

(1) 10^4 × the number of transformants per ml.
(2) Integration efficiency ratio of the transformant yield for a given marker to the yield for the marker str-r41.

Using 4H-labeled DNA from strain 119, we determined the yield of transformants as a function of the quantity of DNA taken up for the hex+ and hex− strains (Fig. 2).

The yields of transformants increase linearly with the amounts of DNA taken up. For a given amount of donor DNA taken up, the yield of sulf-d (very high efficiency) transformants is about the same in both strains. The HE marker (str-r41) is introduced with about 1/3 of the efficiency of the sulf-d marker in the hex− strain and at about 1/2 the efficiency in the hex+ strain. As expected, the LE marker sty-gF is introduced with less than one-tenth the efficiency of the sulf-d marker in the hex+ strain and with equal efficiency in the hex− strain. The hex− alteration results in a marked increase in the efficiency with which LE markers are introduced, as well as a modest increase in the efficiencies of some other markers. Thus, the moderate increase in efficiency of the str-r41 marker is largely responsible for the apparent "lowering" of the sulf-d efficiency observed in the hex− strain (Table 1). What is presumably a single mutational difference between the two strains results in the loss of capacity to discriminate against LE markers.

### Spontaneous Mutation Rates in hex− and hex+ Strains

When the donor and recipient strains of pneumococcus differ by a point mutation, the product of transformation is a heteroduplex structure containing a base-pair mismatch. It seems likely that the hex+ function is responsible for the elimination of most of the LE transformants as a consequence of the particular base-pair mismatch that they harbor. If newly occurring mutations involved the transient existence of such base-pair mismatches, they would also be subject to elimination in the hex+ strain but would survive in the hex− strain. On the basis of this expectation, mutation rates in the two strains were determined for a number of loci (Table 2).

The hex− strain R6x exhibits substantially higher mutation rates than does the hex+ strain. The strain R6H also displays the same high mutability as R6x to streptolydigin resistance, the only kind of mutation tested.

### Distribution of Markers with Respect to Integration Efficiency

If this difference in spontaneous mutation rates is indeed due to the elimination of certain classes of LE mutants in the hex+ strain, we would expect that HE mutants should
be relatively more abundant among hex\(^+\) mutants than among hex\(^-\) mutants.

The efficiency classes of spontaneously arising hex\(^+\) mutants and hex\(^-\) mutants are displayed in Figs. 3–5. Most of the Str-resistant mutants in either strain appear to be of the LE class. Among Rif-resistant mutants, HE mutants appear to be relatively more abundant in the hex\(^+\) strain. The most striking case is seen among fusidic acid mutants (Fig. 4). In this case virtually all of the mutants appearing in the hex\(^+\) strain are HE while more than half of those appearing in the hex\(^-\) strain are LE. It is not possible to make a quantitative interpretation of these observations, but it seems clear that LE mutants are indeed more abundant among mutants appearing in the hex\(^-\) strain.

**DISCUSSION**

In pneumococcus, a mechanism seems to exist which can eliminate 90\%, or more, of the products of transformation with LE markers. The LE and HE markers that have been studied are mainly single-site mutations (7, 8, 21), and among these, a likely candidate for the basis of elimination of most of the LE transformants could be the composition of the basepair mismatch present in the DNA of these transformants. This discrimination between LE and HE markers in the hex\(^+\) strains could be accounted for by the existence of one or several nucleolytic enzymes capable of distinguishing among the different mismatched base pairs. The enzyme could be an endonuclease that acts on only one strand of the heteroduplex, excising the portion of the DNA harboring the appropriate mismatch. Subsequent repair synthesis would make the surviving LE transformants homogenous, a distinguishing property of LE transformants (21, 22). In this case, we would have to further assume that the enzyme preferentially attacked

**Table 2. Comparative spontaneous mutation rates**

<table>
<thead>
<tr>
<th>Loci</th>
<th>Mutation Rates</th>
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<tbody>
<tr>
<td></td>
<td>R6(hex(^+))</td>
</tr>
<tr>
<td>Rifampin</td>
<td>(10*^-7])</td>
</tr>
<tr>
<td>(10 (\mu)g/ml)</td>
<td>(25/25)</td>
</tr>
<tr>
<td>Streptolydigin</td>
<td>0.80 (52/80)</td>
</tr>
<tr>
<td>(15 (\mu)g/ml)</td>
<td>0.30 (25/25)</td>
</tr>
<tr>
<td></td>
<td>0.35 (26/60)</td>
</tr>
<tr>
<td>Fusidic acid</td>
<td>0.23 (48/60)</td>
</tr>
<tr>
<td>(75 (\mu)g/ml)</td>
<td>0.40 (39/60)</td>
</tr>
<tr>
<td>Optochin (2 (\mu)g/ml)</td>
<td>2.9 (25/25)</td>
</tr>
<tr>
<td>Thymidine(^-) thyA(_1) (\rightarrow) thymidine(^+)</td>
<td>0.38 (53/60)</td>
</tr>
<tr>
<td>Thymidine(^-) (\rightarrow) thymidine(^-) (2–10 (\mu)g/ml)</td>
<td>0.73 (41/60)</td>
</tr>
<tr>
<td>Thymidine(^-) (\rightarrow) thymidine(^-) (5 (\mu)g/ml)</td>
<td>0.68 (45/60)</td>
</tr>
</tbody>
</table>

Numbers in parentheses correspond to the number of tubes without mutants over the total number of tubes. Where 25 tubes were examined, bacteria were grown to high density and the mutation rates were estimated graphically (19). In all other cases the mutation rates were determined by the P(o) method (19).

* R6H.

† Mutants requiring 2–10 \(\mu\)g/ml of thymidine for growth were selected by plating Thy\(^-\) bacteria (50 \(\mu\)g/ml) in nutrient medium containing 10 \(\mu\)g/ml of thymidine and 4 \(\mu\)g/ml of aminopterin.
the donor DNA strand (22) or that an attack on the recipient strand was frequently lethal. The enzyme could also be a nuclease that frequently cuts both strands of the DNA heteroduplex harboring the appropriate mismatch, thus killing the transformants. Whatever mechanism might eventually provide a complete description of the behavior of LE markers, the action of an enzyme that recognizes and eliminates certain mismatched bases seems essential.

Strain R6 is has, as the result of a single mutation, lost the property of discriminating between HE and LE markers. It appears to be deficient in such an enzyme and integrates LE markers with high efficiency.

The strains R6 and R6x are indistinguishable with regard to their growth rates and their sensitivities to ultraviolet light and alkylating agents such as nitrosoguanidine and methyl methane sulfonate (data not shown). The time required to resume growth after exposure to the above agents is also indistinguishable in the two strains.

The mechanism of discrimination that acts during the transformation process apparently also intervenes in the survival of spontaneously occurring mutants. Tiraby (11) has observed that the spontaneous mutation rate to aminopterin resistance in the ami A locus was higher in a mutant strain similar to R6x than in the parent, discriminating strain. We have shown that the nondiscriminating strain R6x exhibits a higher spontaneous mutation rate than the discriminating strain for a number of loci. Depending on the locus examined, differences between 5- and 30-fold have been observed. Loci could exhibit characteristic sensitivity to the presence of the discriminating function that depends on their polynucleotide sequences as well as on the kinds of amino-acid substitutions that would permit the manifestation of the mutant property selected for. The distribution of the integration efficiencies of independently occurring spontaneous mutations in various loci substantiates the expectation that mutations of the lower efficiency classes occur less frequently in the discriminating strain than in the nondiscriminating strain. Although difficult to quantitate, the differences in mutation rates between the two strains could be accounted for by the elimination of a fraction of the mutations that fall into the LE class. It should be noted that LE mutations are by no means absent in the discriminating strain.

The function responsible for discrimination is pleiotropic in that it is responsible for a reduction of the spontaneous mutation rate as well as the elimination of certain classes of transformants. It seems likely that this reduction is due to the elimination of a large fraction of the products of spontaneous mutation. The nondiscriminating strain could, therefore, be described as a mutator strain in which the increased mutation rate was due to its failure to eliminate those products.

This work was supported by Grant no. AI-05388-10 from the U.S. Public Health Service. G.T. was partially supported by fellowships from the Pierre Philippe Foundation and the Foundation for Research in Medicine and Biology.