Studies on Transformations of Hemophilus influenzae

III. The genotypes and phenotypic patterns of three streptomycin-resistant mutants

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ABSTRACT A general method of determining the nature of the genotypes in mutants of transformable bacteria with similar phenotypes is discussed. The method is used to identify the genotypic patterns of three mutants of Hemophilus influenzae which are resistant to different levels of streptomycin. A mutant resistant to 700 μg per ml of the antibiotic was found to be made up of two unlinked, independent loci—presumably on different molecules of transforming DNA. These loci, when in separate cells, render them resistant to maximum levels of 10 and 100 μg per ml streptomycin respectively and are therefore designated as Sm10 and Sm100. When they enter the same cell they produce a resistance up to 700 μg per ml streptomycin, so the cells are noted as Sm700. This multiplicative action is more easily visualized as due to two independent processes of combating the antibiotic which enhance each other rather than two identical processes.

Bacterial transformation systems are well suited for analysis of genetic components and processes because decisive experiments can be performed easily. The number of loci involved in a genotype can be analyzed by using the DNA from this cell line to transform unmarked cells and screening the transformants at many levels of expression. Replica plating or clonal isolation permits a study of the progeny of single transformants at any level of expression. Varying at will the number of transforming DNA molecules entering recipient cells allows a quantitative correlation between this number and the number of transformants showing a particular level of expression. Such a correlation is important in distinguishing between linked and unlinked genetic units (1, 2).
At least three different genotype patterns are now recognized which are responsible for different mutants with a given phenotype. These are: (a) a single non-disseminating locus (3–5), (b) a multi-locus unit in which the loci are linked, but disseminate in some of the progeny by recombination (6), and (c) multi-locus unit of independent genetic loci (3, 8, 9). A single locus DNA produces transformants having a uniform phenotypic pattern like the cell line donating the transforming DNA, and reexposure of these transformed cells to the same donor DNA will not alter the pattern of expression. Multi-locus units produce transformants exhibiting a mixed pattern of expression. If the pattern contains two, instead of three or more different levels of expression, it will suggest that the two individual loci produce equivalent levels of expression. The transformants from a multi-locus DNA showing low levels of expression probably contain single loci, and if so, they will be converted to higher levels of expression by reexposure to the donor cell DNA. This latter step is a second transformation which introduces a different locus. The loci of a multi-locus cell may be linked together on the same DNA molecule or unlinked and on separate DNA molecules. Linked loci which are responsible for the high level of expression in a multi-locus unit are distinguished from unlinked markers by the way in which the numbers vary with transforming DNA concentration. The number of cells having the highest level of expression will vary directly with the DNA concentration if the loci are linked, and as the square (or some power) of the DNA concentration if the loci are unlinked.

Many transformable properties have been described (10), but not all of them lend themselves to extensive quantitative analysis. Many single loci are known and a few linked loci have been studied—particularly the sulfonamide resistances in pneumococcus analyzed in detail by Hotchkiss and Evans (6, 7). Sirotnak, Lunt, and Hutchison (9) reported a number of one and two locus mutants of pneumococcus resistant to amethopterin, but they did not distinguish between linked and unlinked units.

The present paper describes an analysis of two unlinked genetic units responsible for streptomycin resistance in three mutants of Hemophilus influenzae. These mutants are all different from the single locus high level mutant described much earlier by Alexander and Leidy (4).

MATERIALS AND METHODS

In general, the experimental methods were those described previously (1). The (SM700) cells were derived by two stepwise selections for increased resistance commencing with Rd Hemophilus influenzae sensitive to streptomycin concentrations above 3 μg per ml. In the symbol Sm700, Sm = streptomycin, 700 = the maximum

1 Linked loci of qualitatively different phenotypes are not considered here.
concentration in micrograms per milliliter of Sm to which the culture is resistant. The Sm$^{700}$ DNA was prepared from a culture of cells with this resistance pattern grown from an isolate in Elev broth (1) containing 700 µg per ml of Merck's Sm base.

Transformations were carried out as follows: an aliquot of Rd Sm sensitive H. influenzae (2 X 10$^9$ per ml) made competent by an aerobic-non-aerobic procedure (1) was diluted tenfold into 0.14 M NaCl containing the transforming DNA. After shaking 30 minutes at 37°C, an equal volume of Elev broth was added and the agitation continued for 135 minutes, after which an aliquot was “pour plated” with Elev agar containing the indicated concentration of Sm.

EXPERIMENTAL RESULTS

The Resistance Pattern of Cells Transformed with Sm$^{700}$ DNA

Transforming Rd Sm sensitive H. influenzae cells with DNA obtained from an isolate of cells resistant to 700 µg per ml of Sm and screening the transformed culture with increasing concentrations of Sm led to the resistance profile shown in Fig. 1. It is apparent that cells with three different levels of resistance were obtained from this one homogeneous donor strain. Whereas virtually no receptor cells were resistant to Sm concentrations above 4 to 5 µg per ml (points not shown), about 0.4 per cent of the cells receiving the Sm$^{700}$ DNA became resistant to 4 to 10 µg per ml of the antibiotic and hereafter are designated Sm$^{10}$; 0.04 per cent became resistant to 20 to 100 µg per ml, and are designated Sm$^{100}$; and 3 per million, or 0.0003 per cent, were resistant to 200 to 700 µg of Sm. The last are designated Sm$^{700}$. It is apparent from Fig 1 that the resistance pattern of transformants within each group was quite uniform and easily distinguishable from the other groups.

Reciprocal Transformations of Sm$^{10}$ and Sm$^{700}$ Cells

The results in Table I show that DNA from Sm$^{10}$ cells will not change the resistance of competent Sm$^{10}$ cells, but will raise the resistance of Sm$^{100}$ cells to a level of Sm resistance of 700 µg per ml. Likewise, the DNA from the Sm$^{100}$ cells will not raise the resistance of competent Sm$^{10}$ cells, but will raise Sm$^{10}$ cells to a level of Sm resistance of 700 µg per ml.

Resistance Patterns of Sm$^{10}$ and Sm$^{700}$ DNAs

To determine whether the Sm$^{10}$ or Sm$^{700}$ loci would fractionate or show any signs of interrelationship, their resistance patterns were examined separately and in combination. The results shown in Fig. 2 leave little doubt about the uniformity and independence of the two individual genetic loci. Neither pro-
Figure 1. The resistance pattern of transformants obtained with DNA from Sm\textsuperscript{200} cells. Effect of streptomycin concentration on the number of resistant cells per 8 \times 10^8 recipient cells.

| TABLE I |
| TRANSFORMATIONS OF Sm\textsuperscript{19} AND Sm\textsuperscript{100} CELLS BY 0.1 \mu g per ml Sm\textsuperscript{19} AND Sm\textsuperscript{100} DNAs LEADING TO Sm\textsuperscript{200} CELLS |

<table>
<thead>
<tr>
<th>Recipient cell</th>
<th>Resistance of cells donating DNA</th>
<th>Transformants resistant to 700 \mu g Sm per ml per 8 \times 10^8 recipient cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sm\textsuperscript{19}</td>
<td>Sm\textsuperscript{19}</td>
<td>0 \times 10^4</td>
</tr>
<tr>
<td></td>
<td>Sm\textsuperscript{100}</td>
<td>1.2 \times 10^4</td>
</tr>
<tr>
<td></td>
<td>No DNA</td>
<td>0 \times 10^4</td>
</tr>
<tr>
<td>Sm\textsuperscript{100}</td>
<td>Sm\textsuperscript{19}</td>
<td>1.4 \times 10^4</td>
</tr>
<tr>
<td></td>
<td>Sm\textsuperscript{100}</td>
<td>7.8 \times 10^3</td>
</tr>
<tr>
<td></td>
<td>No DNA</td>
<td>7.4 \times 10^3</td>
</tr>
</tbody>
</table>

* The 700 \mu g per ml Sm refers to the "level" of resistance. 300 \mu g per ml was used for screening this level. (See Fig. 1.)
produced progeny having a resistance different from that of the parent cell, i.e. DNA from Sm$^{19}$ cells produced transformants resistant to concentrations of Sm up to 10 μg per ml but not beyond and DNA from Sm$^{105}$ cells led to resistance up to 100 μg per ml, but not beyond. In combination, Sm$^{19} +$ Sm$^{105}$ DNA produced transformants, some of which were resistant only to 10 μg per ml and some to 100 μg per ml. There was still a third group of cells resistant to 700 μg per ml of Sm. As may be seen by comparing Figs. 1 and 2, the pattern of resistances developed by transforming with a mixture of Sm$^{19} +$ Sm$^{105}$ was essentially the same as the pattern shown in Fig. 1 using the DNA from Sm$^{105}$ cells.

In Fig. 2 the fewer number of cells becoming resistant to 10 μg per ml Sm after transformation with Sm$^{105}$ DNA compared to the number when Sm$^{10}$ DNA was used is due, perhaps, to the difference in locus size and hence, the probability of incorporation into the host. In support of this, the Sm$^{105}$ marker is more sensitive to ultraviolet radiation than is Sm$^{19}$ DNA.

![Figure 2. The resistance pattern of transformants obtained with DNAs from Sm$^{19}$, Sm$^{105}$ cells, and a mixture of DNAs from these two cells.](image-url)
The Effect of DNA Concentration from Sm$^{700}$ Cells on the Transformations to Sm$^{700}$

As noted earlier in this paper, the distinguishing characteristic between linked and unlinked loci in transforming DNA is not whether they produce transformants having the higher resistance of a double locus unit, but whether the number of these higher resistance transformants varies linearly with the

\[ \text{No. of transformations to DNA Concentration Sm}/\text{ml} \]  

\[ \begin{array}{cccc}
\text{DNA Concentration} & \text{Sm}^{10}/\text{ml} & \text{Sm}^{100}/\text{ml} & \text{Expected doubles*} \\
0.05 & 4.8 \times 10^6 & 5.3 \times 10^5 & 3.2 \times 10^3 \\
0.02 & 4.9 \times 10^5 & 6.0 \times 10^4 & 3.7 \times 10^3 \\
0.01 & 3.5 \times 10^4 & 4.3 \times 10^3 & 1.9 \times 10^3 \\
0.005 & 2.0 \times 10^3 & 2.9 \times 10^2 & 7.3 \times 10^2 \\
0.002 & 0.9 \times 10^2 & 1.1 \times 10^1 & 1.2 \times 10^1 \\
0.001 & 3.8 \times 10^1 & 5.4 \times 10^0 & 2.6 \times 10^0 \\
0.0005 & 2.4 \times 10^0 & 3.0 \times 10^{-1} & 0.9 \times 10^{-1} \\
0.0002 & 1.2 \times 10^{-1} & 1.2 \times 10^{-2} & 1.8 \times 10^{-2} \\
\end{array} \]

*The expected number of double transformants (Tde) was calculated from frequencies of the individual transformants as follows:

\[ \frac{Tde}{C} = \frac{T_{10}}{C} \times \frac{T_{100}}{C} \text{ or } Tde = \frac{T_{10} \times T_{100}}{C} \]

Where $T_{10}$ and $T_{100}$ are the number of transformants at the level of 10 $\mu$g per ml and 100 $\mu$g per ml Sm resistance and $C$ is the number of recipient cells. $C$ was obtained by direct analysis after the 135 minute incubation necessary to permit the expression to the 700 $\mu$g per ml level of Sm resistance and was 8 $\times$ 10$^8$ per ml. This number includes cells formed during the 135 minutes of incubation, but since the individual or low level transformants were also sampled at this time, the effect of long incubation is not so serious.

DNA concentration or as the square (or some power function). If, in Sm$^{700}$ cells, the Sm$^{10}$ and Sm$^{100}$ loci are linked, the number of Sm$^{700}$ cells formed, regardless of what fraction of the total, should vary directly with the DNA. If these loci are independent and are taken up randomly, the number of Sm$^{700}$ cells should vary with the square of the DNA concentration, or, in more applicable terms, the frequency of Sm$^{700}$ transformations will be the product of the frequencies of the Sm$^{10}$ and Sm$^{100}$ transformations.

The results of experiments in which various concentrations of DNA from Sm$^{700}$ cells were used to transform sensitive Rd cells and then scored for the number of cells resistant to three levels of Sm are shown in Table II. In-
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eluded also is the number of double transformations expected from the frequencies of the individual transformations. The good agreement between the observed number of Sm\textsuperscript{7\textastisk} cells and the calculated number of double transformations leads to the conclusion that the resistance to 700 \(\mu\)g Sm per ml is due to two unlinked factors.

**Effect of Sm\textsuperscript{10} plus Sm\textsuperscript{100} DNA Concentration on Sm\textsuperscript{7\textastisk} Transformants**

The effect of varying the concentration of a mixture of Sm\textsuperscript{10} and Sm\textsuperscript{100} DNAs on the number of Sm\textsuperscript{7\textastisk} transformants is shown in Table III along with the number expected from the individual transformants. As was anticipated, the agreement between observed and expected is good. The similarity of results in Tables II and III suggests that the Sm\textsuperscript{7\textastisk} DNA is a mixture of Sm\textsuperscript{10} and Sm\textsuperscript{100} DNAs.

**Discussion**

The resistance pattern of transformed cells when Sm\textsuperscript{7\textastisk} DNA was used (Fig. 1) suggests that Sm\textsuperscript{7\textastisk} cells contain at least two, and perhaps three loci, Sm\textsuperscript{10}, Sm\textsuperscript{100}, and Sm\textsuperscript{7\textastisk}. The Sm\textsuperscript{10} and Sm\textsuperscript{100} cells were isolated from these plates and their DNAs failed to raise the resistance of homologous strains to a 700 \(\mu\)g level, but each raised the complementary strain to this level (Table I). Thus it is not necessary to assume that there is a Sm\textsuperscript{7\textastisk} locus, though it is not yet excluded. The resistance pattern produced by transformation with Sm\textsuperscript{10} or Sm\textsuperscript{100} DNAs (Fig. 2) failed to show any evidence of subfractionation of these loci, so that it is tentatively considered that each is a single locus.
The agreement in Table II between the observed number of transformants resistant to 700 μg Sm per ml and those calculated as random double transformants of Sm\(^{100}\) and Sm\(^{100}\) at the different concentrations of DNA from Sm\(^{700}\) cells shows clearly that these two loci behave as though they were independent. It also eliminates the evidence of a Sm\(^{700}\) locus. Thus, the genotype of the Sm\(^{700}\) cell fits the description, noted earlier, of two independent loci.

It now becomes necessary to distinguish in *Hemophilus influenzae* the various genetic loci for streptomycin resistance. The first locus isolated and identified by Alexander and Leidy (4) is designated as Sm\(^{2000}\) α, and the two units reported in this paper are Sm\(^{10}\) β and Sm\(^{100}\) γ. The cell line resistant to 700 μg per ml of Sm contains the two unlinked units β and γ and might be indicated as Sm\(^{700}\) β·γ.

The multiplicative effect of Sm\(^{10}\) and Sm\(^{100}\) loci on each other may indicate that the resistance to the antibiotic is accomplished differently by the two loci. If they produced the resistance by identical methods, *i.e.* producing the same enzyme, their effects might be expected to be more nearly additive.

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