A STUDY OF STREPTOMYCIN RESISTANCE IN MICROCOCCUS PYOGENES VAR. AUREUS1

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There are two theories as to the origin of microbial resistance to toxic agents; namely, (a) resistance is the result of some interaction between microorganism and toxic agent, resulting in an increased ability of the cell to withstand the deleterious effects of the drug, and (b) the toxic agent merely serves to select from the total population pre-existing mutant cells that are resistant. For example, Seligmann and Wassermann (1947), in support of the first theory, reported the induced resistance of several bacteria to the action of streptomycin. Demerec (1949), on the other hand, recently reported spontaneous mutation and subsequent selection of resistant strains of Staphylococcus aureus to streptomycin, aureomycin, and penicillin.

The experiments reported in this paper offer further support to the spontaneous mutation and biological selection theory.

MATERIALS

Most of the streptomycin used in these studies was the commercial preparation (streptomycin sulfate) of the Abbott Laboratories. In some instances, Merck's calcium chloride complex streptomycin was used. This material was dissolved in sterile, distilled water to give a stock solution of 100,000 units per ml. Other concentrations were made by further dilutions of this stock. These were always maintained at refrigerator temperature until used.

The organism studied was Micrococcus pyogenes var. aureus, the standard assay strain 209P. Stock cultures were maintained on nutrient agar slants by transfers every other month. Storage was at refrigerator temperature.

Because plain nutrient broth (0.3 per cent beef extract and 0.5 per cent peptone) does not contain in excess any of the various factors known to inactivate streptomycin (Donovick et al., 1948), it was the medium used throughout the experiments reported. The reaction before sterilization was pH 6.8 to 7.0.

EXPERIMENTAL RESULTS

Derivation of resistant cells from progeny of a single cell. A single-celled strain was derived from Micrococcus pyogenes var. aureus for use as the streptomycin-

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sensitive parent culture. All resistant mutants were subsequently derived from this streptomycin-sensitive culture.

To determine whether a few naturally occurring, resistant cells could be detected in the progeny derived from a single cell, a method similar to that devised by Klein and Kimmelman (1946) was used. One hundred tubes each containing 9.6 ml of nutrient broth, 0.3 ml inoculum, and 0.1 ml of antibiotic stock to give streptomycin at 200 units per ml were prepared. The 200-units-per-ml level was 100 times the minimum inhibiting concentration established for the parent susceptible culture. The inoculum was taken from a shake flask culture containing 50 ml of nutrient broth. Immediately prior to the test, the number of cells per ml of the inoculum was determined by plate counting. Visible growth in any of the tubes after 24 hours' incubation at 37 C was considered as resulting from one or more naturally occurring resistant mutants in the 0.3-ml inoculum for that tube. For comparisons of resistant mutants obtained in another way, several were developed by serial transfers in progressive concentrations of the antibiotic. The inoculum was always one standard loopful of a 24-hour culture. This was taken from the tube that had shown growth in the greatest concentration of antibiotic in the previous series. The first tube in the next series contained that same concentration of the antibiotic to serve as a verification. The levels of antibiotic tested in each series differed usually by a 10-fold factor (0.03, 0.1, 1, 10, etc., units per ml).

One tube out of 100 containing similar incula of the single-celled culture at 200 units per ml of streptomycin showed heavy growth after 24 hours of incubation. This mutant, although selected by a 200-units-per-ml level, was later found to be resistant to at least 1,000 units per ml of streptomycin. Therefore, although the homogeneity assumed for the descendants of a single cell is often cited in support of adaptation (Himshelwood, 1944), it is evident that the clone studied here was not homogeneous with respect to streptomycin susceptibility. This heterogeneity is best explained as the result of mutation.

The data also indicate the mutation to streptomycin resistance to be independent of the action of the antibiotic. Although a 2-units-per-ml level of streptomycin completely inhibited visible growth of the parent organism, a 100-fold increase (200 units per ml) was used in this selection procedure. This margin, of course, eliminated the possibility of growth of the parent population occurring after the addition of streptomycin; therefore, the mutant cell must have originated some time during growth prior to testing for resistance. On the basis of plate counts, it was calculated that each 0.3-ml inoculum contained approximately 67,200,000 cells, and thus in 100 tubes some 6,729,000,000 cells had been added as inocula. If it is assumed that growth resulted from the least possible number, i.e., one mutant cell, the incidence of mutant cells to normal was 1 to 6.72 x 10^9 organisms.

It would be extremely difficult to explain these data as a result of adaptation due to a chemical interaction between antibiotic and microorganism. Each of the 100 tubes examined contained comparable numbers of microorganisms and the same level of streptomycin; therefore, on an adaptation basis each culture
would have the same opportunity to become resistant. The more logical explanation is that random mutation accounts for the one resistant cell in the huge bacterial population and that it originated before contact with streptomycin.

Table 1 shows the development of resistance by a culture serially transferred in progressive levels of streptomycin. The culture was resistant to 1 but not to 10 units in the third transfer of this series; with the sixth, to 10 but not to 100; with the seventh, to 100 but not to 1,000; and with the eleventh, to at least 1,000 units per ml.

It is apparent from the data presented in table 1 that if a concentration of streptomycin lower than 200 units per ml had been used in the single-step selection method, a greater number of resistant mutants might have been selected. The appearance of mutants initially resistant to levels lower than 200 units per ml was unavoidably eliminated by the use of this level of antibiotic.

Further evidence for a mutational origin of resistance is the fact that the resistant cells have the characteristics commonly assigned to mutants. They appeared suddenly in a low frequency in a huge population and have remained stable after nearly 2 years of transfers in the absence of the antibiotic.

**TABLE 1**

*Resistance development of Micrococcus pyogenes var. aureus by serial transfer in increasing concentrations of streptomycin*

<table>
<thead>
<tr>
<th>INITIAL STREPTOMYCIN RESISTIVITY</th>
<th>GROWTH IN STREPTOMYCIN CONCENTRATION AT VARIOUS TRANfers</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 u/ml</td>
<td>2  3  4  5  6  7  8  9  10  11</td>
</tr>
<tr>
<td></td>
<td>1  1  1  1  10  100  100  100  100  1,000</td>
</tr>
</tbody>
</table>

Attempts to develop resistance excluding biological selection. Most workers at the present time assert random mutation and biological selection to be the unique method for the origin of resistance. This assertion was challenged by an attempt to develop resistant cultures under conditions excluding selection. If that could be done, it might be indicative of adaptation.

A level of streptomycin permitting uninhibited growth as shown by the development of the usual growth curve was determined and utilized in an experiment to exclude the possibility of selection of resistant mutants. Such a curve would still allow a possible interaction between cell and antibiotic that might lead to resistance. A series of tubes containing the medium plus streptomycin at 0.01, 0.033, 0.07, 0.1, and 0.3 units per ml was inoculated with one standard loopful of a 24-hour culture of the parent. Incubation was at 37 C for 24 hours. The level permitting the development of a normal growth curve as compared with the control was determined by turbidity readings made with the Evelyn photocolorimeter.

An attempt was next made to increase the resistance of the organism by repeated transfers in the presence of that level of streptomycin which allowed usual growth, i.e., 0.01 unit per ml. The inoculum was a single standard loopful of a 24-hour culture; incubation was at 37 C for 24 hours. After the incubation
period, the organism was again inoculated into medium containing the same concentration of antibiotic. A parallel series of inoculations from each tube was made into medium containing 1, 1.5, and 3 units per ml of streptomycin to assay for increased resistance. These concentrations represented a 3-, 5-, and 10-fold increase over the minimum inhibiting concentration (0.3 units per ml as determined by the method previously given).

Biological selection can function only if the cell to be selected differs in some characteristic that gives it an advantage over the dominant organism. One may assume that experimental conditions permitting the development of the usual growth curve would negate chances for a biological selection, although such conditions would not prevent mutation per se, for this ability lies inherent in the cell. However, if selection were prevented, such mutants would remain an ex-

![Figure 1. The effect of various sublethal concentrations of streptomycin on the growth of Micrococcus pyogenes var. aureus.](http://jb.asm.org/)

tremely small segment of the population. As shown in figure 1, it was found that 0.01 unit per ml of streptomycin was without effect on the usual growth curve of the sensitive organism, but greater concentrations of the antibiotic produced longer lag phases and depressions of total growth. Although selection was ruled out by the use of this low level of antibiotic, the possibility that streptomycin was capable of producing specific cellular changes in the direction of greater resistance was still allowed. However, after 11 serial transfers in the medium plus 0.01 unit per ml of streptomycin, no increased resistance could be detected. Assays for such increases were made with small increments of streptomycin since it appeared likely that resistance (if at all quantitative in response) would be of a low order.

**Biological selection of resistant mutants in sublethal concentrations of streptomycin.** The logical extension of the foregoing reasoning is, first, that any level of
the antibiotic capable of altering the usual growth curve of the sensitive organism would aid in the selection of mutants, which in the altered conditions have a growth advantage over the usual type of cells, and, second, that a different group of mutants will be selected according to the level of streptomycin set as determining resistance. Such a biological selection would necessarily favor those cells having some advantage in growth in this level of streptomycin over the common cell type (usual type) in the original population. A culture with these characteristics would be either streptomycin-resistant or dependent.

A series of experiments was therefore undertaken to demonstrate that resistance could be developed by serial transfers in such a sublethal concentration of streptomycin. Four Evelyn tubes, each containing 9.9 ml of medium plus 0.1 ml of antibiotic stock to give a final concentration of 0.1 unit per ml, were inoculated with one standard loopful of the organism. At various times during the incubation period Evelyn readings were taken to determine the length of the lag phase and subsequent growth levels. When each culture was 24 hours old,

<table>
<thead>
<tr>
<th>TABLE 2</th>
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<tbody>
<tr>
<td><strong>Increased resistance of Micrococcus pyogenes var. aureus due to transfer in a sublethal concentration of streptomycin</strong></td>
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</table>

<table>
<thead>
<tr>
<th>CULTURE</th>
<th>INCREASED RESISTANCE AFTER TRANSFERS IN PRESENCE OF 0.1 U/ML STREPTOMYCIN</th>
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<tbody>
<tr>
<td></td>
<td>Resistance (u/ml) at each transfer</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>2</td>
<td>&lt;1</td>
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<tr>
<td>3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>4</td>
<td>&lt;1</td>
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* The figure 1 represents an increase of 3-fold over that of the minimum inhibiting concentration (0.3 units per ml); 1.5 represents a 5-fold increase.

another similar series containing the same level of streptomycin was inoculated from the preceding one. At the same time, any increase in resistance was determined by inoculation into a side series of tubes containing, respectively, 1, 1.5, 5, and 10 units per ml of the antibiotic. After each increase in resistance was detected, it was confirmed by subsequent testing of a loopful of inoculum introduced into the increased level of antibiotic.

Increased resistance was readily demonstrated after a serial transfer procedure in this low level of the antibiotic. Such increases were of low magnitude ranging from 3-fold in 3 cultures to a 5-fold increase in 1 culture (table 2). A control series carried through the same procedure in the absence of streptomycin failed to demonstrate any increases in resistance. Mutations may have occurred, but, if so, the lack of the selective agent (streptomycin) in the control explains the failure of resistant cells to emerge in the population.

In a series of experiments to be reported later (English and McCoy), it was found that the streptomycin-resistant mutants were capable of much better growth than the streptomycin-sensitive parent organism in a semisynthetic me-
dium (Frieden and Frazier, 1947). This capacity for increased growth in the re-
sistant mutant strains was found to be associated with biotin synthesis. It was
assumed that if an intimate relationship did exist between the capacity for ex-
cellent growth in semisynthetic medium and streptomycin resistance, the rela-
tionship might be demonstrated to be reciprocal, i.e., excellent growth of the
sensitive parent would be indicative of an increased resistance to streptomycin.
Such a mutual relationship was shown by the selection of one culture of the
parent (out of 121 attempts) that produced much heavier growth in plain, semi-
synthetic medium in the absence of streptomycin than the usual sensitive cul-
ture. Evelyn comparison made between this culture and the other resistant var-
iants showed them to be very similar in growth. This culture was then tested
for resistance to streptomycin and was found to be resistant to at least 1,000
units per ml of the antibiotic. This finding may be cited as additional evidence
for the mutational origin of resistance.

SUMMARY

Evidence is presented in favor of the random mutation and biological selec-
tion theory for the origin of microorganisms resistant to streptomycin within a
population of susceptible cells. The evidence includes the following:

(a) Mutants resistant to at least 1,000 units per ml of streptomycin were ob-
tained by a single-step selection procedure from a single-celled strain of Micro-
coccus pyogenes var. aureus.

(b) When sensitive cells were grown in the presence of streptomycin under
conditions excluding selection, yet allowing the possibility of adaptation, resist-
ance failed to develop.

(c) In contrast, under conditions in which selection was allowed to exert its
influence, resistance was readily obtained.

(d) A mutant was selected by a serial transfer procedure in semisynthetic me-
dium in the absence of streptomycin; it was later found to be resistant to at
least 1,000 units per ml of streptomycin.

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