Mutation to High-level Streptomycin-resistance in R⁺ Bacteria

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SUMMARY

Bacteria carrying an R factor conferring resistance to low concentrations of streptomycin frequently give highly resistant variants. Resistance to high concentrations of streptomycin can arise in a sensitive bacterium either from a single mutation or as the result of successive mutations in different genes (Demerec, 1948; Hsu & Herriott, 1961). The resistance genes may be either in the chromosome or in an R factor.

METHODS

R₁ (Meynell & Datta, 1966) confers resistance to sulphonamide, streptomycin, chloramphenicol, kanamycin and ampicillin. The bacteria in which it was tested were *Escherichia coli*, K₁₂ strains 15-3 *pro⁻ met⁻* and 16-2 *pro⁻ his⁻ try⁻* (Clowes & Rowley, 1954), and the defined media and methods used for R factor transfer were as described in Pearce & Meynell (1968).

Isolation and detection of R⁻ segregants. Unlike the F factor (Hirota, 1960), R₁ is not eliminated by growth in acridine broth, and since it carries an ampicillin-resistance gene, which determines the production of penicillinase (Datta & Kontomichalou, 1965), R⁻ bacteria cannot be isolated by the penicillin-screening method in the ordinary way (Watanabe & Fukasawa, 1961). Nevertheless, the screening technique could be successfully applied when cephalosporin, a penicillin which is relatively insensitive to penicillinase, was substituted for ampicillin. An overnight broth culture of R₁⁺ bacteria was diluted in fresh broth to a concentration of 10⁴ bacteria/ml and incubated for 2 hr until the bacteria were growing exponentially; chloramphenicol was then added to 25 µg./ml and the culture re-incubated for 1 hr. At this time, cephalosporin (Cephaloridine, Glaxo) was added to 20 µg./ml and the culture further incubated for 4 hr, after which the number of viable bacteria was found to be 0·3–0·05% of that present when the cephalosporin was added. Dilutions were spread on nutrient agar plates which were incubated overnight; next day R⁻ clones were detected as penicillinase-negative colonies by the starch–iodine method (Foley & Perret, 1962, modified by Hennessey; T. Hennessey, personal communication). For this method, the plates were overlaid with 3 ml of 0·6% water agar containing 0·3 ml of a 2% solution of starch; and when the layer had set, 3 ml of a mixture of 3·2 M-potassium iodide and

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0.08 M-iodine solutions containing 100 mg./ml. benzylpenicillin was poured on the surface. In 2–3 min., all the R+ colonies were surrounded by a white halo, and it was possible very quickly to subculture R- clones before all the bacteria were killed. When strain J5-3 (R I) and three streptomycin-resistant mutants, H53, H54 and H55 were treated in this way, penicillinase-negative colonies amounted to 22, 13, 23 and 85%, respectively.

**RESULTS AND DISCUSSION**

R I conferred only low-level resistance to streptomycin and growth was inhibited on nutrient agar by concentrations exceeding streptomycin 20 μg./ml. It was therefore surprising to find in crosses with R I+ donor strains (Pearce & Meynell, 1968) that on defined medium containing streptomycin 250 μg./ml., a 0.1 ml. inoculum of a broth culture of *Escherichia coli* K12 strain J5-3 (R I) gave about 500 medium-sized colonies as well as many more smaller ones. The presence of the R factor was responsible for the appearance of these colonies, since a similar inoculum of either strain J5-3 before infection or an R- segregant of strain J5-3 (R I) gave no growth. The colonies produced by strain J5-3 (R I) contained bacteria with various degrees of increased streptomycin-resistance. Thus the limited streptomycin-resistance conferred by R I was evidently enough to allow the inoculated bacteria to multiply in the presence of streptomycin at 250 μg./ml. to a population density where resistant mutants appeared.

The degree of streptomycin resistance of the variants was determined by streaking one loopful of a fully grown broth culture across a series of plates of defined medium containing graded concentrations of streptomycin increasing by steps of \(\sqrt{2}\) from 1 μg./ml. to 16,384 μg./ml., the degree of resistance being recorded as the highest concentration of streptomycin which permitted confluent growth (Reeve, 1966). This method had the advantage of giving a sharp end-point, although the estimated degree of resistance was higher than that obtained by assessing the numbers and appearance of individual colonies. As others have noted (Tzagoloff & Umbreit, 1963; Gundersen, 1965) on defined medium the bacteria grew in the presence of higher concentrations of streptomycin than on nutrient agar.

Mutation to a low degree of streptomycin resistance occurs comparatively often (Demerec, 1948) and, if the mutated chromosomal gene acted synergistically with the resistance determinant of the R factor, a highly resistant organism would result. Several variants of greater resistance were isolated as individual colonies on plates where growth was no longer confluent. Table 1 shows that R- segregants of H53 grew only on streptomycin 22.4 μg./ml. and, when the R factor was transferred to the sensitive strain J6-2, all of 5 individual R+ recipient colonies showed only the usual degree of resistance, i.e. to 128 μg./ml. Thus the variant, H53, derived from strain J5-3 (R I) and resistant to streptomycin 2048 μg./ml. was the product of a sensitive bacterium mutating to resistance to only 22.4 μg./ml. while carrying an R factor conferring no more than its original resistance to 128 μg./ml. Again, when the R factor was introduced into H56, a mutant of J5-3 resistant to streptomycin 128 μg./ml., the result was a culture resistant to as much as 11,500 μg./ml. Thus, the resistance genes on the chromosome and the R factor clearly co-operated to produce a bacterium of disproportionately high overall resistance. To see whether the conditions for mutation to high-level streptomycin-resistance could be reproduced equally well when the determinant for low-level resistance was in the chromosome instead of in the R factor,
Streptomycin resistance in \( R^+ \) bacteria

A mutant of J5-3 whose resistance level was as nearly as possible the same as that conferred by \( R^+ \) was isolated on streptomycin agar. This mutant, H56, gave confluent growth, like J5-3 (\( R^+ \)), on concentrations of streptomycin up to 128 \( \mu \text{g.}/\text{ml} \) and when 0.1 ml volumes of overnight broth cultures of the two strains were plated in parallel on defined medium containing streptomycin 250 \( \mu \text{g.}/\text{ml} \), both gave about 70 full-sized colonies of bacteria later shown to be resistant to streptomycin 16,000 \( \mu \text{g.}/\text{ml} \). The similar behaviour of the mutant and the \( R^+ \) strain in increasing the rate at which highly resistant variants arose strongly suggested that the function of the \( R \) factor was simply to confer an initial degree of resistance. In each case, a further mutation of the kind that occurs relatively frequently and confers only a small degree of resistance on

Table 1. \textit{Escherichia coli K12. Mutation to streptomycin-resistance in \( R^+ \) bacteria}

<table>
<thead>
<tr>
<th>Strain or variant</th>
<th>Obtained From</th>
<th>By</th>
<th>Resistance to streptomycin (( \mu \text{g.}/\text{ml} ))</th>
<th>Further procedure</th>
<th>Resulting derivative</th>
<th>Resistance to streptomycin resistance (( \mu \text{g.}/\text{ml} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>J5-3</td>
<td></td>
<td></td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H56</td>
<td>J5-3</td>
<td>Selection on streptomycin</td>
<td>128</td>
<td>Infection with ( R^+ )</td>
<td>H56 (( R^+ ))</td>
<td>11,500</td>
</tr>
<tr>
<td>J5-3 (( R^+ ))</td>
<td>J5-3</td>
<td>Infection with ( R^+ )</td>
<td>128</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H55</td>
<td>J5-3 (( R^+ ))</td>
<td>Selection on streptomycin</td>
<td>180</td>
<td>Curing of ( R^+ )</td>
<td>H69</td>
<td>22.4</td>
</tr>
<tr>
<td>H53</td>
<td>J5-3 (( R^+ ))</td>
<td>Selection on streptomycin</td>
<td>2048</td>
<td>Curing of ( R^+ )</td>
<td>H67</td>
<td>22.4</td>
</tr>
<tr>
<td>H54</td>
<td>J5-3 (( R^+ ))</td>
<td>Selection on streptomycin</td>
<td>2867</td>
<td>Transfer of ( R^+ ) to J6-2</td>
<td>16-2 (( R^+ ))</td>
<td>5 isolates</td>
</tr>
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</table>

A sensitive bacterium then results in a highly resistant organism. There still remained some slight difference between J5-3 (\( R^+ \)) and the mutant H56, which may perhaps only have reflected a difference in the mechanism of resistance (Okamoto & Suzuki, 1965), for while the plates inoculated with the \( R^+ \) strain had by 48 hr developed a large number of tiny colonies, no such colonies appeared on the plates spread with the mutant.

\( R^+ \) confers resistance to streptomycin 128 \( \mu \text{g.}/\text{ml} \), but, at the same time, it can be seen greatly to increase the rate of appearance of highly streptomycin-resistant variants. Gundersen (1963, 1965a) and Ginoza & Painter (1964) observed what may be the same phenomenon, which was attributed to 'genetic instability' resulting from a 'mutator gene' present on an episome. The particular episome studied by Gundersen itself gave resistance to low concentrations of streptomycin (Gundersen, 1965a), and Ginoza & Painter (1964) noted that the apparently mutagenic effect of \( R \) factors was limited to drugs where the \( R \) factor already conferred some degree of resistance. The chromosomal mutation was not in the gene where high-level resistance is ordinarily acquired by a single mutational step (Gundersen, 1963); and in the bacterial variants examined by Ginoza & Painter (1964) two genes, one present in the \( R \) factor and the
other in the chromosome, and each of which singly confer resistance to streptomycin 25 μg./ml., co-operated to give an organism resistant to 1000 μg./ml. Ginoza & Painter concluded from this result that the chromosomal gene was acquired as a direct result of genetic recombination between chromosome and R factor. On the other hand, the similar rates of mutation to high-level resistance in bacteria carrying R1 or a chromosomal mutation giving the same degree of resistance implies rather that the R factor acts simply by providing an initial degree of resistance sufficient for the bacterial population to reach a concentration at which one of the comparatively frequent mutations which ordinarily gives low degrees of resistance can occur. When the bacterium already carries one resistance determinant, either an R factor or chromosome, the synergistic action of the two resistance genes produces an organism which is highly resistant.

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