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

R_1 (Meynell & Datta, 1966) confers resistance to sulphonamide, streptomycin, chloramphenicol, kanamycin and ampicillin. The bacteria in which it was tested were *Escherichia coli*, K12 strains J5-3 pro^-met^- and J6-2 $pro^-his^-try^-$ (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), R_1 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 R_1^+ bacteria was diluted in fresh broth to a concentration of 10^4 bacteria/ml. and incubated for 2 hr until the bacteria were growing exponentially; chloramphenicol was then added to 25 $\mu\text{g./ml.}$ and the culture re-incubated for 1 hr. At this time, cephalosporin (Cephaloridine, Glaxo) was added to 20 $\mu\text{g./ml.}$ 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 (R1) 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

R1 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 R1⁺ 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 (R1) 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 (R1) gave no growth. The colonies produced by strain J5-3 (R1) contained bacteria with various degrees of increased streptomycin-resistance. Thus the limited streptomycin-resistance conferred by R1 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 (Tzagaloff & Umbreit, 1963; Gundersen, 1965*b*) 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 (R1) 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,

a mutant of J5-3 whose resistance level was as nearly as possible the same as that conferred by R1 was isolated on streptomycin agar. This mutant, H56, gave confluent growth, like J5-3 (R1), on concentrations of streptomycin up to 128 $\mu\text{g./ml.}$ and when 0.1 ml. volumes of overnight broth cultures of the two strains were plated in parallel on defined medium containing streptomycin 250 $\mu\text{g./ml.}$, both gave about 70 full-sized colonies of bacteria later shown to be resistant to streptomycin 16,000 $\mu\text{g./ml.}$ 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. *Escherichia coli* K12. Mutation to streptomycin-resistance in $R1^+$ bacteria

Strain or variant	Obtained		Resistance to streptomycin ($\mu\text{g./ml.}$)	Further procedure	Resulting derivative	Resistance to streptomycin resistance ($\mu\text{g./ml.}$)
	From	By				
J5-3	.	.	4	—	—	—
H56	J5-3	Selection on streptomycin	128	Infection with R1	H56 (R1)	11,500
J5-3 (R1)	J5-3	Infection with R1	128	—	—	—
H55	J5-3 (R1)	Selection on streptomycin	180	Curing of R1	H69	22.4
H53	J5-3 (R1)	Selection on streptomycin	2048	Curing of R1	H67	22.4
				Transfer of R1 to J6-2	J6-2 (R1) (5 isolates)	128
H54	J5-3 (R1)	Selection on streptomycin	2867	Curing of R1	H68	22.4

a sensitive bacterium then results in a highly resistant organism. There still remained some slight difference between J5-3 (R1) 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.

R1 confers resistance to streptomycin 128 $\mu\text{g./ml.}$, but, at the same time, it can be seen greatly to increase the rate of appearance of highly streptomycin-resistant variants. Gundersen (1963, 1965*a*) 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, 1965*a*), 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 $\mu\text{g.}/\text{ml.}$, co-operated to give an organism resistant to 1000 $\mu\text{g.}/\text{ml.}$ 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 R I 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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