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Modification by an R determinant for streptomycin of his^{sd} phenotype in a mutant strain of *Escherichia coli* B

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1. INTRODUCTION

We reported elsewhere (Molina, Schito, Calegari & Romanzi, 1965) the results of conjugation experiments between *Escherichia coli* B streptomycin-dependent (*Sm^d*)† recipient strain and *E. coli* K12 Hfr R1 donor strains harbouring an R factor of which the four known R determinants confer low-level resistance to streptomycin (*Sm^r*) and tetracycline (*Tc^r*), and restriction of phages T1 (*T1^{rt}*) and P1 (*P1^{rt}*). It was observed that the yield of recombinants endowed with the R factor was greatly reduced as compared with control crosses made with Sm-sensitive/resistant recipient strains; when, however, transfer occurred, a shift from Sm dependence to Sm resistance was noticed. The use of a complete resistance factor, *R(SmTcT1P1)*, and of a resistance factor, *R(TcT1P1)*, lacking the R determinant for Sm demonstrated that the latter (of the four R determinants) is the one responsible for the limited appearance of the R factor among the recombinants, and is somehow involved in the transition observed from Sm dependence to Sm resistance. The apparent reduction in frequency of transfer of the R determinant for Sm may be interpreted either as due to limited acceptance by the *Sm^d* recipient strain or as the consequence of an inability to grow which the determinant itself confers upon the *Sm^d* strain.

In order to verify these hypotheses, and with the purpose of getting some information on the actual effect of the Sm determinant on streptomycin dependence, the R factor was transferred, both by conjugation and transduction, to a conditionally streptomycin-dependent (*CSD*) strain. We considered a strain of this type would be suitable for our purpose, on the basis of Gorini and Kataja's assumption (1964), that in both classes of mutants—*Sm^d* and *CSD*—an Sm-suppressible defect may be present. Unlike the classical mutant, which depends upon

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† The following abbreviations are used: Sm, streptomycin; *Sm^s*, streptomycin-sensitive; *Sm^r*, streptomycin resistance/resistant; *Sm^d*, streptomycin-dependent; *CSD*, conditionally streptomycin-dependent; *his^{sd}*, CSD-histidine auxotroph; Tc, tetracycline; *Tc^r*, tetracycline resistance/resistant; *T1^{rt}*, restriction of phage T1; *P1^{rt}*, restriction of phage P1; *R(SmTcT1P1)*, resistance factor conferring resistance to Sm and Tc and restriction of phages T1 and P1; *R(TcT1P1)*, incomplete resistance factor without the R determinant for Sm; MM, liquid minimal medium; MA, minimal agar; NA, nutrient (Brain Heart Infusion) agar; *his$^-$*, histidineless; *met$^-$*, methionineless; *thr$^-$*, threonineless; *leu$^-$*, leucineless; *NTG*, *N*-methyl-*N*-nitroso-*N'*-nitroguanidine.

Sm under all conditions of growth, *CSD* strains require in minimal medium either the specific metabolite or the drug (Gorini & Kataja, 1964).

The results here reported show that the R determinant for Sm, when present in the *CSD* strain, prevents repair by streptomycin, so that, assuming the same mechanism in both cases, its inhibitory effect on Sm^d recombinants could be predicted from the failure of the *CSD* strains carrying it to grow without its required metabolite.

Furthermore, the modification of the *CSD* mutant's observed behaviour enables some conclusions to be drawn as to the nature of resistance to Sm conferred by an episomic determinant.

2. MATERIALS AND METHODS

(i) *Media*

Difco Brain Heart Infusion was used as nutrient broth for the growth of cultures and for conjugation experiments. Difco Brain Heart Infusion agar was used as nutrient solid medium (NA). NA containing Tc at a final concentration of 25 μg/ml was employed to assess the number of donor bacteria present in mating mixtures, for genetic analysis of recombinants, and as selective medium in transduction experiments. Sm was added to NA at final concentrations of 100 μg/ml for isolating Sm^r mutants, and of 10000 μg/ml for the assay of Sm resistance of recombinants and transductants.

Lederberg's liquid minimal medium (MM) and minimal agar (MA) (Lederberg, 1950) were used, with the addition of vitamin B 1 (25 μg/ml). The following supplements were added where indicated: histidine (50 μg/ml), penicillin (50 u./ml) streptomycin (5, 50, 500, 1000, 5000, 10000 μg/ml), tetracycline (25 μg/ml).

The soft agar for propagation of phage *P1kc* was prepared by adding 7 g of Difco Bacto agar per litre of Brain Heart Infusion broth. L broth (Lennox, 1955) was the medium for the growth of cultures to be employed in transduction experiments.

(ii) *Bacterial strains*

The donor strains used were:

E. coli K 12, Hfr R1/11: obtained by transferring the resistance factor *R(SmTcT1P1)* into the Sm^s, met^- Hfr R1 strain.

E. coli K 12, Hfr R1/30: unlike the previous strain, provided with the resistance factor *R(TcT1P1)*, without the R determinant for Sm (Molina, Calegari & Schito, 1965).

Klebsiella pneumoniae, spher 13: thr^-, leu^-; strain in which the R factor was originally discovered (Molina, 1964).

E. coli K 12, W 945, 42/45: F^-, thr^-, leu^-, carrying *R(SmTcT1P1)*.

E. coli K 12, 58–161, 176/5: F^-, met^-, carrying *R(SmTcT1P1)*

The recipient strains used were:

E. coli B 25: CSD-histidine auxotroph (his^{sd}), obtained with the following procedure from strain B, wild type: (1) a spontaneous mutant with high resistance to Sm (up to 10000 μg/ml), the survivor on NA containing 100 μg/ml of Sm, was

selected; (2) a washed cell suspension from an exponentially growing culture of the *Sm*^{r} mutant was exposed for 15 min at 37 °C to the mutagenic action of NTG, at a concentration of 700 μg/ml in tris-maleic buffer (Adelberg, Mandel & Chein Ching Chen, 1965) at pH 6 (0·003 % survivors). The cells were then washed and after an intermediate overnight growth in nutrient broth a suitable inoculum, washed free of broth, was seeded into liquid minimal medium (MM) plus penicillin (50 u./ml) and incubated for 24 hr. The survivors were plated on NA, and the *CSD* mutants identified by replica plating onto minimal (MA) and also minimal plus Sm (50 μg/ml) agar. The colonies growing on MA plus Sm and on NA but not on MA, were isolated, and their specific requirements in the absence of Sm identified. The influence of Sm and histidine on the growth of this mutant in MM is set out in Table 2. On MA growth occurred with the addition of either his (50 μg/ml) or Sm at the following concentrations: 50, 1000, 5000 and 10000 μg/ml.

E. coli B 26: *thr*$^{-}$, *leu*$^{-}$, was obtained from strain B 25 by repeated exposure to NTG, followed by penicillin treatment.

E. coli B: wild-type strain was used in control crosses in which recombinants from the conjugation and transduction experiments were employed as donors.

(iii) *Phage strains*

The phage strains used in transduction experiments were:

P1kc/B9, propagated by soft-agar layer technique on *E. coli* strain B 9, which was obtained by transferring the resistance factor *R(SmTcT1P1)* into strain B wild type.

P1kc/B23, propagated on *E. coli* strain B 23, which carries the resistance factor *R(TcT1P1)*, without Sm determinant.

Phage lysates were sterilized with chloroform vapour.

(iv) *Mating experiments*

Crosses were carried out by mixing equal volumes of exponentially growing broth cultures of donor and recipient strains. After incubation for 60 min at 37 °C, the mixture was washed twice and resuspended to half of the original volume in buffer; 0·1 ml volumes of suitable dilutions were plated on selective media for the desired class of recombinants. The parental cells were controlled by plating them separately on the same selective medium. In any cross, 100 recombinants were purified by isolating them, and by restreaking one colony from each recombinant clone, on the same medium as that used for their selection. The recombinants were then checked for inheritance of the R factor, for resistance on NA to Sm (10000 μg/ml), and for growth response on MA to the specific metabolite required (his: 50 μg/ml) and to Sm (50 and 1000 μg/ml, and for some of them even 5000 and 10000 μg/ml.) Only Tc resistance and T1 restriction were used as a sign of the presence of R factor, P1 restriction being too difficult to detect by the cross-streaking technique, and cytoplasmic Sm resistance being masked by high-level chromosomal resistance.

(v) *Transduction experiments*

Exponentially growing cultures of the recipient bacteria in L broth were mixed with equal volumes of phage lysate diluted so as to obtain phage multiplicities of 2–4. $CaCl_2$ was added ($2{\cdot}5 \times 10^{-3}$ M), and after 30 min incubation at 37 °C the mixture was plated on NA containing Tc (25 μg/ml) for selection of inheritance of the R factor. In each experiment sixty transductants—after having been restreaked on the same selective medium—were scored for $T1^{rt}$, Sm^r, and growth response on MA to Sm and to the specific requirement.

(vi) *Loss of the R factor*

This was obtained spontaneously in ageing cultures (25 days) on NA slants of transduction recombinants.

3. RESULTS

(i) *Conjugation experiments*

Table 1 summarizes the results obtained in four different conjugation experiments. Each of these was repeated at least three times and gave similar results.

Table 1. *Transfer by conjugation of the resistance factors* R(SmTcT1P1) (*crosses 1 and 3*) *and* R(TcT1P1) (*crosses 2 and 4*) *into a* his^{sd} *mutant*

		Analysis of recombinants					
			Growth response on				
Cross	Selection	No. tested	MA	MA + his	MA + Sm	Tc^r, $T1^{rt}$*	Resistance to Sm
1. HfrR1/11 × B25	MA + his + Tc	100	−	+	−	+	+
2. HfrR1/30 × B25	MA + his + Tc	100	−	+	+	+	+
3. HfrR1/11 × B26	MA + his	100					
		A 95%	−	+	−	+	+
		B 5%	−	+	+	−	+
4. HfrR1/30 × B26	MA + his	100					
		A 94%	−	+	+	+	+
		B 6%	−	+	+	−	+

In crosses 1 and 2, inheritance of Tc^r carried by the R factor was selected. In crosses 3 and 4, inheritance of the donor chromosomal markers thr^+ and leu^+ was selected: A = recombinants also inheriting the donor R factor, B = recombinants not inheriting the donor R factor.

MA = minimal agar; his = histidine (50 μg/ml); Tc = tetracycline (25 μg/ml).

* + indicates both Tc resistance and T 1 restriction; − indicates absence of both characters.

Crosses 1 and 2 were plated on MA containing histidine (50 μg/ml) and Tc (25 μg/ml), the selective marker therefore being cytoplasmic resistance to tetracycline. Transfer frequencies were respectively $4{\cdot}7 \times 10^{-4}$ and 5×10^{-4} for the first and the second cross. Analysis of recombinants gave different results depending on the type of R factor carried by the donor strain. While in fact transfer of the resistance factor *R(TcT1P1)* had no effect on the *CSD* phenotype of the recipient

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strain (cross 2), the transfer of the resistance factor *R(SmTcT1P1)* (cross 1) prevented the *CSD* phenotype from appearing, and all the recombinants behaved like ordinary *his*$^-$ strains; therefore it seemed as if Sm were no longer able to repair their nutritional requirement. The actual presence of the Sm determinant in recombinants of cross 1 was proved by crossing five of them with the *Sm*^{s} *E. coli* B strain. Together with Tc resistance (the selected marker), low-level Sm resistance and T1 restriction were transferred.

Identical results were obtained in further conjugation experiments not reported in Table 1 in which either *F*$^-$ strains of *E. coli* K 12 (42/45 and 176/41) or the spher 13 *Klebsiella* strain were used as donors. The same selection as that of crosses 1 and 2 was used. The absence of chromosomal transfer which characterizes this type of cross provides additional proof of the role of the R determinant for Sm in preventing this drug from suppressing the gene defect of the strain. Growth characteristics in MM of one recombinant (B 33) from cross spher 13 × B 25 are reported in Table 2.

Table 2. *Growth of strains B 25 and B 33 under different conditions in minimal medium*

Strain tested	Addition to MM:	Yield (O.D.) after 18 h at 37 °C*					
	his (μg/ml)	.	50	50	.	.	.
	Sm (μg/ml)	.	.	500	5	50	500
B 25		0·010	0·500	0·480	0·155	0·190	0·215
B 33		0·010	0·445	0·470	0·011	0·015	0·010

* Gorini & Kataja's (1964) method was employed. Minimal medium enabled the wild type to reach an optical density (O.D.) of 0·465. Optical density was measured at 490 mμ in a Beckman DU spectrophotometer.

In crosses 3 and 4, *thr*$^+$ *leu*$^+$ recombinants were selected. As in the foregoing experiments, counterselection of the donor was made by omission of its required growth factor. Recombination frequencies were 2×10^{-3} for cross 3 and $4{\cdot}6 \times 10^{-3}$ for cross 4. In both experiments two recombinant classes were identified on the basis of the inheritance of the R factor. As shown in Table 1, analysis of recombinants confirms the effect of the Sm determinant on the *CSD* phenotype. In fact, only those recombinants of cross 3 which acquired the resistance factor *R(SmTcT1P1)* are *his*$^-$, while recombinants of cross 4 maintain their *CSD* phenotype, whether transfer of the resistance factor *R(TcT1P1)* had occurred or not. The Sm determinant was proved to be present in class A recombinants of cross 3, as they were able to transfer low-level Sm resistance to *E. coli* B.

Cross 3 was repeated 4 times. On one occasion only, a new class of recombinants (4 out of the 100 checked) appeared, which maintained the *CSD* phenotype, in spite of being *Tc*^{r}*T1*^{rt}. However, when used as donors in control crosses with *E. coli* B, the four strains transferred the resistance factor *R(TcT1P1)* without Sm determinant. The loss of a single R determinant, either spontaneously or during transfer, is not an unusual event.

It may be noted that no reduction in transfer frequency of the resistance factor *R(SmTcT1P1)* into the *CSD* strain can be observed when comparing the frequencies of inheritance of the two R factors among recombinants of crosses 3 and 4.

(ii) *Transduction experiments*

The effect of the R determinant for Sm on the *his^{sd}* phenotype was analysed also by transduction of the two resistance factors *R(SmTcT1P1)* and *R(TcT1P1)* into the *E. coli* B 26 strain. Both types of experiment were repeated three times. Selection was made on NA + Tc. The frequency of transduction was about one transductant for every 10^5 *P1kc/B9* particles—*R(SmTcT1P1)*—and about one for every 10^4 *P1kc/B23* particles—*R(TcT1P1)*. Analysis of transductants (Table 3) showed that in the first experiment the phenotype of the recipient strain changed from *his^{sd}* to *his$^-$*, while no modification of *CSD* phenotype followed the transmission of an R factor devoid of the Sm determinant (second experiment).

Table 3. *Transfer by transduction of the resistance factors* R(SmTcT1P1) *(Expt. 1) and* R(TcT1P1) *(Expt. 2) into a* his^{sd} *mutant*

		Analysis of transductants					
			Growth response on				
Lysate	Selection	No. tested	MA	MA + his	MA + Sm	Tc^r, T1^{rt}*	Resistance to Sm
1. P1kc/B9	NA + Tc	80	−	+	−	+	+
2. P1kc/B23	NA + Tc	80	−	+	+	+	+

In both experiments inheritance of Tc^r carried by the R factor was selected. NA = nutrient (BHI) agar; Tc = tetracycline (25 μg/ml).

* See note for Table 1.

As with the conjugation recombinants, Sm determinant was proved to be present in transductants from the first experiment by crossing five of them with *E. coli* B.

Data on transduction follow those of conjugation experiments and stress the correlation between *CSD* phenotype modification and acquisition of Sm determinant. That the suppression mechanism resulting in conditional Sm dependence is merely prevented from acting was proved by the reappearance of the *his^{sd}* phenotype when *his$^-$* transductants spontaneously lost their R factor.

It may be pointed out (data not published) that the resistance factor *R(Sm Tc T1P1)* can be transduced at the same frequency into both *CSD* and *Sm^s* strains of *E. coli* B, while attempts to transduce it into an *Sm^d* strain have so far been unsuccessful. On the other hand, the resistance factor *R(TcT1P1)* could be transduced with about equal frequencies into the three types of strain: *Sm^s*, *Sm^d* and *his^{sd}*.

4. DISCUSSION

These results show that when harbouring an R determinant for Sm, the *his^{sd}* mutant behaves like *his$^-$*. Consequently, Sm seems no longer able to correct the

suppressible gene defect which, together with the allele present at the Sm locus, may be considered to be involved in determining the *CSD* phenotype (Gorini & Beckwith, 1966).

A reduction in permeability of the cell membrane—the basis of episomal Sm resistance according to Rosenkranz (1964)—might be held responsible for the effect observed. This, however, seems unlikely as even Sm concentrations up to 10000 μg/ml, though well beyond the resistance level conferred by the Sm determinant, cannot replace the specific requirement. Alternatively, the cytoplasmic Sm determinant could behave like those alleles present at the Sm locus which do not permit the Sm correction of an Sm-suppressible defect. If this were so, the R determinant would have proved itself able to interfere in the translation processes, thus indicating that ribosomes may be involved in determining cytoplasmic resistance to Sm. Further experiments with *CSD* mutants obtained from *Sm^r* mutants of independent origin will make it clear whether only a particular type of correction is prevented, or whether the R determinant is unable to permit the correction of whatever Sm-suppressible mutation may occur.

Unlike the *Sm^d* mutant, no difficulty was encountered when transferring the Sm determinant into the *CSD* strain. The reduced transfer, previously observed with the *Sm^d* recipient strain, can be interpreted, on the basis of the present data, as due to a lethal phenotype conferred by this R determinant. The prevention of the Sm repairing action in fact prevents the *Sm^d* mutant from growing, as no medium, however rich, enables its Sm-suppressible gene defect to be overcome, whereas this is possible for the *CSD* mutant. Actually, in crosses with *Sm^d* mutant, no recombinant harbouring the Sm determinant while retaining the *Sm^d* phenotype has ever been found.

Studies are now in progress to ascertain if the high-level Sm resistance, which seems to be related to the entry of the R determinant into the *Sm^d* mutant, is a consequence of the transfer itself or rather a prerequisite for the viability of the recombinants.

SUMMARY

When an R determinant for streptomycin is transferred into a conditionally streptomycin-dependent *E. coli* B mutant—which requires in minimal medium either histidine or streptomycin—the latter behaves like a histidineless strain. This phenotype modification shows that the repairing action of streptomycin is prevented. The specific requirement of the strain is not now replaced even by streptomycin concentrations up to 10000 μg/ml at which the conditionally streptomycin-dependent mutant could originally grow, and which are well beyond the resistance level characteristic of the R determinant itself. These data seem to suggest that a reduction in permeability of the cell membrane cannot be held responsible for the phenomenon observed.

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