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Trimethoprim resistance controlled by a combination of plasmid and chromosomal genes

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SUMMARY

R388s is a mutant of the R factor, R388. Unlike its parent R388s does not confer trimethoprim resistance, though it does confer sulphonamide resistance and the ability to transfer. It is shown that when bacteria harbour R388s mutations to trimethoprim resistance occurred at a high frequency ($3{\cdot}5 \times 10^{-4}$), whereas such mutations never occurred when the host lacked the R factor. Bacteria harbouring R388s did not mutate to resistance to any other antibiotics at a detectable frequency.

The expression of trimethoprim resistance in bacteria possessing R388s appears to require two genes, one located on the R factor and the other situated presumably on the chromosome. The mechanism of trimethoprim resistance in these bacteria is probably due to reduced permeability and is thus quite unlike the insusceptible target site mechanism of the parent R factor, R388.

1. INTRODUCTION

Trimethoprim (Tm) prevents the bacterial production of tetrahydrofolate derivatives, by inhibiting the enzyme dihydrofolate reductase (5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase EC 1.5.1.3) (Hitchings & Bushby, 1961). Sulphonamides act at an earlier stage in folate biosynthesis and both drugs are synergistic when used in combination. Three years after the clinical introduction of the trimethoprim–sulphonamide combination, bacterial strains resistant to it appeared in hospital isolates. This resistance was of a high level and conferred by transmissible resistance plasmids (R factors) (Datta & Hedges, 1972).

The first R factor found to code for resistance to both drugs was R388 which we designate R388wt in this paper. We have shown that the trimethoprim resistance mechanism conferred by this R factor is due to the production of a dihydrofolate reductase that is insusceptible to trimethoprim (Amyes & Smith, 1974*a*). This was the first report of an insusceptible target site being the mechanism of R factor mediated drug resistance. A mutant of R388wt (which we have designated R388s) has been isolated. This mutant no longer confers resistance to trimethoprim but has retained sulphamethoxazole (Sx) resistance and ability to transfer.

In this paper we show that a high proportion of bacteria harbouring R388s mutate to Tm resistance. The Tm resistance of these mutants possessing R388s

differs genetically and biochemically from the resistance conferred by R388wt. With R388s, Tm resistance seems to require the presence of both the plasmid and of the chromosomal genes, and impermeability would seem to be the mechanism by which bacteria resist Tm. With R388wt the plasmid alone is sufficient to confer Tm resistance, namely the insusceptible target site mechanism (Amyes & Smith, 1974*a*).

2. MATERIALS AND METHODS

The bacterial strains and the R factors used in this study are listed in Table 1.

Table 1. *Bacteria and R factors*

Strain	Relevant characters	Reference or source
(*a*) Bacteria		
Escherichia coli J6-2	*pro*, *his*, *trp*, F^-	Clowes & Rowley (1954)
Escherichia coli strain 28	*thr*, *leu*, *pro*	W. Hayes
Escherichia coli PA309	*thr*, *leu*, *trp*, *his*, *arg*, *thi*	Wood (1968)
Escherichia coli J5-3	*pro*, *met*	N. Datta
(*b*) R-factor		
R388*	Tm^R, Sx^R	Datta & Hedges (1972)
R388s	Sx^R	Amyes & Smith (1975)
R7K	Am^R	P. Kontomichalou

Tm^R confers resistance to trimethoprim
Sx^R confers resistance to sulphamethoxazole
Am^R confers resistance to ampicillin

* For the sake of clarity this R factor is referred to as R388wt (wild type) in the text.

(i) *Media.* The minimal medium used was that described by Davis & Mingioli (1950) supplemented with 0·28% (w/v) glucose. Solid Davis–Mingioli (DM) medium was made as described by Smith (1967). The nutrient broth used was Oxoid No. 2.

(ii) *Reagents.* Amino acids were the L-form, obtained from the Sigma Chemical Company, London, and aqueous solutions sterilized by membrane filtration. Sulphamethoxazole and trimethoprim lactate were kindly supplied by the Wellcome Research Laboratories. Results are expressed in terms of trimethoprim base (Tm). Aminopterin was kindly donated by Lederle Laboratories. All drugs were sterilized by membrane filtration.

(iii) *Mutational frequency determinations.* Overnight nutrient broth cultures were washed with DM base and resuspended in DM medium. Serial dilutions were made and 0·1 ml amounts spread on DM agar containing Tm (5 μg/ml) or Sx (100 μg/ml). The plates were incubated at 37 °C for 66 h. Viable counts on drug-free media were also done.

(iv) *Other procedures.* R-factor transfer was performed as described by Smith (1969), using Sx (100 μg/ml) or Tm (5 μg/ml) as selection and minimum inhibitory concentrations (M.I.C.) were determined as described by Amyes & Smith (1974*b*).

3. RESULTS

(i) *Reversion to trimethoprim resistance*

When the R factor R388s was received from Dr Naomi Datta it was transferred to *E. coli* J6-2 using Sx selection. The R^+ recipient was purified by two single-colony isolations on Sx. To check for the absence of Tm resistance the R^+ strain was then streaked on DM medium containing Tm. Although at first sight this procedure confirmed that the isolates of strain J6-2 had not received Tm resistance, closer inspection of the plates revealed that a few colonies had grown in the area of the first streak. As this is normally regarded as an indication of reversion, a single colony isolate of *E. coli* J6-2(R388s) was taken after two subcultivations on Sx, grown overnight in Oxoid No. 2 medium and tested for its frequency of mutation to Tm resistance.

The results were that, while the bacteria were completely Sx-resistant, Tm resistance was exhibited at a frequency of $3{\cdot}52 \times 10^{-4}$ per cell plated. When the test was repeated using the R^- parent strain neither Sx nor Tm resistance occurred at a detectable frequency ($< 10^{-9}$). These results at first sight seemed to suggest that R388s was reverting to its original character with respect to its ability to confer Tm resistance.

(ii) *Transfer of trimethoprim resistance*

To check this point a Tm^R isolate of strain J6-2(R388s) was mated with *E. coli* strain 28 as recipient. R^+ recipients were selected using either Tm or Sx. As controls additional matings were made using strain J6-2(R388s) that had not been exposed to Tm selection, and strain J6-2(R388wt), subcultured either on Tm or Sx, as donor. The results (Table 2) show that with all four donors the transfer of Sx resistance occurred at approximately similar rates. Surprisingly transfer of Tm resistance seemed to occur at about 0·03 % of this frequency when either donor harboured R388s irrespective of whether the donor was Tm^R or Tm^S. However, there was linked transfer of resistance to both drugs from either donor that contained R388wt.

These results were not expected because they show that the Tm-resistance character of the Tm-resistant isolate of *E. coli* J6-2(R388s) did not co-transfer with Sx resistance. The low frequency of Tm-resistance transfer that seemed to be occurring with this donor strain occurred at the same rate with which trimethoprim resistance was observed in *E. coli* J6-2(R388s) when the 'reversion frequency' experiment was performed.

As a further check colonies of strain 28 taken from these matings were cultured in nutrient broth and tested for sensitivity to Tm and to Sx. The results (Table 3) show that any exconjugant obtained from mating with R388wt retained stably resistance to both drugs. However, when exconjugants obtained from matings with R388s were isolated on Sx and then tested for antibiotic resistance it can be seen that Tm resistance was observed in only about 0·04 % of the bacteria, which nevertheless retained stably their Sx resistance. In addition, it can be seen that Tm resistance in strain 28, when it did occur, was retained stably by the bacteria.

Table 2. *Frequency of trimethoprim resistance transfer*

Donor	Frequency of resistance found in strain 28 per input donor cell		Ratio of Tm resistance to Sx resistance found in strain 28
	To Sx	To Tm	
E. coli J6-2(R388s)*	$1{\cdot}6 \times 10^{-2}$	$7{\cdot}5 \times 10^{-6}$	$2{\cdot}8 \times 10^{-4}$
E. coli J6-2(R388s)†	$8{\cdot}1 \times 10^{-3}$	$2{\cdot}4 \times 10^{-6}$	$4{\cdot}6 \times 10^{-4}$
E. coli J6-2(R388wt)*	$2{\cdot}0 \times 10^{-2}$	$2{\cdot}2 \times 10^{-2}$	1·12
E. coli J6-2(R388wt)†	$1{\cdot}1 \times 10^{-2}$	$1{\cdot}0 \times 10^{-2}$	0·96

* The culture of the donor strain was inoculated from a single colony growing on Sx.
† The culture of the donor strain was inoculated from a single colony growing on Tm.

Table 3. *Frequency of Tm and Sx Resistance in strain 28 harbouring various R factors*

Donor	Viable count on Tm ÷ viable count on Sx R+ recipient isolated on	
	Tm	Sx
E. coli (R388s)*	1·20	$5{\cdot}0 \times 10^{-4}$
E. coli (R388s)†	0·85	$2{\cdot}7 \times 10^{-4}$
E. coli (R388wt)*	1·15	0·97
E. coli (R388wt)†	1·20	0·92

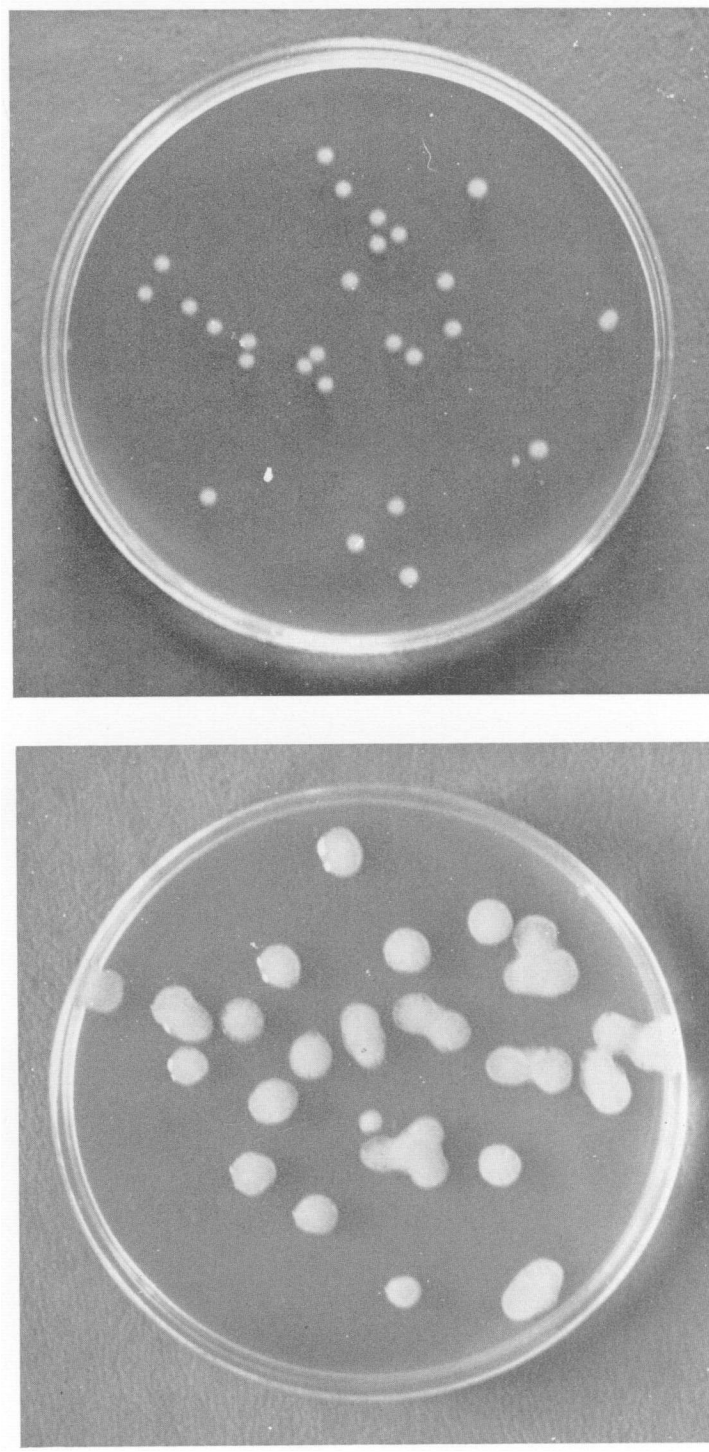
* The culture of the donor strain was inoculated from a single colony growing on Sx.
† The culture of the donor strain was inoculated from a single colony growing on Tm.

(iii) *Colonial morphology*

It was noticed throughout the experiments that colonies of bacteria harbouring R388wt grown on Sx, Tm or media lacking antibiotics were of normal appearance. The same was true for bacteria harbouring R388s, which had not been exposed to Tm selection. However, all clones of strain J6-2(R388s) which were Tm-resistant exhibited a larger than normal colony size and mucoid appearance (see Plate 1). This abnormal colonial morphology was observed with these Tm-resistant bacteria even after subcultivation in media containing Sx or lacking antibiotics.

(iv) *R-factor elimination*

The transfer experiments showed that although Tm resistance was not transferred with R388s the R-factor had to be present in strains before they could exhibit a high-frequency of Tm resistance. In order to test whether the continued presence of R388s was necessary for the expression of Tm resistance, elimination studies were made. One thousand and sixteen clones of *E. coli* J6-2(R388s) that had not been exposed to Tm selection were replica-plated on to drug-free media and on to media containing Sx. Four of these clones, which we term strain J6-2(R^-) were found to be lacking Sx resistance and had presumably lost R388s. When broth cultures of J6-2(R^-) were tested for mutation to Tm resistance none was



(*a*)

(*b*)

Colonial morphology of *Escherichia coli*. J6-2(R388s) on nutrient agar plates after 24 h incubation. (*a*) Trimethoprim-sensitive type. (*b*) Trimethoprim-resistant type.

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observed (frequency $< 10^{-9}$), indicating that the ability to mutate to Tm resistance required the presence of the plasmid. No clones of *E. coli* J6-2(R388s) that had mutated to Tm resistance were found to have lost the R factor even though a large number of colonies was tested.

As the R388s plasmid was not lost spontaneously at a detectable rate from the Tm-resistant isolate of strain J6-2 (R388s) another procedure was adopted. R388 is a member of the W compatibility group (Datta & Hedges, 1972) and thus cannot coexist in a cell with another W plasmid. The R factor R7K is a W plasmid that confers ampicillin resistance. Hence *E. coli* J5-3(R7K) was used as donor in a mating with a trimethoprim-resistant isolate of *E. coli* J6-2(R388s) using ampicillin to select for R7K transfer. Exconjugants were purified by three successive subcultivations on agar containing ampicillin. When viable counts were performed it was found that exconjugants exposed to this treatment, while gaining ampicillin resistance had lost sulphonamide resistance. This was taken to indicate that R7K had been gained while R388s had been lost and hence we term this organism J6-2-(R7K)(R^-).

An observation which lent support to this view was that the mucoid colonial appearance was absent from this strain.

Strain J6-2(R7K)(R^-) was then tested for resistance to trimethoprim and perhaps surprisingly, found to be trimethoprim sensitive. In case some residual Tm resistance had been retained by these bacteria a closely graded M.I.C. test using single cell inocula was carried out. It was found that J6-2 (R7K)(R^-) and strain J6-2 were inhibited by 0·4 μg/ml Tm but grew in the presence of 0·2 μg/ml Tm. Thus is would seem that while trimethoprim resistance does not transfer with R388s the continued presence of the plasmid is necessary for the expression of any Tm resistance in these bacteria.

To follow up this point another experiment was performed. *E. coli* J6-2(R^-) and *E. coli* J6-2(R7K)(R^-) were taken as recipients and R388s transferred into them. The donor strains used were a Tm-resistant isolate of *E. coli* 28(R388s) and an isolate of strain 28(R388s) that had not been exposed to Tm selection. Sulphamethoxazole was used to select for the transfer of R388s. The results with strain J6-2(R^-) as recipient were that neither donor transferred linked Tm resistance although, as usual, the bacteria now harbouring R388s again exhibited Tm resistance at a frequency of $4{\cdot}70 \times 10^{-4}$. However, when strain J6-2(R7K)(R^-) was used as recipient it was found that, irrespective of the donor phenotype, Tm resistance was exhibited by all the bacteria that had received Sx resistance. Moreover, all clones had regained their mucoid colonial appearance which thus correlates perfectly with the presence of Tm resistance in all cell lines harbouring R388s.

Thus when R388s is eliminated from a strain exhibiting the Tm resistance the bacteria become Tm sensitive and non-mucoid and when R388s is reintroduced the bacteria regain Tm resistance and a mucoid colonial morphology.

These experiments may be subject to the criticism that the strains used are not strictly isogenic because the strain J6-2(R^-) which lost R388s spontaneously has

not been exposed to R7K. To check this point, R388s was eliminated from an isolate of J6-2(R388s) that had not been exposed to Tm-selection, by mating with strain J5-3(R7K) using ampicillin selection and passage as before. The resultant strain was taken as recipient and R388s reintroduced by mating with a strain 28(R388s) donor of each type (i.e. Tm-resistant and Tm-sensitive). It was found that, irrespective of the donor used, the introduction of R388s did not confer Tm resistance, though Tm-resistant bacteria were as usual detected at a frequency of about $2{\cdot}0 \times 10^{-4}$. Thus R7K could not be responsible for the effects observed with strain J6-2(R7K)(R^-).

(v) *Specificity of resistance*

It has been reported that some ampicillin-resistant mutants of *E. coli* K_{12} have a mucoid character similar to that observed here with the Tm-resistant isolates of strains possessing R388s (Greaves, 1972). Therefore, the drug sensitivity of the strains harbouring R388s was examined to determine whether the specificity of its kind of Tm resistance extended to include resistances to other antibiotics. Broth cultures of *E. coli* J6-2(R388s) that were Tm-resistant and those that had not been exposed to Tm selection were diluted to 10^{-6} with DM base and 0·1 ml amounts spread on DM agar containing just sufficient drug to inhibit the growth of the R^- strain. As controls, the R^- strain and *E. coli* J6-2(R388wt) were also tested. After incubation the plates were examined for growth. The results (Table 4) show that neither strain exhibited a resistance to any of the drugs tested.

In addition, the mutational frequency to resistance to the antibiotics listed in Table 4 in the Tm-sensitive and the Tm-resistant isolates of *E. coli* J6-2(R388s) was also examined by the method described in the Materials and Methods. It was found that neither strain increased the level of mutation to resistance to any of the antibiotics shown in Table 4 at the concentrations listed.

(vi) *Other mutations*

A more specific method to test whether R388s conferred any ability to increase the frequency of mutation was tried. *E. coli* PA309 is known to revert to tryptophan independence at a detectable rate. An R^+ isolate was constructed using Sx selection and *E. coli* J6-2(R388s) as donor. It was found that the R^- strain mutated to tryptophan independence at a rate of $5{\cdot}2 \times 10^{-8}$, while the R^+ strain mutated to tryptophan independence at a rate of $6{\cdot}2 \times 10^{-8}$. Thus the presence of the R388s plasmid appears to have no significant effect on reversion.

(vii) *Levels of Tm resistance*

Minimum Inhibitory Concentration (MIC) determinations were performed to compare the level of Tm resistance in *E. coli* (R388wt) with that of a Tm-resistant isolate of *E. coli* (R388s). As controls the R^- strain and an isolate of *E. coli* (R388s) not exposed to Tm selection were included. As we have previously shown that R388wt confers resistance to 2,4-diaminopteridines as well as to Tm (Amyes & Smith, 1976) the level of resistance to aminopterin was also determined. The

results (Table 5) show that R388wt increases the Tm-resistance of the host by almost 10000-fold while the resistance to aminopterin was increased by at least 75-fold. The Tm-resistant strain harbouring R388s exhibited a level of resistance to Tm 1000 times greater than that found in the R^- strain. This, however, was about ten times lower than the resistance conferred by the R388wt plasmid. In addition, the Tm-resistant strain of J6-2(R388s) exhibited no increase in resistance

Table 4. *Resistance Pattern of R-factors in* E. coli *J6-2*

Antibacterial compound conc. (μg/ml)		J6-2	J6-2 (R388wt)	J6-2 (R388s)*	J6-2 (R388s)†
None		+	+	+	+
Tm	5	−	+	−	+
Sx	100	−	+	+	+
Polymyxin	10	−	−	−	−
Kanamycin	10	−	−	−	−
Cephlosporin	10	−	−	−	−
Ampicillin	10	−	−	−	−
Tetracycline	3	−	−	−	−
Streptomycin	10	−	−	−	−
Chloramphenicol	10	−	−	−	−
Rifampicin	10	−	−	−	−
Nalidixic acid	10	−	−	−	−
Spectinomycin	100	−	−	−	−

\+ Growth.
− No visible growth.
* Not exposed to Tm selection.
† Exposed to Tm selection.

Table 5. *Minimum inhibitory concentrations of trimethoprim and aminopterin against strains of* E. coli *J6-2*

	J6-2	J6-2 (R388wt)	J6-2 (R388s)*	J6-2 (R388s)
Tm	0·4	3000	1·25	400
Tm + 0·5 mM EDTA	0·4	3000	1·25	40
Aminopterin	40	> 3000†	100	40
Aminopterin + 0·5 mM EDTA	40	> 3000†	100	40

* Not exposed to Tm selection.
† The highest concentration that could be tested due to insolubility.

to aminopterin. This indicates that the resistance mechanism to Tm in this strain is unlike that conferred by R388wt on at least two counts. Indeed, when cell extracts of Tm-resistant strains of J6-2(388s) were examined for the presence of a dihydrofolate reductase insusceptible to the drug, none could be found. This is the mechanism of resistance in bacteria harbouring the parent R388wt (Amyes & Smith, 1974*a*, 1976).

The mucoid appearance of the Tm-resistant isolates of *E. coli* J6-2(R388s)

could be taken to indicate that the outer layers of such bacteria differ from, for example, that of *E. coli* J6-2(R388wt). Moreover, this hypothesis may lead further to the suggestion that such a surface change could explain the mechanism of resistance to Tm as being that of impermeability. One possible way of implicating impermeability is to investigate the effects of EDTA treatment, as this compound has been shown to increase the permeability of bacteria to certain drugs including aminopterin (Lieve, 1965; Smith, 1967; Smith & Wyatt, 1974). The sensitivity of strain J6-2 harbouring the various types of R388 was therefore tested in the presence and absence of 0·5 mM EDTA, which was found in pilot experiments to have no effect on the ability of the bacteria to form colonies on solid media.

The results (Table 5) show that only the level of Tm resistance in the Tm-resistant isolate of strain J6-2(R388s) was affected by such treatment. Neither the Tm nor the aminopterin resistance levels of the strain harbouring R388wt was affected by EDTA treatment. This not only confirms that the Tm-resistance mechanism in the Tm-resistant isolates harbouring R388s is different from that of bacteria containing R388wt, but may also be suggestive that impermeability could be the mechanism of resistance in the former strain.

4. DISCUSSION

The presence of the plasmid, R388s, in *Escherichia coli* confers sulphonamide resistance and mating ability but in addition a high proportion of the bacteria exhibit trimethoprim resistance. We show this latter resistance differs from the mechanism of trimethoprim resistance conferred by R388wt and hence reversion cannot explain these results. Indeed, reversion has also been ruled out in genetic tests where Tm resistance did not exhibit co-transfer with Sx resistance.

If, as we suggest, the Tm resistance of the Tm-resistant isolates of bacteria that harbour R388s is due to reduced permeability to the drug, then this mechanism is quite unlike any other Tm-resistance mechanism so far reported in *E. coli*, resembling that of *Pseudomonads* and *Klebsiella* (Hitchings, Burchall & Ferone, 1966). It is almost impossible to test Tm uptake directly using radioactiveity labelled drugs, as it binds only transiently to the target enzyme in Tm-sensitive cells (Amyes, 1974). This type of resistance mechanism has been suggested to account for chloramphenicol resistance by certain R factors in Pseudomonads (Kono & O'Hara, 1976).

Two further sets of observations are relevant. (1) Tm-resistant isolates containing R388s exhibited a mucoid colonial morphology as well as Tm resistance and both these traits were lost together when the R factor was eliminated. (2) When R388s was reintroduced by mating both the mucoid character and the Tm resistance returned irrespective of whether the donor strain had been resistant or sensitive to Tm. Thus it would seem that Tm resistance in these circumstances is dependent absolutely on two sets of genes: one set plasmid borne and the other presumably located on the chromosome. Other workers have observed that high-

level drug resistance can be attributed to a combination of plasmid and chromosomal genes (Gunderson, 1963, 1965; Ginoza & Painter, 1964; Pearce & Meynell, 1968; Roberts & Reeve, 1970). However, in all these cited cases the R factor, on its own, could confer a significant degree of protection which when added to a strain exhibiting low-level chromosomal resistance resulted in a synergistically high level of resistance. In our study the chromosome did not give any detectable Tm resistance and it was only when the R factor was added that a significant level of resistance to Tm could be detected.

An aspect deserving comment concerns the manifestation of Tm-resistant clones of bacteria which harbour R388s; a phenomenon never observed to occur in the same strain lacking the R factor. The simplest explanation of this is that a chromosomal mutation also occurs in the R^- strain but that it is 'silent' in the absence of the plasmid. However, a disturbing feature is the extremely high frequency with which Tm-resistant clones occur (about 3×10^{-4} per cell plated) which is orders of magnitude higher than is normally accepted for chromosomal mutations. Is it possible that the R factor itself increases the mutational frequency as has been concluded by other workers (Ginoza & Painter, 1964; McCann *et al.* 1975)? We examined this possibility experimentally with negative results. However, despite this, other work concerning the fine structure of R factors may have a bearing on our findings.

Following our discovery that the gene for TEM β-lactamase production was ubiquitous among R factors we concluded that it possessed a special ability to translocate between replicons (Hedges *et al.* 1974). Later it was shown that this gene could translocate between plasmids as a discrete DNA segment (Hedges & Jacob, 1974). Subsequently it was found that the DNA segment containing TEM β-lactamase gene was flanked on either side by inverted complementary base sequences (Heffron, Rubens & Falkow, 1975). This DNA segment belongs to the class of elements now termed transposons (Hedges & Jacob, 1974) of which at least one is known to confer Tm resistance (Barth *et al.* 1976). The inverted complementary base sequences which flank some, but not all transposons, have been related to insertion sequences (Berg *et al.* 1975; Gottesman & Rosner, 1975; Heffron *et al.* 1975) which in turn are known to be mutagenic (Reif & Saedler, 1975) at frequencies of $1\text{–}4 \times 10^{-4}$ (Foster, 1976). Thus the possibility remains that R388s could contain DNA sequences of this nature which may promote the occurrence of mutation to Tm resistance to occur in the chromosome by, for example, an insertion process. However, DNA studies are necessary before this point can be tested experimentally.

Irrespective of the mechanism whereby the putative chromosomal mutation has occurred we have shown that the continued presence of the R factor is necessary for the expression of Tm resistance by these bacteria and hence the mutation is 'silent' in the absence of the plasmid. As far as is known this is the first report of this type of essential co-operation between R factors and their host.

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