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Characteristics of some single-step mutants to chloramphenicol resistance in *Escherichia coli* K12 and their interactions with R-factor genes

By E. C. R. REEVE*

Institute of Animal Genetics, Edinburgh 9

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1. INTRODUCTION

Single-step mutants to Chloramphenicol resistance (*Cm-r*†) are easily selected in *Escherichia coli* by plating about 10^7 cells on nutrient agar containing 5 μg./ml. of antibiotic. Only low-level resistance is obtained, and several independent mutations selected in this way were found also to give low levels of resistance to Aureomycin and Puromycin, the degree of resistance to each antibiotic varying from one mutant to another. The method of assay in these comparisons was to measure the RNA synthesis induced when each antibiotic was added to cells starved of required amino-acids (Reeve & Bishop, 1965*a*,*b*).

Attempts to detect differences in resistance levels among the same mutants by plating on nutrient agar plus antibiotic were unsuccessful, although resistant mutants could always be distinguished in this way from the sensitive parent strain. More recently, surprisingly good discrimination has been obtained by streaking the mutants on minimal agar containing antibiotic and any amino-acids required for growth. The results of a series of tests of this kind are presented below.

R-factors causing resistance to Chloramphenicol or to Tetracycline but not to both have also been introduced into a number of resistant mutants and the resulting changes in level of resistance measured. One mutation may have its resistance effect completely masked by an R-factor, while another causes a striking increase in the level of resistance mediated by the same factor.

2. MATERIALS AND METHODS

Bacterial strains

Escherichia coli K12 strain J62 F$^-$ *pro*$^-$ *try*$^-$ *his*$^-$ *str-r* and the six independent *Cm-r* mutants 1a–c and 2a–c derived from it have already been described (Reeve & Bishop, 1965*b*). In addition a number of single-step *Cm-r* and *Tc-r* mutants of *E. coli* K12 HfrH *met*$^-$ *str-r* have been selected by plating independently grown samples of about 10^7 cells on nutrient agar containing 5 μg./ml. of Cm or 1 or 2 μg./ml. of Tc. One colony was picked per sample and purified by repeated streaking on nutrient agar. The parent strain was supplied by Dr W. Hayes.

R-factors

These were supplied by Dr Naomi Datta and are numbered according to her system (see Meynell & Datta, 1966).

* Agricultural Research Council Unit of Animal Genetics.

† Abbreviations: Cm = Chloramphenicol, Tc = Tetracycline, Pm = Puromycin. *Cm-r* and *Tc-r* indicate mutants selected for 1-step resistance to Cm and Tc, respectively.

Plating tests

Strains were fully grown in nutrient broth from single colonies, and were diluted 10^{-2} in buffer before being streaked on minimal antibiotic agar. Using a template, ten 1-in. streaks were put on each plate, with a wire loop of about 2·5 mm. internal diameter. The plates were used within 24 hours and generally on the day of pouring. Antibiotics in powder form were dissolved in sterile distilled water and passed through an Oxoid filter membrane before use. Tetracycline was used instead of Aureomycin, since the latter decays during plate incubation. Antibiotic concentrations were increased in steps of $\sqrt{2}$ or about 1·4, so that two steps doubled the concentration. This was found convenient both for preparation and for distinguishing between mutants.

Scoring of plates

After incubation for about 40 hours at 37°C, streaks were scored as + + (a thick streak representing full growth), + (a thin streak indicating partial inhibition of growth) and − (complete or nearly complete inhibition). Generally these classes were quite definite for each mutant and growth passed through the stages + +, +, − in three successive steps. In any test, one mutant is classed as more resistant than another (symbol >) if it registers + + on a higher antibiotic level. Where there was doubt, one mutant was classed as ($\geqslant$), i.e. at least as resistant, and probably more resistant than the other. (=) indicates that no difference in resistance could be detected between two strains. The concentration of antibiotic giving + growth is taken as MIPC, The Minimal Inhibitory Plate Concentration for that mutant. Tests in which a strain was plated at two concentrations differing by a factor of 2 showed that a variation in cell density of this magnitude had no effect on resistance grading.

3. RESULTS

Repeated plating tests have been made with the six *Cm-r* mutants of J62 and the parent strain, to assess their resistance to Cm, Tc and Pm; and Table 1 gives the order of resistance to the different antibiotics in each test. It also gives the order of resistance grading obtained in the RNA biosynthesis assays of Reeve & Bishop (1965*b*).

In the case of Chloramphenicol, the four plate tests are completely consistent with each other and with the RNA assay, and show clearly the occurrence of three levels of resistance. These are, in decreasing order of resistance: (1a), (1b, 2a, 2b), (1c, 2c), all more resistant than J. Two resistance levels are shown quite consistently for Tetracycline, 1b, 2a and 2b being more resistant than 1a, 1c and 2c. But the RNA assay, made with Aureomycin, almost reverses this relationship, showing 2c as more resistant than 1b. The results for both antibiotics are based on several mutually consistent tests, so this difference in ordering must be taken as a real one. It suggests that resistance to Tetracycline and to Aureomycin are not always closely correlated.

The tests with Puromycin are also very consistent, and bring out the novel fact that one mutant (2a) is actually more sensitive than the parent strain. 1a, 1c and 2c show increased resistance to Pm while 1b and 2b do not. The RNA assay agrees approximately with this grading, but shows 2c as appreciably more resistant than 1a.

Table 2 summarizes the results of the plating tests, giving both the Minimal Inhibitory Plate Concentrations (MIPC) and a set of resistance grades from which the resistance pattern of each mutant can be seen at a glance. Mutants (1a), (1c, 2c) and (1b, 2b) clearly form three groups with different resistance characteristics, and seem likely to result from mutations at three different loci. Mutant 2a is similar to 1b and 2b, except for its increased sensitivity to Pm.

Table 1 *Summary of plating tests with* Cm-r *mutants of* E. coli *J*62

	Date of test
Resistance to Chloramphenicol	
1a > 1b = 2a = 2b > 1c = 2c > J	21/9/1965
1a > 1b = 2a > 2c > J	19/10/1965
1a > 1b > 2c > J	27/10/1965
1a > 1b = 2a = 2b > 1c = 2c > J	21/1/1966
[1a > 1b > 2c > J]*	
Resistance to Tetracycline	
1b = 2a = 2b > 2c = 1a = 1c > J	21/9/1965
1b = 2a > 2c > J	19/10/1965
1b = 2a > 2c > 1a > J	27/10/1965
1b = 2a = 2b > 2c = 1a = 1c > J	21/1/1966
[2c > 1b > 1a > J Aureomycin]*	
Resistance to Puromycin	
1a = 2c > 1b ⩾ J > 2a	19/10/1965
1a = 2c > 1b ⩾ J > 2a	27/10/1965
1a = 2c = 1c > 1b = 2b = J ⩾ 2a	13/1/1966
1a = 2c = 1c > 1b = 2b = J > 2a	21/1/1966
[2c > 1a ⩾ 1b ⩾ J]*	

All comparisons except those marked [ ]* are based on growth of cells at about 10^7/ml. streaked on M9 minimal antibiotic agar supplemented with amino acids required for growth, and incubated about 40 hr at 37°C. Antibiotic concs. were in steps of $\sqrt{2}$.

J: sensitive strain J62. 1a, 1b, ...: *Cm-r* mutants of J62. > more resistant than, ⩾ probably more resistant than, = no detectable difference in resistance level.

* Assay by measuring induction of RNA synthesis by antibiotic in cells starved of required amino-acids. Mean of several tests. Data from Reeve & Bishop (1965*b*).

Three R-factors were introduced individually into some of these strains: R1 carrying resistance to Cm but not to Tc, and R46 and R57, both carrying resistance to Tc but not to Cm. The three R-factors carried resistance to various other antibiotics, as indicated in Table 3. Resistance levels to Cm, Tc and Pm were measured by streak tests in the same way as for strains not carrying these factors.

None of the R-factors had a detectable effect on the resistance of any mutant to Pm, while R1 had no effect on resistance to Tc and the other two factors had no effect on resistance to Cm. This confirms the very specific nature of R-factor resistance previously reported. Table 3 shows the effect of R1 on resistance to Cm and of R46 and R57 on resistance to Tc, in the strains tested most thoroughly.

Table 2. *Resistance levels and grades of J*62 *mutants*

	Minimal inhibitory plate conc. (MIPC) in μg./ml.			Resistance grade		
Strain	Cm	Tc	Pm	Cm	Tc	Pm
J	3·5	0·5	140	0	0	0
1a	14	0·7	200	4	1	1
1c, 2c	5	0·7	200	1	1	1
1b, 2b	7	1·4	140	2	3	0
2a	7	1·4	100	2	3	−1

MIPC is defined and measured as explained in Materials and Methods. Resistance grade is expressed in steps of $\sqrt{2} \times$ MIPC of the sensitive strain.

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Table 3. *Effect of R-factors on resistance levels of J62 strains*

	(MIPC of antibiotic in μg./ml.)				
	Chloramphenicol		Tetracycline		
R-factor present	None	R1	None	R46	R57
Strain J	3·5	226	0·5	7	113
1a	14	226	0·7	7	113
1b	7	450	1·4	42	320
2a	7	320	1·4	42	320
R-factor resistance:		ACKSu		ASTSu	STSu
RTF character		*fi*+		*fi*−	*fi*−

R-factor resistance characters: A(Ampicillin), C (Chloramphenicol), K (Kanamycin), S (Streptomycin), Su (Sulphonamide), T (Tetracycline).

Factor R1 increases the resistance of the sensitive strain to Cm some 60-fold, raising MIPC from 3·5 to 226 μg./ml. Mutation 1b causes a doubling of MIPC both in the absence and presence of this R-factor, so it clearly expresses its resistance effect to Cm when the R-factor is present. 1a behaves quite differently, its high mutational resistance to Cm being completely masked by R1. Mutation 2a causes some increase in resistance in cells carrying R1, but its effect may be partially masked.

Resistance to Tc was increased in the sensitive strain some 14-fold by R46 and over 200-fold by R57. Mutations 1b and 2a express their resistance to Tc in the presence of each R-factor, whereas no effect of 1a could be detected when either R46 or R57 was present, possibly because it has only a small effect on resistance to Tc.

Less extensive tests have been made with the other three J mutants, but all appear to express some measure of resistance to both antibiotics when the corresponding R-factors are present. Mutant 1a thus differs from all the rest, not only in its pattern of resistance to the three antibiotics, but in the fact that its resistance effects on Cm and Tc are completely masked by an R-factor carrying the corresponding resistance genes.

Recently a number of single-step mutants for resistance to Cm or Tc have been selected in HfrH *met*−, and a preliminary survey has been made of their resistance characteristics and of the effect of infecting them with R1 or R57. It is not proposed to present the results of this survey in detail here, but some points relevant to the data given above are of interest. Most of the mutants show appreciable resistance to both Cm and Tc, and their effects are not masked by either R-factor, so they would be classed as of the 1b type described above. Two mutants resemble 1a in being more resistant to Cm than to Tc and in having their resistance to Cm entirely masked when R1 is introduced. Another mutant, selected for resistance to Tetracycline, grows much more slowly than the parent strain, has a low level of resistance to Tc, unchanged sensitivity to Cm and much increased sensitivity to Pm. These mutants are all non-mucoid in character, as are the mutants of J62 tested. Mucoid mutants were also obtained by selecting on antibiotic plates and will be described in another paper. Attempts are now being made to establish the chromosomal locations of at least those mutations giving the higher resistance levels.

4. DISCUSSION

Failure to extract enzymes which inactivate Tc or Cm from *E. coli* made resistant to these drugs by selection or the presence of an R-factor, and the finding that cell-free systems from resistant and sensitive strains were equally sensitive to inhibition of protein-synthesizing activity by the antibiotics, led to the conclusion that both mutational and R-mediated resistance to each antibiotic were due to permeability changes in the cell membrane (Okamoto & Mizuno, 1964; Watanabe, 1963).

Recently, however, by modifying their procedure, Okamoto & Suzuki (1965) have succeeded in extracting from R-infected cells an enzyme which rapidly inactivates Cm and is not present in sensitive cells. This enzyme is thought to be responsible for R-mediated resistance to Cm. The authors have so far failed to demonstrate the presence of a Tc-inactivating enzyme in R-infected cells, but consider that such an enzyme may nevertheless be responsible for R-mediated Tc resistance. Enzymes were also extracted which inactivated Dihydrostreptomycin and Kanamycin, so it seems possible that all resistance due to R-factors is caused by drug-inactivating enzymes. None of these enzymes could be detected in sensitive strains of *E. coli*, but tests do not appear to have been made on cells selected for mutational resistance, so the nature of this type of resistance remains an open question.

The present results have some bearing on this problem. Mutations such as those described, which alter the levels of resistance to two or three different antibiotics, can hardly do so by producing inactivating enzymes, except on the unlikely supposition that each is a mutation of a regulator gene, leading to an alteration in the cell concentrations of several enzymes. An alternative hypothesis, that the *Cm-r* mutations modify the sensitivity of the protein biosynthetic system to the three antibiotics, appears contrary to the evidence of Okamoto & Mizuno (1964), referred to above. This leaves us with the original hypothesis that these mutations modify the permeability of the cell membrane to the drugs. Most of our mutants, when combined with R-factors, behave as might be expected on this theory, assuming that R-mediated resistance to Cm and to Tc is due to enzymes which break down these antibiotics internally. The mutations increase the resistance of cells carrying an R-factor, presumably by increasing the maximum external concentration of antibiotic at which the enzyme can be effective.

Mutant 1a is an exception to this pattern, since its rather large effect on resistance to Cm is not expressed at all in the presence of the R-factor. This result would follow if the mutant produced a small concentration of an inactivating enzyme which became superfluous in the presence of the R-factor enzyme, but this would not explain the effect of the mutant in increasing resistance to Tc and Pm. Possibly an unusual type of permeability effect is also involved in this case.

SUMMARY

Six one-step Chloramphenicol (Cm)-resistant mutants of *Escherichia coli* K12 were graded for resistance to Cm, Tetracycline (Tc) and Puromycin (Pm) by streaking on minimal agar plates containing antibiotic. They fell into at least three distinct groups on the basis of their resistance patterns. One mutant showed increased sensitivity to Pm. Most of the mutants expressed their effect on resistance to Cm and Tc in the presence of R-factors carrying resistance genes for these antibiotics, but one mutant with a relatively high level of resistance to Cm had its resistance effect completely masked in the presence of R-mediated resistance. Similar cases were found among mutants selected for Cm-resistance in another strain of K12.

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