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Genetic analysis of some mutations causing resistance to tetracycline in *Escherichia coli* K12

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1. INTRODUCTION

Single-step mutations to Cm-resistance† in *Escherichia coli* K12 vary in the levels of resistance they give to different antibiotics (Reeve & Bishop, 1965; Reeve, 1966). One of these mutations (*cmlA1*), which gives a relatively high resistance to Cm and little if any to Tc has a chromosomal location between λ-prophage and *pyrD*. It is readily transduced by P1 with very little phenotypic lag, and gives similar resistance levels in the different strains of *E. coli* K12 to which it has been transferred (Reeve & Suttie, 1968). This paper provides evidence that mutations in at least four other genes give some degree of resistance to Cm and Tc or to Tc alone. Two of these genes give mucoid antibiotic-resistant mutants.

2. MATERIAL AND METHODS

(i) *Bacterial and phage strains*

The bacterial strains employed were all derivatives of *Escherichia coli* K12, with the following genotypes and origins:

RE1: J62, *proB*‡ *try his str-r* $\lambda^+\lambda^r$ Cm-s F^- (from Dr W. Hayes).

RE23: HfrH *met str-r* $\lambda^+\lambda^r$ (from Dr. W. Hayes).

Derivatives of RE23 carrying Tc-r mucoid mutants *tce*(*M*) 1–9, respectively (see text):

RE82: B11, *metB str-r* $\lambda^-\lambda^r$ Cm-s Hfr (from Dr W. Hayes, see Broda, 1967).

RE103–5, 107: Cm-r mutants *1a*, *1b*, *1c* and *2b* of RE1 (Reeve, 1966). These mutations have been renamed *cmlA1*, *cml-2*, *cml-3*, and *cml-5* (Reeve & Suttie, 1968):

RE110: MS3, *gal pyrD str-r* $\lambda^-\lambda^s$ Cm-s F^- (from Dr J. Shapiro).

RE122: RE82 made *cmlA1* by transduction with P1 (Reeve & Suttie, 1968).

Phage P1Kc was used for transduction.

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† Abbreviations: Cm = chloramphenicol, Tc = tetracycline, Pm = puromycin. Cm-r and Cm-s indicate phenotypes (partially) resistant and sensitive to chloramphenicol. *Cml* and *tce* are symbols for genes giving mutants with increased resistance to Cm and/or Tc. Other gene symbols generally follow Taylor & Trotter (1967).

‡ This mutation was wrongly given as *proC* by Reeve & Suttie (1968).

(ii) *Media and technical methods*

These were as described in Reeve (1966) and Reeve & Suttie (1968), unless otherwise stated in text and tables.

3. RESULTS

(i) *Mutations* cml-2 *and* cml-5, *giving relatively high Tc-resistance*

In contrast to *cmlA1*, these two mutations cause a relatively large increase in resistance to Tc and only a small increase in Cm-resistance, as shown in Table 1. *CmlA1* can be easily transduced by Phage P1 into Cm-s strains, by plating cells treated for 20 min with donor phage immediately on minimal agar containing Cm at 10 μg/ml (Reeve & Suttie, 1968). However, attempts to transduce *cml-2* or *cml-5* by selecting on agar containing various levels of Tc were unsuccessful, whether or not time was allowed for phenotypic expression of resistance.

Table 1. *Antibiotic resistance levels of J62 mutants*

Cm-r mutation in RE1 (J62)	Minimal inhibitory plate concn. (μg/ml.) Cm	Tc	Pm
None (sensitive parent)	3·5	0·5	140
cmlA1 (= 1a)	14	0·7	200
cmlB2, *cmlB5* (= 1b, 2b)	7	1·4	140
cml-3, *cml-6* (= 1c, 2c)	5	0·7	200

Estimates based on table 2 of Reeve (1966). Old designations of mutations are given in parentheses.

Information was obtained on *cml-5* by mating RE122 (Hfr B11 *cmlA*) to RE107 (J62 *cml-5*). RE122 feeds in *cmlA* as an early marker closely linked to λ, and *try* and *his* are injected later, at about 15 and 30 min at 37 C (Fig. 1). Try$^+$Str-r and Try$^+$His$^+$Str-r recombinants were selected after interruption of mating at 30 and 50 min, respectively, and purified clones were tested for resistance to Cm and Tc and for λ-lysogeny. All recombinants fell into four distinct resistance patterns with no overlap or danger of misclassification, and the same patterns occurred whether interruption was at 30 or 50 min, and whether the clones were His$^+$ or His$^-$. The experimental results are summarized in Table 2.

The resistance patterns of the original Cm-s strain and of the two mutant types are shown at the top of the table, and may be compared with those of the four recombinant types shown below. The resemblance between particular recombinant and parental types is so close that evidently type 1 is Cm-s, type 2 carries *cmlA* and type 3 carries *cml-5*, while type 4 is *cmlA*, *cml-5*. This conclusion is strengthened by the data in the lower part of the table on the distribution of λ among the recombinants. The great majority (80 %) of the progeny are either the donor type (*cmlA*, λ^-) or the recipient type (*cml-5*, λ^+), as we should expect, since *cmlA* and λ are near enough on the chromosome to be co-transducible with P1.

CmlA shows the same recombination frequency (10 %) with *cml-5* as with λ, while *cml-5* and λ recombined in 20 % of the progeny. These figures suggest that the two resistance mutations are rather closely linked but in different cistrons, and that the order is λ, *cmlA*, *cml-5*. This order is confirmed by further evidence given below.

Table 2. *Patterns of antibiotic resistance in recombinants from mating*

RE122 (Hfr B11 *cmlA*) × RE107 (J62 *proB try his cml-5* F⁻)

	Growth on Cm (μg/ml)						Growth on Tc (μg/ml)					
Strain	*3·5*	*5*	*7*	*10*	*14*	*20*	*0·5*	*0·7*	*1·0*	*1·4*	*2·0*	*2·8*
J62 Cm-s	+/−	−	−	−	−	−	+/−	−	−	−	−	−
J62 *cmlA*	++	++	++	+/−	−	−	+	+/−	−	−	−	−
J62 *cml-5*	++	+	−	−	−	−	++	++	++	+/−	−	−
Recombinants												
Type 1	+/−	−	−	−	−	−	+/−	−	−	−	−	−
2	++	++	++	+/−	−	−	+	+/−	−	−	−	−
3	++	+	+/−	−	−	−	++	++	++	+	−	−
4	++	++	++	++	++	+	++	++	++	++	+	+/−

Distribution of recombinant types (numbers)

Assumed genotype			λ⁺	λ⁻	Total
1	+	+	3	0	3
2	A	+	4	26	30
3	+	5	14	1	15
4	A	5	0	2	2
		Total	21	29	50

Log phase broth-grown cells of donor and recipient strains were mixed at 37 °C. After 30 and 50 min, samples were diluted and mating interrupted with an M.S.E. Microhomogeniser, before plating on selective plates. Recombinant clones were purified twice on agar before being tested. They were then grown up in broth and diluted 1/50 in buffer for streaking on antibiotic and other test plates, using our standard test procedure. Growth after 42 hr at 37 C was scored as follows: ++, thick confluent growth; +, thin confluent growth; +/−, slight growth; −, no visible growth or a few colonies only.

Table 2 also shows that, when both resistance genes are present together, they raise the resistance level by about 100 % for Cm and 50 % for Tc above the highest given by either gene alone.

Failure to achieve direct transduction of either *cml-2* or *cml-5* makes further analysis difficult, but fortunately it was discovered that both mutations could be co-transduced by P1 with *pyrD*, located between *cmlA* and *try* (Fig. 1). Table 3 summarizes several co-transduction tests, and shows that both *cml-2* and *cml-5* have co-transduction frequencies of 50–60 % with *pyrD*. Since the two mutations also give identical patterns of antibiotic resistance (Reeve, 1966), it may be assumed that they are mutations in the same cistron, which will be designated

cmlB. *PyrD* and *cmlA* are not co-transducible with P1 (Reeve & Suttie, 1968), so the order of the chromosomal region containing the resistance genes must be

gal, λ, *cmlA*, (*cmlB*, *pyrD*),

where the order of the two genes in the brackets remains to be determined.

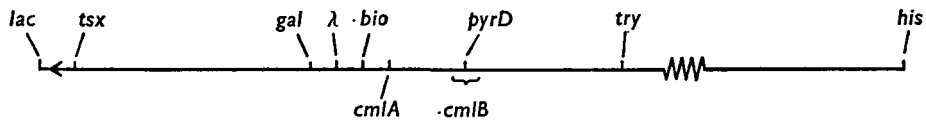

Fig. 1. Approximate chromosomal locations of genes referred to in text. The arrow gives origin and direction of transfer by Hfr strains RE82 and RE122. The bracket above *cmlB* indicates that its position with respect to *pyrD* is not known.

Table 3. *Co-transduction of* pyrD *with* cml-2 *and* cml-5 *by phage P1*

	Transductants				
		Numbers		Co-transduction (%)	
Expt no.	U+ tested	*cml-2*	*cml-5*	U+ *cml-2*	U+ *cml-5*
1	390	224	.	57	.
2	80	.	51	.	64
3	120	.	42	.	35
4	100	50	.	50	.
4	100	.	62	.	62
		Overall frequencies:		56	52

RE110 (*gal pyrD* Cm-s) was transduced to Pyr+ (symbol U+ in table) by P1 grown on RE104 (*cml-2*) or RE107 (*cml-5*). U+ clones were purified by streaking on nutrient agar, and two colonies from each clone were tested for Gal+, Pyr+ and Tc-resistance by streaking sequentially on suitable test plates. The presence of *cml-2* or *cml-5* could be detected without ambiguity by streaking on minimal agar containing Tc at 2 μg/ml. No U+ transductants were Gal+.

(ii) *Mutation* cml-3, *giving slight resistance to both Cm and Tc*

This mutant gives too low a resistance to Cm and Tc for transduction of the mutation to be effective, as Table 1 shows. A small-scale mating test, parallel to one described above for *cm-5*, showed that *cmlA* and *cml-3* are not closely linked and that *cml-3* is not located in the *gal-try* region. The *cml-3* locus is evidently distinct from *cmlA* and *cmlB*, and will be designated *cmlC*. The mutation *cml-3* combines with *cmlA* to increase the resistance levels for Cm and Tc by about 50% above the highest level given by either mutation separately.

(iii) *Mucoid mutations giving resistance to Tetracycline*

When sufficient antibiotic-sensitive bacteria are plated on agar containing Tc, mucoid as well as non-mucoid colonies appear, and the former obviously possess resistance to Tc. The mutants previously discussed are all non-mucoid and of the same colony type as the Cm-s parent. Mutants of RE23 (HfrH *met str-r* λ+) were

selected by plating about 10^7 cells on minimal agar containing methionine and 1 μg/ml of Tc. Nine independent mucoid colonies were selected and purified. They are referred to as *tce*(*M*) 1–9. Eight of the mutant lines were tested for resistance to Cm, Tc and Pm by our usual test procedure, and the results are given in Table 4.

Table 4. *Growth of RE23 (HfrH* met *Tc-s) and its mucoid Tc-r mutants on various antibiotics*

Antibiotics in medium (μg/ml)	RE23 Tc-s	Mucoid Tc-r mutants	
		Group 1	Group 2
None	++	++	++
Cm 3·5	+/−	+	+/−
5	−	+/−	−
7·1	−	−	−
Tc 0·5	+/−	++	++
0·7	−	+	++
1·0	−	−	+
1·4	−	−	+/−
2·0	−	−	−
Pm 35	++	++	+
50	++	++	−
71	+	++	−
100	+/−	+	−
142	−	+/−	−
200	−	−	−

Each strain was grown overnight in broth, diluted 1/50 in buffer, and streaked onto minimal agar containing methionine and the antibiotic concentrations indicated. The streaks were scored after 42 hr at 37 °C. Symbols as in Table 2. Group 1 mutants included *tce*(*M*) 1, 2, 3 and 6. Group 2 included *tce*(*M*) 4, 5, 7 and 9.

The parent non-mucoid strain shows slight growth on Cm 3·5, Tc 0·5 and Pm 100 (units in μg/ml), so its resistance pattern closely resembles that of J62 Cm-s used in previous mutant studies (see Table 1). The eight mucoid mutants fall into two well-defined groups which show striking contrasts in resistance pattern. Group 1 are about 50 % more resistant than the parent strain to all three antibiotics, while group 2 show no increase in Cm-resistance, a marked increase in Tc-resistance, and a remarkable increase in *sensitivity* to Pm (they are inhibited by half the Pm concentration needed to inhibit growth of the parent strain). The different mutants within each group had identical resistance patterns within the limits of discrimination of the test.

Attempts to locate each mutant type by mating tests proved difficult, since the mutant strains appeared to have reverted to the F^+ state. Table 5 gives the results of mating 7 of the mutants to RE1 (*proB try his*) for 60 min and plating for recombinants on minimal agar containing either no amino acids, proline or histidine, the number of colonies and the proportion which were mucoid being given in each case. Per donor cell plated, there were about 10^{-6} recombinants on Min agar and about 10^{-5} when histidine or proline was present, suggesting that the matings were

of F+-type. Nevertheless, the mucoid frequencies in Table 5 divide the mutants into two distinct classes. All group 1 mutants give a high proportion of mucoid colonies (over 50 %) when there is enforced inclusion of the *proB*+ gene from the donor strain, while the mucoid frequency is 10 % or less when there is no selection for *pro*+. This shows that the mucoid gene of the group 1 mutants is close to *proB*, and makes it probable that it is the mucoid locus symbolized as *capR* by Markovitz & Baker (1967) and as *lon* by Taylor & Trotter (1967).

Mutants of group 2 gave a very low proportion of mucoid colonies on all three types of selective plate, suggesting that these mutations are not closely linked to *his* or *proB*, and are not in the *capR* locus. This conclusion is supported by the very different resistance patterns of the two groups. Further work on these mutant types is in progress.

Table 5. *Mating test on RE23 mucoid Tc-r mutants*

RE 23 (HfrH *met*) tce(M) × RE1 (J62 *proB try his*) Tc-s

	No. of recombinant colonies			% colonies mucoid			
Supplement...	None	His	Pro	None	His	Pro	Mutant group*
Mutant tce(M) 1	11	174	265	73	63	3	1
2	13	203	223	85	58	10	1
3	20	181	336	80	55	3	1
6	12	165	233	92	76	6	1
4	8	128	222	0	0	1	2
5	19	410	204	0	2	2	2
9	7	360	281	0	3	2	2

Donor and recipient cells were grown to log. phase in broth and mixed at about 5×10^7 per ml of each sex. After washing and resuspension in buffer, samples were plated to give about 2×10^7 donor cells on each plate type. The plates contained M 9 minimal agar, supplemented where indicated by 30 μg/ml of histidine or proline. Incubation was for 45 h at 37 °C.

* As defined in Table 4.

4. DISCUSSION

Mutations in five different genes have now been shown to affect resistance to Tetracycline, Chloramphenicol or both antibiotics, and in some cases also to alter resistance to Puromycin. Two have been located as the chromosomal genes *cmlA* near λ, and *cmlB* near *pyrD*. Two others give mucoid antibiotic-resistant mutants, one at an unidentified locus and the other probably at the *capR* locus near *lac*. *CapR* (*lon*) is a regulatory gene for several enzymes of capsular polysaccharide synthesis (Markovitz & Baker, 1967), and it is not at all clear why mutations at this locus should cause increases in resistance to Tc, Cm and Pm, nor why mucoid mutations at another locus should cause a sharp decrease in resistance to Pm. On the basis of the data accumulated so far, it is not yet possible to say anything about the nature of the resistance mechanisms. But the picture now emerging is that mutations at different loci give characteristically different patterns of resistance to the three antibiotics; and though the effect on resistance to each antibiotic is always small, the pattern is clearly distinguishable for different groups

of mutants. Unpublished evidence also suggests that mutations selected to give the same resistance pattern as that of *cmlA* or *cmlB* tend to be mutations in the same locus.

SUMMARY

Mutations in at least five different genetic loci cause some degree of resistance to Chloramphenicol, Tetracycline or both antibiotics in *Escherichia coli* K12. Two have been identified as chromosomal genes: *cmlA* near λ prophage and *cmlB* near *pyrD*. A third, giving mucoid antibiotic-resistant mutants, is probably *capR* (*lon*) near *lac*. Another group of mucoid mutants gives marked increases in resistance to Tetracycline and in sensitivity to Puromycin; these mutants do not occur at *capR*. Although all the mutations have rather small effects on resistance level, the patterns of resistance to the three antibiotics appear to be characteristically different for mutations at different loci.

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REFERENCES

Broda, P. (1967). The formation of Hfr strains of *Escherichia coli* K 12. *Genet. Res.* **9**, 35–48.

Markovitz, A. & Baker, B. (1967). Suppression of radiation sensitivity and capsular polysaccharide synthesis in *Escherichia coli* K 12 by Ochre suppressors. *J. Bact.* **94**, 388–395.

Reeve, E. C. R. (1966). Characteristics of some single-step mutants to chloramphenicol resistance in *Eschericria coli* K 12 and their interactions with R-factor genes. *Genet. Res.* **7**, 281–286.

Reeve, E. C. R. & Bishop, J. O. (1965). Variations in resistance to three antibiotics among some single-step mutants to Chloramphenicol resistance in a strain of *Escherichia coli* K 12. *Genet. Res.* **6**, 310–315.

Reeve, E. C. R. & Suttie, D. R. (1968). Chromosomal location of a mutation causing chloramphenicol resistance in *Escherichia coli* K 12. *Genet. Res.* **11**, 97–104.

Taylor, A. L. & Trotter, C. D. (1967). Revised linkage map of *Escherichia coli*. *Bact. Rev.* **31**, 332–353.