Genet. Res., Camb. (1965), **6**, *pp.* 310–315
Printed in Great Britain

Variations in resistance to three antibiotics among some single-step mutants to Chloramphenicol resistance in a strain of *Escherichia coli* K 12

By E. C. R. REEVE* and J. O. BISHOP

Institute of Animal Genetics, Edinburgh 9

(*Received* 6 *May* 1965)

1. INTRODUCTION

A multi-step chloramphenicol resistant (*CM-r*)† strain of *Escherichia coli* K 12, obtained by selection in CM, was found to possess a considerable degree of resistance also to AM† and PM†, as indicated both by its ability to grow on agar containing these antibiotics, and by the change in the effect of each antibiotic on RNA synthesis under amino acid starvation, compared with that in the sensitive strain. CM, AM and PM all produce what may be called the 'RNA response' in *RC-stringent* auxotrophs, i.e. each derepresses RNA synthesis, to an extent depending on the concentration of antibiotic, when added during starvation for a required amino acid. Characteristic patterns of response were found for each antibiotic in the CM-sensitive (*CM-s*) strain, and much greater concentrations of each drug were required to induce a given level of RNA synthesis in the *CM-r* strain (Reeve & Bishop, 1965).

These results indicate that at least some of the *CM-r* mutations accumulated during the selection for CM-resistance must also give a measure of resistance to one or both of the other antibiotics; and in this paper we describe an attempt to characterize the resistance patterns of some single step *CM-r* mutants. Preliminary tests suggested that the method of streaking on antibiotic plates, even with careful standardization of streak size and cell density, while quite adequate for distinguishing *CM-r* mutants from the (*CM-s*) parent strain, was not sensitive enough to detect variations in level of resistance between different 1-step mutants (Reeve & Smith, unpublished). Tests using the 'RNA response' as an assay method proved to be more sensitive, and enabled us to show that there were significant differences in resistance level among a group of mutants selected in a single strain.

2. MATERIALS AND METHODS

Bacterial strain. Escherichia coli K12 strain J 62, F− *pro*− *try*− *his*− *str-r RC-stringent*, obtained from Dr W. Hayes. This strain was used with the intention later of transducing the *CM-r* mutations into various Hfr strains in order to locate their chromosomal positions.

Media. M 9 is M 9 minimal medium (Adams, 1959); M 9/PTH is the same medium supplemented with 40 μg./ml. each of proline, tryptophan and histidine.

Selection of mutants. Two single colonies of J 62 were streaked for single clones on nutrient agar. Three clones from each (1a-c, 2a-c) were grown up in broth and plated at about 8×10^6 cells per plate on nutrient agar containing 5 or 10 μg./ml. of CM. An

* Agricultural Research Council Unit of Animal Genetics.

† Abbreviations: CM = Chloramphenicol, AM = Aureomycin (Chlortetracycline), PM = Puromycin, *CM-s* and *CM-r* indicate Chloramphenicol sensitive and resistant strains or mutants.

average of 10^{-6} of the cells plated formed colonies on the CM-5 plates but none on the CM-10 plates, during 2 days' incubation at 37°C. One colony was picked originating from each of the six clones, to ensure selection of independent mutations, and, after purification by streaking, each was found to have increased resistance to CM. They were labelled R1a, R1b, . . . R2c, according to the parent clone. The mutants and the sensitive strain were kept on nutrient agar plates at 3°C., on which they had been streaked for single colonies, and were transferred to fresh plates at intervals of about 2 months. No reversions to sensitivity have so far been detected in colonies picked from these plates. Mutant R3, used in some of the tests, was obtained in precisely the same way as the other *CM-r* mutants, by selection from J 62.

Measurement of RNA synthesis. The protocol described by Reeve & Bishop (1965) was followed, except that each line was grown up for testing in M 9/PTH medium (without CM), and, when growing in log phase, was washed and resuspended in cold M 9 medium. Amino acids, when added in the test, consisted of proline, tryptophan and histidine at 75 μg./ml. final concentration. Each test started from single colonies of the parental and resistant strains.

3. RESULTS

Two exploratory tests were made to compare the 'RNA response' to various levels of CM and AM of the sensitive strain and the six resistant mutants R1a-c, R2a-c. Their results are summarized in Table 1. The parental strain (here labelled S) was compared with mutant R2a in Experiment 1 and with the other five *CM-r* mutants in Experiment 2, using the same antibiotic concentrations. Other concentrations, also tested in Experiment 1, added no useful information and are not included. Incubation was for 1 hour at 37°C., which is about two-thirds of the cell doubling time for all strains. The first two rows of the table give ^{14}C-Uracil uptake with no supplement and with a supplement of the three required amino acids, and the difference between these quantities is taken as the control level. In the rest of the table the excess uptake above the 'no supplement' level induced by each antibiotic concentration is expressed as a percentage of this control.

Table 1. *Induction of RNA synthesis by CM and AM in J62 and single-step* CM-r *mutants of J62*

Supplement	Uptake of ^{14}C-Uracil in 1 hr. at 37°C.: c.p.m./10							
	Experiment 1		Experiment 2					
	S	R2a	S	R1a	R1b	R1c	R2b	R2c
None	52	44	60	49	50	42	54	34
Amino acids	817	624	843	927	966	752	1044	803
	Uptake as % of controls ('amino acids'—'none')							
CM 8	9·1	3·2	13	2·7	9·8	7·0	1·9	4·2
32	69	30	74	6·4	24	21	11	15
128	66	73	60	60	49	62	61	50
AM 1	20	5·7	16	7·1	5·4	4·4	2·5	2·6
4	63	40	73	48	36	28	24	18
16	64	69	75	61	53	57	53	48

Incubation for 1 hr. at 37°C. in basal medium of M 9 salts + glucose at 0·20% and ^{14}C-Uracil at 20 μg./ml. with specific activity 0·05 μc./ml. Amino acid supplement was proline + tryptophan + histidine at 75 μg./ml. final conc. Antibiotic concs. in μg./ml. S is CM-sensitive strain J62. R1a, R1b, etc., are single-step *CM-r* mutants.

The corresponding sets of figures for strain S in the two tests are in good agreement, and show that induced RNA synthesis is near a maximum with 32 μg./ml. of CM or 4 μg./ml. of AM: when the dose of either antibiotic is quadrupled, there is no further increase in uptake. The six *CM-r* mutants all show relatively less response than the sensitive strain to the two lower concentrations of each antibiotic, suggesting that all have increased resistance to both CM and AM.

The best antibiotic concentrations for distinguishing between the different strains appear to be the intermediate levels of 32 μg./ml. CM and 4 μg./ml. AM, both giving partial induction of RNA synthesis in the mutants and maximum induction in the sensitive parental strain. At these levels the *CM-r* mutants show a considerable range of response: 6–30% with CM 32 and 18–48% with AM 4. This range of variation could reflect either real differences in resistance or a rather large experimental error variability, and further tests were made to settle this point. The three mutant strains R1a, R1b and R2c, which appeared to differ in resistance pattern as judged by Table 1, were chosen for repeated tests with CM 32 and AM 4, together with strain S. The results of these tests, including the data from Experiment 2, are summarized in Table 2*a*, which also gives the mean response of each strain over the set of tests.

Table 2. *^{14}C-Uracil uptake by* 1-*step* CM-r *mutants: analysis of repeated experiments with CM and AM*

(*a*) Uptake as percent controls

	CM 32				AM 4			
Expt.	S	R1a	R1b	R2c	S	R1a	R1b	R2c
2	74	6	24	15	73	48	36	18
3	79	15	19	40	64	49	22	23
4	64	7	18	14	21	12	11	6
5	71	12	25	28	44	21	17	10
6	†	16	23	44	†	20	15	11
7	65	26	34	41	38	27	20	8
Average	71	13·8	23·9	30·3	55	29·5	20·2	12·7

† Sample of S strain discarded because of probable contamination.

(*b*) Analysis of variance of responses of R strains after transformation to degrees

Source of variance	Degrees of freedom	Mean squares CM 32	Mean squares AM 4
Between Experiments (E)	5	92*	137**
Between Mutants (M)	2	215**	214**
Error (E × M)	10	17·8	13·0

* Significant at P = 0·05. ** Significant at P = 0·01.

(*c*) Mean responses in transformed units with significance levels

Treatment	Mean response by R1a	R1b	R2c	Standard error	Least significant difference at P = 0·05	P = 0·01
CM 32	21·1	29·1	32·1	± 1·72	3·8	5·4
AM 4	32·3	26·4	20·4	± 1·47	3·3	4·7

https://doi.org/10.1017/S0016672300004183 Published online by Cambridge University Press

The responses of the different strains show a fairly consistent pattern from test to test, but there is a good deal of variation between tests in the level of response to AM. The reason for this variation is unknown. Aureomycin decomposes rather rapidly in solution, and a fresh solution was always prepared immediately before each test. Possibly a titration error occurred in the preparation of the solution for Experiment 4 which gives very low responses to all four strains.

There is no doubt that the three R mutants are more resistant than the parent strain to both antibiotics, and there is also a strong suggestion that they differ among themselves in resistance level. This possibility is tested by the analysis of variance given in Table 2*b*, which is applied to the responses transformed to degrees (Fisher & Yates, Table 10, 1957). Clearly there are significant differences between the mutants in response level to both antibiotics.

Finally, Table 2*c* gives the mean transformed responses for each strain, together with their standard errors (from Table 2*b*) and the differences which are just significant at the 5% and 1% probability levels.

All three strains obviously differ in resistance to AM, and R1a certainly differs from the others in resistance to CM while R1b is also probably more resistant than R2c to CM. We thus have the remarkable result that the three strains differ in their resistance to the two drugs in such a way that the strain most resistant to CM (R1a) is least resistant to AM, and vice versa. Table 1 suggests that the other R mutants probably fall into one or other of these patterns.

Tests of the response to Puromycin were included in the last three experiments, using 800 μg./ml. in Experiment 5 and 1600 μg./ml. in Experiments 6 and 7 (duplicate samples were tested in the latter), and the results are analysed in Table 3. Experiment 7 includes tests on an extra *CM-r* mutant, R3, which appeared to have rather low resistance to both CM and AM on the basis of a single test with each (38% response to CM 32 and 30% responses to AM 4, tested in Experiment 7).

Table 3. *Induction of RNA synthesis by Puromycin (PM)*

(*a*) % response to PM 800 and PM 1600

Experiment	PM: μg./ml.	S	R1a	R1b	R2c	R3
5	800	11·5	5·0	10·2	2·6	—
6	1600	—	30·9	35·2	20·5	—
7†	1600	43·5	29·9	35·5	17·7	28·6
		38·3	29·1	31·8	16·5	25·8
Mean of 1600		40·9	30·0	34·8	18·0	27·2

† Two samples of each strain were tested at PM 1600.

(*b*) Analysis of transformed responses to PM 1600

	S	R1a	R1b	R2c	R3
Mean	39·8	33·2	35·8	25·3	31·4
Deviation from S		−6·6**	−4·0*	−14·5**	−8·4**
Deviation from R1a			2·6*	−10·5**	1·8
Deviation from R1b					−4·4**

Standard errors: mean of 2: ± 0·95.
mean of 3: ± 0·78.

* Significant at P = 0·05. ** Significant at P = 0·01.

https://doi.org/10.1017/S0016672300004183 Published online by Cambridge University Press

Table 3*a* gives the individual percentage responses and the strain means for PM 1600. There is very little response to 800 μg./ml. of PM even by the *CM-s* strain (11·5%), and the responses to 1600 μg./ml. only range from 18 to 41%. The different estimates for the same strain in Experiments 6 and 7 show remarkable consistency, and these have been used to test the significance of differences between various strains in the PM 1600 responses in Table 3*b*. After transforming the responses to degrees as in Table 2, a mean variance between replicates is calculated as 1·82 with 8 degrees of freedom (Experiment 6 is treated as adding a third replicate value for three of the strains tested in Experiment 7). Table 3*b* gives the transformed means, the significance levels of differences among them, and the standard errors of means of two and three samples. The four mutant strains all give significantly lower responses than the parent strains to PM, and are all clearly resistant to this antibiotic. There are at least three different resistance levels among them, since R2c, R3 and R1b all differ significantly from each other at the 1% probability level. R1a is intermediate in response between R3 and R1b, and might have the same resistance as either of them.

A summary of the resistance picture for the four mutants on which repeated tests have been made, as far as it has been determined, is given in Table 4, grades being used to distinguish levels of resistance. It will be seen that the four mutants all appear to differ in their resistance patterns to the three drugs.

Table 4. *Distribution of resistance levels in different mutants*

	Grade of resistance*				
Antibiotic	S	R1a	R1b	R2c	R3
CM	0	3	2	1	1 or 2
AM	0	1	2	3	0 or 1
PM	0	1 or 2	1	3	2

* 0 = Same susceptibility as *CM-s* strain.
1, 2, 3 = Increasing levels of resistance, distinguished by statistical tests.

Different Grades indicate significant differences in response, based on Tables 2*c* and 3*b*.

A final point of interest is that Strain J 62 ,the *CM-s* strain used in this paper, appears to be much more resistant to PM than the *CM-s* strain AB 311 examined by Reeve & Bishop (1965). Both are auxotrophs, though with different nutritional requirements, derived from *E. coli* K 12, but while AB 311 gave nearly maximum response to 800 μg./ml. of PM, J 62 gave only 12% response to this level and 40% to 1600 μg./ml. The two strains appear to show equal susceptibility to each of the other two antibiotics. The significance of this difference is unknown, and is receiving further study.

4. DISCUSSION

The results presented indicate that single-step *CM-r* mutations may give several different levels of resistance to CM, AM and PM. Four different patterns of resistance have been distinguished among the seven mutants compared, and other patterns are likely to occur since our method of selecting *CM-r* mutants by plating on 5 μg./ml. CM probably excludes some mutants which grow very slowly on this concentration. While there are some grounds for believing that resistance results from genetically controlled changes in the cell membrane, affecting its permeability (Watanabe, 1963), nothing is

known as to the nature of these changes. Our results suggest the likelihood that pattern of resistance to the three antibiotics is correlated with the location of the mutants on the chromosome, but this remains to be tested directly.

SUMMARY

Chloramphenicol (CM), Aureomycin (AM) and Puromycin (PM) induce RNA synthesis in *RC-stringent Escherichia coli* starved of a required amino acid. This fact has been used to develop a method for comparing the levels of resistance of single-step *CM-r* mutants to the three antibiotics. Three levels of resistance to each antibiotic were found among four mutants selected in a single *CM-s* strain. The mutant with the highest CM resistance has the lowest AM resistance, and vice versa, while the level of PM resistance was not correlated with that of either CM or AM. The four mutants all differed from each other in their patterns of resistance to the three antibiotics.

We wish to thank Mrs Norma Robertson and Mrs Sandra Levitt for technical assistance, and Miss Katherine Smith for help with some of the experiments and for valuable discussion.

REFERENCES

Adams, M. H. (1959). *Bacteriophages*. New York: Interscience Publishers.

Fisher, R. A. & Yates, F. (1957). *Statistical Tables for Biological, Agricultural and Medical Research*, 5th ed. Edinburgh: Oliver & Boyd.

Reeve, E. C. R. & Bishop, J. O. (1965). Multi-step resistance to Chloramphenicol in RC-stringent *Escherichia coli* K 12—its effect on the induction of RNA synthesis by antibiotics under amino acid starvation. *Genet. Res.* **6**, 304-309.

Watanabe, T. (1963). Infective heredity of multiple drug-resistance in bacteria. *Bact. Rev.* **27**, 87–115.