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Chromosomal location of a mutation causing chloramphenicol resistance in *Escherichia coli* K 12

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1. INTRODUCTION

Mutants of *Escherichia coli* K 12, resistant to a low level of chloramphenicol, are easily selected by plating about 10^7 bacteria on antibiotic agar. Such mutants probably occur at more than one genetic locus, since they show several different patterns of resistance to Cm‡, Tc and Pm (Reeve & Bishop, 1965; Reeve, 1966). In certain instances genetically determined resistance to a variety of antibiotics in the Enterobacteriaceae depends on extra-chromosomal genes, attached to or at least transferred by RTF agents (Watanabe, 1963; Anderson & Lewis, 1965*a*, *b*); and the question arises whether low-level resistance to Cm is due to chromosomal or extra-chromosomal genes. In this paper we show that the mutation previously referred to as *1a* (Reeve, 1966), giving a relatively high level of resistance to Cm in comparison to other one-step mutants, has a chromosomal location and is closely linked to the chromosomal attachment site of phage λ. Transduction tests with P1 indicate that there is little phenotypic lag in the expression of this mutant gene after transfer to a new strain.

2. MATERIAL AND METHODS

(i) *Nomenclature of resistant mutants*

Following current usage (Demerec, Adelberg, Clark & Hartman, 1966) we shall give three-letter symbols to resistance mutations as follows: *cml-1*, *cml-2*, etc., will identify mutations selected for resistance to Cm, and *tce-1*, *tce-2*, etc., will identify those selected for resistance to Tc. Separate loci, as they are designated, will be labelled *A*, *B*, *C*, etc., but since some mutations have been found to give resistance to both antibiotics, it may be necessary later to identify two loci, e.g. *cmlX* and *tceY*, as the same. Table 1 gives the old and new symbols for the mutants described previously.

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‡ Abbreviations: Cm = chloramphenicol, Tc = tetracycline, Pm = puromycin. Cm-r and Cm-s indicate phenotypes resistant and sensitive to chloramphenicol. M.O.I. = multiplicity of phage infection.

Table 1. *Symbols for mutants to Cm-resistance in Escherichia coli K12 strain J62*

Old symbol*	New symbol
1a	*cml-1 = cmlA1*
1b	*cml-2*
1c	*cml-3*
2a	*cml-4*
2b	*cml-5*
2c	*cml-6*

* Reeve (1966); also called *R1a*, *R1b*, etc., in Reeve & Bishop (1965).

(ii) *Bacterial and phage strains*

The bacterial strains, all derivatives of *E. coli* K12, had the following genotypes and origins:

J62	*proC try his str-r* $\lambda^+ \lambda^r$ Cm-s F^- (from Dr W. Hayes)
RE 103	mutant of J62 carrying *cmlA 1* (*1a* of Reeve, 1966).
MS 3	*gal pyrD str-r* $\lambda^- \lambda^s$ Cm-s F^- (from Dr J. Shapiro)
M25U	*pyrD his str-r* $\lambda^- \lambda^r$ Cm-s F^- (from Dr E. Meynell)
HfrH	*thi str-s* $\lambda^- \lambda^s$ Cm-s (from Dr W. Hayes)
B11	*metB str-r* $\lambda^- \lambda^r$ Cm-s Hfr (from Dr W. Hayes)

B11 injects the chromosome in the order: origin, *gal*, λ, *pyrD*, *try* (Broda, 1967).

The bacteriophages used were P1Kc and T4rII1272.

(iii) *Media and culture methods*

Minimal agar was M9 minimal medium (Adams, 1959) with Difco Bacto agar at 1·5 %. When required, amino acids were added at 50 μg/ml, uracil at 20 μg/ml, Cm at 10 μg/ml and streptomycin at 200 μg/ml. Antibiotic solutions were sterilized by filtration and added to the melted agar before pouring. Nutrient broth was made with 8 g Difco dehydrated nutrient broth and 5 g NaCl/l. of water. Difco Bacto agar was included at 1·5 % to give nutrient agar.

(iv) *Mating and transduction methods*

Details of the mating procedure are given in Table 2. P1 transduction generally followed the methods of Lennox (1955), modified according to the protocols in Tables 3–6.

(v) *Scoring of recombinant clones*

Clones were purified once by streaking on nutrient agar, and two colonies from each were picked with sterile loops and streaked serially on the required test plates, using templates to give twenty streaks per plate. If the antibiotic plate (minimal agar containing 10 μg/ml Cm) was third or fourth in the sequence of test plates, the loop carried over insufficient cells to allow visible growth of a sensitive strain, and excellent discrimination between sensitive and resistant clones was obtained

after 3 days incubation. To test for λ-lysogeny, the last streak was made onto nutrient agar previously spread with T4rII 1272. Replica-plating was not found reliable in testing for Cm resistance.

3. RESULTS

(i) *Mating of Cm-s Hfr to Cm-r F⁻ strains*

HfrH Cm-s was mated to J 62 *cmlA 1*, and samples were shaken to interrupt mating at 30, 45 and 65 min, and plated on selective plates for recombinants as shown in Table 2. At each blending time parallel samples were plated on media

Table 2. *Mating of HfrH to J 62 cmlA 1*

Time of interruption (min)	Characters selected ♂	Characters selected ♀	Analysis of recombinants: Total scored*	Per 10^4 ♂♂	Cm-s (%) †
30	pro	str-r	4440	1270	0
45	pro try	str-r	2733	234	71
65	pro try his	str-r	684	39	80

Broth-grown log-phase HfrH cells were mixed with stationary phase F^- cells in warm broth at about 10^8 Hfr and 2×10^8 F^- cells per ml. The mixture was diluted 0·1 ml into 20 ml fresh warm broth after 5 min to stop further pair formation. Samples were diluted 1/10 (30 min), 1/3 (45 min) or 1/2 (65 min) in buffer, and shaken on a Griffin flask shaker before plating to interrupt mating. Mating and incubation were at 37 °C.

* On plates without Cm.

† Based on the relative numbers of recombinants on plates with and without Cm.

with and without Cm (10 μg/ml), to estimate the proportion of recombinants which had derived the *Cm*-gene from each parent. HfrH feeds in the chromosome clockwise in the order, *thr*, *leu*, *pro*, *lac*, *gal*, *try*, *his*,..., and selection was made for recombinants carrying donor genes *pro* at 30 min, *pro try* at 45 min and *pro try his* at 65 min.

At 30 min virtually all recombinants retain the *Cm-r* gene of the recipient strain, but the proportion carrying the donor *Cm-s* has risen to 70 % by 45 min and 80 % by 65 min. This indicates that Cm-sensitivity is transferred by HfrH between 30 and 45 min after mating pair formation, some time after the transfer of *pro* and probably before *try*. The result strongly suggests that *cmlA* has a chromosomal location, though an extra-chromosomal gene recessive to the sensitive allele could perhaps provide an alternative explanation.

Hfr B 11 also transfers its chromosome clockwise, but with origin shortly after *lac* (Broda, 1967). On mating B 11 Cm-s to J 62 *cmlA 1* and selecting for *try*$^+$, the earliest marker available, 65 % of recombinants obtained after blending at 20 or 25 min were Cm-s, so that the time of transfer of the gene for Cm-sensitivity from the Hfr strain is correlated with the point at which chromosomal transfer begins. This confirms that *cmlA* has a chromosomal location within or close to the *gal–try* segment.

(ii) *Mating of Cm-r Hfr to Cm-s F⁻ strains*

More accurate location of the gene required the construction of Hfr strains carrying *cmlA*, so that resistance could be used as a selective marker. This was achieved in two ways: (*a*) by mating Hfr B11 to J62 *cmlA*, selecting simultaneously for a late donor marker (*pro*⁺) and for *Cm*-resistance and then selecting recombinant clones with Hfr state; and (*b*) by transduction of *cmlA* into B11 with P1. Matings to Cm-s F⁻ strains of B11-type Hfr strains carrying *cmlA*, derived by both methods, again gave a chomosomal location for *cmlA*, between *gal* and *try*, and close to *pyrD*. Details of these tests will not be given, since co-transduction studies with P1 later allowed the location of *cmlA* to be determined with greater accuracy.

(iii) *Transduction of* cmlA1 *by P1*

The first attempts to transduce *cmlA* were unsuccessful, apparently because it was assumed that there would be a considerable phenotypic lag in expression of resistance after transfer. The best results were obtained by plating on antibiotic minimal agar immediately after a short treatment of the recipient strain with phage. A number of successful transduction experiments have now been carried out, in which *cmlA 1* was transferred from its original mutant strain back and forth between various strains.

Table 3 gives the details of an experiment in which uracil-independence and Cm-resistance were transferred from J62 *cmlA 1* into strain M25U (*pyrD*, Cm-s). Phage treatment was for 60 min at 37 °C, with an M.O.I. of about 1, and the recipient cells were then spun down and resuspended in buffer for plating. Both genes were transferred with about the same frequency of 1–2/10^5 surviving cells, which is more than $\times 100$ the frequency with which mutations appeared on control plates receiving untreated cells.

Table 3. *Transduction of* pyrD⁺ *and* CmlA *into strain M25U*

Selection for:	*pyr*⁺	Cm-r
Viable cells plated:	3×10^7	$1{\cdot}5 \times 10^7$
Transductants:	308	311
Transduction frequency:	$1{\cdot}0 \times 10^{-5}$	$2{\cdot}1 \times 10^{-5}$
Controls (5×10^7 cells plated):	0	0

Cells of recipient strain M25U (*pyrD his-* Cm-s), grown in broth to about 5×10^8/ml, were infected at M.O.I. of about 1 with PlKc grown on J62 *cmlA 1*. After 1 hr at 37 °C the cells were resuspended in buffer + sodium citrate 0·5 %, and plated on selective plates containing Citrate at 0·5 % to reduce further phage adsorption. Viable counts were made to measure the number of viable cells plated. Selection for Cm-resistance was on supplemented minimal agar containing Cm at 10 μg/ml. Plates were incubated 40 hr to give *pyr*⁺ and 64 hr to give Cm-r transductants.

A Cm-r transductant clone was then purified, and this and the parent M25U Cm-s strains were used as donors for the transfer of Cm-r and *pro*⁺ by P1 to J62 Cm-s. The results of this test are given in Table 4.

P1 grown on both donor strains transferred *pro*⁺ with about equal frequencies,

but Cm-resistance was only transferred at a rate significantly above the control (mutation) level by P1 from the resistant donor, and in this case the frequency of transduction is again about the same for the two characters ($4/10^5$ viable treated cells).

Table 4. *Transduction of* cmlA *and* pro$^+$ *into strain J 62 Cm-s*

Donor strain	M.O.I.	Viable recipient cells plated	Transductants per 10^7 viable cells	
			pro$^+$	*Cm-r*
M25U Cm-s	1·7	12×10^6	270	4
M25U Cm-r	1·3	7×10^6	390	420
None	0·0	2×10^7	0·5*	1·5*

Methods similar to those in Table 3.
* Mutant frequencies on control plates.

These experiments show that the *CmlA* mutation may be consistently transferred from one strain to another by P1, and that it is only transferred by phage grown on a resistant donor strain. The use of 10 μg/ml of Cm in minimal agar containing any required nutritional supplements allowed Cm-r colonies to develop during 3–4 days incubation at 37 °C, and nevertheless gave a very low background mutation frequency of about 1/10 that obtained when cells are plated on antibiotic nutrient agar (Reeve & Bishop, 1965). This suggests that antibiotic minimal agar containing 10 μg/ml Cm prevents most Cm-r mutations except those similar to *cmlA 1* from developing into colonies. A similar discrimination has not so far been found possible on antibiotic nutrient agar.

(*iv*) *Co-transduction of* cmlA 1 *with other genes*

Table 5 summarizes the results of several transduction tests with various stocks, and shows a co-transduction frequency between *cmlA* and λ of about 1·3 % when the recipient strain alone is lysogenic, while *cmlA* is apparently not co-transduced with either *gal* ($<$ 1/800) or *pyrD* ($<$ 1/500). λ prophage has been shown to be attached between *gal* and *biotin* and when λ is absent from the donor strain there is a 47 %

Table 5. *Co-transduction tests on* cmlA 1 *and other genes*

Relevant characters of strains		Type and no. of transductants				
		Selected for		Co-transductions		
Donor	Recipient	Type	No.	Type	No.	%
cmlA gal+	Cm-s *gal*$^-$	*gal*+	520	*cmlA*	0	0
		cmlA	286	*gal*+	0	0
cmlA pyr+	Cm-s *pyrD*	*pyr*+	330	*cmlA*	0	0
		cmlA	200	*pyr*+	0	0
cmlA λ^-	Cm-s $\lambda^+\lambda^r$	*cmlA*	872	λ^-	11	1·26

Each set of data represents the sum of two separate tests. To test for co-transductions, transduced clones on the selection plates were streaked directly on suitable test plates.

frequency of P1 co-transduction between *gal* and *bio* (Rothman, 1965). We can therefore place *CmlA* between *bio* and *pyrD*, the order being *gal*, λ, *bio*, *cmlA*, *pyrD*. Difficulties have been found in getting satisfactory transduction results with *bio*, but it is hoped to establish the *cmlA–bio* co-transduction frequency shortly.

(v) *Phenotypic lag in expression of* cmlA *after transduction*

There are some grounds for believing that chromosomal Cm-r mutations increase resistance to Cm, Tc and Pm by reducing the permeability of the membrane to all these antibiotics (Reeve, 1966). If this is so, we should expect a phenotypic lag of perhaps several generations after transfer of such a gene before full resistance was achieved. Several experiments were made to test this hypothesis by growing cells after phage treatment and plating at intervals to measure the change in transduction frequency per viable cell with time. The largest of these experiments (Table 6) compared the transduction frequencies for *metB*$^+$ and *cmlA* at intervals during 180 min of growth in broth after the recipient cells had been treated with P1 for 20 min and then washed to remove most of the free phage, and resuspended in fresh broth containing 0·5 % sodium citrate to reduce further phage attack.

Table 6. *Phenotypic lag test after transduction of* met$^+$ *and* cmlA

Incubation time (min)	Viable cells per ml	Relative no. of cells	Transductants per 10^8 viable cells plated		
			met$^+$	*cmlA*	*cmlA*/*met*$^+$ (%)
0	$2{\cdot}3 \times 10^8$	—	(0)	(3)	—
20	10^8	1	250	44	18
80	$4{\cdot}6 \times 10^8$	4·6	114	27	24
115	$1{\cdot}6 \times 10^8$ ($\times \frac{1}{5}$)	8	59	28	47
150	$3{\cdot}2 \times 10^8$	16	23	15	65
200	$6{\cdot}3 \times 10^8$	31·5	19	22	116

PlKc grown on J 62 *cmlA 1* was added to B 11 *met*$^-$Cm-s (M.O.I. = 0·3) at time 0. Incubation was at 37 °C. After 20 min the cells were washed twice in buffer + 0·5 % sodium citrate at 5 °C and resuspended in broth + citrate. Incubation at 37 °C was continued and the 20 min sample immediately plated. At 115 min, just before sampling, the culture was diluted 5 times in warm broth + 0·5 % sodium citrate. Cell concentration was checked by optical density readings and viable counts. 0·5 ml or more was plated on selective plates for each character at each sampling time. Incubation time includes the period of phage treatment. Numbers in parentheses at time 0 are the frequencies of mutant colonies on control plates receiving untreated cells.

Phage treatment for 20 min at M.O.I. of about 0·3 reduced the viable count from $2{\cdot}3 \times 10^8$ to 10^8 per ml (evidently the phage had a much higher cell-killing than plaque-forming titre). On reincubation after washing, cell division occurred at a fairly constant rate of about twice per hour until the density became too high for log. growth towards the end of the experiment. During the 3 hr from 20 to 200 min, cell number increased 31·5 times, equivalent to 5 divisions per cell. Over this period the frequency of *met*$^+$ transductants fell steadily from 250 to 19 per 10^8 viable cells, while the corresponding frequency for *cmlA* also declined, but much less

dramatically. In consequence, the relative frequency of *cmlA* to *met*$^+$ transductants rose steadily during post-treatment incubation from 18 % at 20 min to over 100 % 3 hr later. The decline in frequency of *met*$^+$ during incubation must be partly due to the multinucleate state of the recipient cells at the time of infection, since the first cell divisions after infection would mainly separate transduced from untransduced genomes. However, the decline is so striking that some delay in integration of the transduced DNA fragment is probably a contributing factor. *CmlA* has been shown to have very little effect on growth-rate (Reeve, unpublished) and the steady rise in the ratio of *cmlA* to *met*$^+$ transductants during incubation suggests that not all *cmlA* transductants are expressed immediately the gene has been incorporated in the recipient cell's genome—in other words, phenotypic lag expresses itself as an increase in the probability that a transduced cell will be able to form a colony on antibiotic agar as post-treatment incubation continues. The extent of this lag varied in different experiments, probably because the effective Cm concentration in the plates varied from one experiment to another (we have not been able to establish conditions in which a constant amount of agar is put in each plate and a constant amount of water is lost by the plate during drying). One experiment gave a good recovery of *cmlA* transductants when cells were plated on antibiotic agar immediately after 10 min treatment with donor phage at 37 °C, so expression of the transduced gene can be very rapid.

4. DISCUSSION

Our experiments have established that at least one of the mutations for low-level resistance to Cm selected in *E. coli* K12 is a chromosomal gene (*cmlA*), and its location has been fairly accurately determined, the gene order being *gal*, *λ*, *bio*, *cmlA*, *pyrD*. *CmlA* gives a higher resistance to Cm than other mutations so far obtained by selecting on a low level of antibiotic, so that it can be detected on antibiotic plates which allow only a low frequency of mutants to develop. Coupled with its apparently rapid phenotypic expression, this allows the mutant gene to be transferred easily by transduction or conjugation, so that it can be used in genetical analysis. It is not recommended, however, as a selective marker in interrupted mating experiments of a resistant male to a sensitive female, since only a small proportion of resistant recombinants form colonies on Cm-agar. This effect may not be a result of phenotypic lag, and is under further study.

SUMMARY

A chloramphenicol-resistant mutation in *Escherichia coli* K12, *cmlA 1* (previously designated *1a*), giving a higher Cm-resistance than other mutations yet examined, has been shown to have a chromosomal location, the gene order being *gal*, *λ*, *bio*, *cmlA*, *pyrD*. *CmlA* can be transduced efficiently into cm-sensitive strains by P1 with little phenotypic lag, and is co-transduced with the *λ*-attachment site (frequency 1·3 %) but not with *gal* or *pyrD*.

REFERENCES

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

Anderson, E. S. & Lewis, M. J. (1965*a*). Drug resistance and its transfer in *Salmonella typhimurium*. *Nature, Lond.* **206**, 579–583.

Anderson, E. S. & Lewis, M. J. (1965*b*). Characterization of a transfer factor associated with drug resistance in *Salmonella typhimurium*. *Nature, Lond.* **208**, 843–849

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

Demerec, M., Adelberg, E. A., Clark, A. J. & Hartman, P. E. (1966). A proposal for a uniform nomenclature in bacterial genetics. *Genetics* **54**, 61–76.

Lennox, E. S. (1955). Transduction of linked genetic characters of the host by bacteriophage P 1. *Virology* **1**, 190–206.

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 *Escherichica coli* K 12. *Genet. Res.* **6**, 310–315.

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

Rothman, June L. (1965). Transduction studies on the relation between prophage and host chromosome. *J. molec. Biol.* **12**, 892–912.

Watanabe, T. (1963). Infective heredity of multiple drug resistance in bacteria. *Bact. Rev.* **27**, 87–114.