A Chromosomal Gene for Chloramphenicol Acetyltransferase in *Streptomyces acrimycini*

By HELEN M. WRIGHT AND DAVID A. HOPWOOD

John Innes Institute, Colney Lane, Norwich NR4 7UH

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INTRODUCTION

Plasmid-borne genes determining antibiotic resistance in eubacteria may have originated in the organisms that produce the antibiotics, notably the streptomycetes (Benveniste & Davies, 1973; Courvalin, Weisblum & Davies, 1977). Although this theory does not require the corresponding genes to be permanently plasmid-linked in their streptomycete hosts, the normal carriage of such genes on plasmids might have favoured their dissemination from such hosts to other bacteria. Of the antibiotic-inactivating enzymes coded by eubacterial plasmids, those catalysing acetylation or phosphorylation of aminoglycosides or acetylation of chloramphenicol are found in various streptomycetes (Benveniste & Davies, 1973; El-Kersch & Plourde, 1976*a*; Shaw & Hopwood, 1976; Nakano *et al.*, 1977). However, nothing appears to be known in these organisms about the genetic determination of these enzymes because they have not been studied in genetically characterized strains. We set out to answer this question for a streptomycete producing chloramphenicol acetyltransferase (CAT).

The genetically well-known *Streptomyces coelicolor* A3(2) is somewhat more resistant to chloramphenicol than most other strains tested, and gives rise to chloramphenicol-sensitive variants at a high frequency, but these do not arise by plasmid loss; nor does absence of CAT explain their phenotype since the parent strain lacks this enzyme (Freeman, Bibb & Hopwood, 1977). In order to study the genetic determination of CAT in a streptomycete, we therefore had to choose one of three genetically uninvestigated species which possess the enzyme (Shaw & Hopwood, 1976). We selected *Streptomyces acrimycini* IPV1610 because of its added interest in yielding a mutant with greatly increased CAT production (Shaw & Hopwood, 1976); this mutant has allowed the CAT produced by the organism to be compared with those of eubacteria, including those determined by plasmids (Zaidenzaig & Shaw, 1977).

METHODS

Media, strains and standard genetical procedures. Complete (CM) and minimal (MM) media and supplementation of MM with growth factors or streptomycin were as described by Hopwood (1967). Soft nutrient agar was Oxoid Nutrient Agar diluted with an equal volume of Oxoid Nutrient Broth. The starting strain was *S. acrimycini* IPV1610 (John Innes stock no. 1201). Auxotrophic and streptomycin-resistant mutants of this strain, isolated during the work, are referred to in the Results. *Escherichia coli* was strain J53 of *E. coli* K12. Incubation of *Streptomyces* cultures was at 30 °C and of *E. coli* at 37 °C. Standard cultural and crossing techniques were those described by Hopwood (1967) and Hopwood *et al.* (1973) for *S. coelicolor*.

Mutagenesis and mutant isolation. Treatments with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (Delić, Hopwood & Friend, 1970) and with ultraviolet light (u.v.) (Harold & Hopwood, 1970) were by standard procedures, as were methods for the isolation of auxotrophic and streptomycin-resistant mutants. Isolation of putative CAT⁻ mutants was attempted by two methods which depended, respectively, on hypotheses that they would, or would not, be more sensitive to chloramphenicol than the starting strain, a proline auxotroph of strain 1201. In the first method, mutagenized spore suspensions were plated on CM to give about 100 colonies per plate. These were replica-plated to MM supplemented with proline and containing 10 µg

chloramphenicol ml⁻¹. Colonies that failed to replicate, representing potential chloramphenicol-sensitive and auxotrophic mutants, were picked to CM and re-tested by replica-plating to MM supplemented with proline, with and without chloramphenicol. The chloramphenicol-sensitive variants were tested for their ability to inactivate chloramphenicol. They were inoculated (20 per plate) on medium lacking chloramphenicol, a plug (7 mm diam.) was cut from each culture, and the plugs were embedded (20 per dish) in MM containing 10 µg chloramphenicol ml⁻¹. After 24 h incubation the plates were overlaid with 5 ml soft nutrient agar containing *E. coli* 153, which was sensitive to chloramphenicol at 2 to 3 µg ml⁻¹, and incubated overnight. Each CAT-containing culture was surrounded by a turbid zone in which destruction of chloramphenicol had allowed the growth of *E. coli*. In the second method, colonies developing on CM from mutagenized spore suspensions were picked (50 per plate) to supplemented MM containing 15 µg chloramphenicol ml⁻¹ and incubated for 24 to 36 h. The plates were overlaid with 5 ml soft nutrient agar containing *E. coli* and incubated overnight. Each CAT-containing culture was covered by a turbid zone of bacterial growth.

Determination of chloramphenicol acetyltransferase. This was done by the autoradiographic procedure of Shaw & Hopwood (1976).

RESULTS

Isolation and characteristics of a CAT⁻ mutant

Since the minimum inhibitory concentration of chloramphenicol in agar for *S. acrimycini* 1201 is about 10 µg ml⁻¹, a concentration tolerated by most of the other wild types tested including those which lacked CAT (Shaw & Hopwood, 1976), we could not predict whether or not a CAT⁻ mutant would be more sensitive to chloramphenicol than the parent strain. We therefore attempted to isolate such mutants by procedures which depended on their being sensitive, or resistant, respectively to the concentration of the antibiotic tolerated by the parent strain.

In the first method, 775 survivors of u.v. treatment yielded 24 (4 %) chloramphenicol-sensitive variants and seven (1 %) auxotrophs. After NTG treatment, 7455 colonies yielded 402 (5.4 %) chloramphenicol-sensitive variants and 172 (2.3 %) auxotrophs. Out of the 426 chloramphenicol-sensitive variants, one (from the NTG treatment) failed to inactivate chloramphenicol. This single putative CAT⁻ mutant (strain 2498), which retained the proline marker of the parent strain, showed no detectable CAT activity when tested by the autoradiographic method. Its sensitivity to chloramphenicol was similar to that of the majority of chloramphenicol-sensitive but CAT⁺ variants, tolerating 1 to 2 µg ml⁻¹. The chloramphenicol-sensitive variants that arose at high frequency by a mechanism not involving loss of CAT were not studied further. In the second method of mutant isolation, 7150 survivors of u.v. treatment yielded no presumptive CAT⁻ mutants.

Tests of reversion to chloramphenicol resistance were done on a serine-requiring mutant (strain 2522) of strain 2498. Spore suspensions treated with NTG yielded colonies at a frequency of about 10⁻⁶ on 10 µg chloramphenicol ml⁻¹. Two such putative revertants, which retained the proline and serine markers, were shown by the autoradiographic method to be CAT⁺.

Genetic analysis of the CAT⁻ mutation

Recombination in S. acrimycini. Recombination was demonstrated in crosses between double auxotrophic derivatives of strain 1201. These four-factor crosses were analysed by the standard procedure of plating on four selective media and characterizing samples of colonies on each medium for the segregation of non-selected markers (Hopwood, 1972). Unambiguous evidence of recombination was provided by the isolation of genotypes combining auxotrophic markers from both parents. Recombinants occurred at rather constant frequencies of a little less than 10⁻⁶. In addition to haploid recombinants, heterokaryons occurred at frequencies representing 2 to 80 % of the total colonies arising on selective media, depending on the markers used for counter-selection. They were readily distinguished from haploid recombinants by their failure to be transferred by replica-plating to a medium of the same composition as that on which they originally developed, and by their giving rise exclusively to parental genotypes. This situation corresponded to that in *S. rimosus*

Fig. 1. Results of a cross between CAT⁻ strain 2564 (outer circle) and CAT⁺ strain 2326 (inner circle). Recombinants inheriting *his*-1⁺ and *str*-1 (indicated by triangles) were selected and classified for the non-selected markers. Allele ratios indicate possible map positions for CAT between *his*-1 and *aur*-1 or between *his*-1 and *leu*-1. Tests of independence between the segregation of CAT^{+/-} and either *aur* or *leu* indicate a position for CAT between *his*-1 and *aur*-1: segregation of CAT^{+/-} is independent of that of *leu* ($\chi^2_1 = 2.97$), but strongly dependent on that of *aur* ($\chi^2_1 = 54.4$). The individual genotype frequencies are in Table 1.

Table 1. Progeny of the cross shown in Fig. 1

CAT ⁺		CAT ⁻	
Genotype*	Number	Genotype*	Number
<i>aur leu ser ath str</i>	37	<i>ser ath str</i>	4
<i>aur leu str</i>	31	<i>leu ser ath str</i>	3
<i>aur ser ath str</i>	27	<i>aur ser ath str</i>	2
<i>aur leu ath str</i>	7	<i>leu str</i>	2
<i>aur str</i>	5	<i>str</i>	1
<i>leu ser ath str</i>	2		
<i>aur ser str</i>	2		
<i>aur ath str</i>	2		
<i>str</i>	2		
<i>aur leu ser str</i>	1		
<i>ser ath str</i>	1		
<i>leu ath str</i>	1		
<i>leu ser str</i>	1		
<i>ser str</i>	1		

* Wild-type alleles omitted.

(Friend & Hopwood, 1971) and *S. glaucescens* (Baumann, Hütter & Hopwood, 1974). When one parent in a cross carried a mutation conferring streptomycin resistance, heterokaryons were largely abolished on selective media containing streptomycin, as expected since streptomycin resistance is usually recessive.

Analysis of the patterns of marker segregation amongst haploid recombinants allowed the construction of a preliminary circular linkage map carrying six markers.

Crosses involving the CAT⁻ mutation. In the first crosses between CAT⁺ and CAT⁻ strains, four heterokaryons were allowed to segregate the two parental genotypes and

samples of several hundred of each were tested for the CAT phenotype. There was no reassortment of CAT phenotype with chromosomal genotype, even when the genotype corresponding to the CAT⁺ parent represented the majority of segregants from a particular heterokaryon. This result suggested chromosomal rather than plasmid linkage of the CAT⁻ mutation. However, it must be remembered that little is known about the behaviour of plasmids in *Streptomyces* heterokaryons. In *S. scabies* the finding that nearly all the segregants from heterokaryons between melanin-positive and melanin-negative strains were melanin-positive provided presumptive evidence for plasmid determination of melanin production (Gregory & Huang, 1964), but unfortunately this remains the only evidence for such a plasmid in their strain.

More direct evidence for chromosomal linkage of the CAT⁻ mutation came from the analysis of haploid recombinants selected from crosses between CAT⁺ and CAT⁻ strains. There was invariably a segregation of CAT⁺ versus CAT⁻ phenotype. The results of several crosses, in which the CAT⁻ mutation was studied in various combinations with other markers, were completely compatible with a position for the CAT locus between *his-1* and *aur-1* (arginine plus uracil) on the circular chromosomal linkage group. An example of such a cross is shown in Fig. 1 and Table 1.

DISCUSSION

The evidence that CAT in *S. acrimycini* IPV1610 is coded by a chromosomal gene is very strong. Variants lacking the activity did not arise with a high frequency such as might have reflected plasmid loss (only one, revertible, mutant occurred out of 8230 survivors of mutagenesis); there was no re-assortment of CAT phenotype with chromosomal genotypes in heterokaryons between CAT⁺ and CAT⁻ strains; and segregation of the CAT⁻ phenotype amongst haploid recombinants from such crosses was that expected for a mutation in a chromosomal gene, which was mapped to the chromosomal linkage group. Further wild-type strains would, of course, have to be studied genetically in order to generalize this conclusion to other examples of antibiotic-inactivating enzymes in streptomycetes. In the strain of *S. griseus* studied by El-Kersch & Plourde (1976*b*) some slight evidence for chromosomal determination of CAT was provided by the failure to induce CAT⁻ variants by treatment with acriflavine or ethidium bromide.

A further interesting finding in the present study was that variants which were 10-fold more sensitive than the starting strain, but still possessing CAT, arose with frequencies of 4 to 5 % after u.v. or NTG treatment, and about 1 % spontaneously. The induced frequency was two- to four-fold higher than that of total auxotrophs, so a general high mutability of the strain does not explain the results. These chloramphenicol-sensitive variants may correspond to those that occur in *S. coelicolor* A3(2), a strain that lacks CAT, in which they were shown not to arise by plasmid loss, but they nevertheless failed to map to the chromosome (Freeman *et al.*, 1977). In *S. acrimycini*, genetic analysis was not refined sufficiently for a serious genetical investigation of these variants. The physiological basis of their chloramphenicol sensitivity is also unknown. It is interesting that, in *S. acrimycini*, the resistance shown by the wild type apparently depends on the simultaneous operation of two factors, a functional CAT enzyme and the wild-type state of the second, unknown, mechanism; in contrast, *S. coelicolor* achieves a higher resistance than that shown by *S. acrimycini* in the absence of CAT.

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