Chloramphenicol Acetyltransferase-independent Chloramphenicol Resistance in *Streptomyces coelicolor* A3(2)

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SUMMARY

Streptomyces coelicolor A3(2) and *S. lividans* 66, which lack chloramphenicol acetyltransferase, gave rise to chloramphenicol-sensitive (Cml^s) variants spontaneously at frequencies of 0.5 to 2 %. The fertility type of *S. coelicolor* in respect of the SCP1 plasmid (SCP1⁺, SCP1⁻ or NF) had no effect on chloramphenicol sensitivity or on the frequency at which Cml^s variants arose.

Cml^s isolates spontaneously reverted to Cml^R at frequencies one to three orders of magnitude lower than the frequency with which Cml^s strains arose from Cml^R. Cml^R revertants obtained spontaneously from Cml^s clones again produced Cml^s isolates at the normal frequency of several per cent. Therefore, Cml^s and Cml^R are reversible phenotypes.

In crosses between marked Cml^R and Cml^s *S. coelicolor* strains, transfer of chloramphenicol resistance into the sensitive strain apparently occurred independently of chromosomal recombination. Mapping experiments excluded the possibility that segregation of a chromosomal locus determines Cml^R versus Cml^s phenotype. In crosses between SCP1⁻ strains, fertility was not significantly different in Cml^R × Cml^s, Cml^R × Cml^R and Cml^s × Cml^s combinations.

Covalently closed circular DNA from Cml^s and Cml^R strains of *S. coelicolor* was indistinguishable in molecular weight and restriction endonuclease cleavage pattern.

It is suggested that chloramphenicol resistance in *S. coelicolor* A3(2) is affected by some kind of transposable genetic element that may be capable of extra-chromosomal existence.

INTRODUCTION

The production of antibiotics and resistance to them in streptomycetes is sometimes controlled by plasmids. The SCP1 plasmid of *Streptomyces coelicolor* A3(2) determines resistance to methylenomycin A, as well as its production (Vivian, 1971; Kirby, Wright & Hopwood, 1975; Wright & Hopwood, 1976), and a plasmid is involved in resistance to oxytetracycline in *Streptomyces rimosus* (Boronin & Sadovnikova, 1972). Since plasmid-determined resistance to antibiotics in streptomycetes is relevant to the theory that the resistance genes now occurring on R factors in the Enterobacteriaceae might have originated in the antibiotic-producing organisms (Benveniste & Davies, 1973), we set out to identify other possible examples of extra-chromosomal determination of antibiotic resistance in the genus *Streptomyces*, whose members produce over 60 % of known antibiotics (Bérdy, 1974). One approach was to test the intrinsic resistance of a collection of wild-type streptomycetes to a variety of antibiotics (Freeman, 1976), and to select as potential candidates for more detailed investigation those strains that were more resistant to a particular antibiotic than were the majority of the collection.

Another approach was to test for the occurrence of known antibiotic-inactivating enzymes in streptomycetes and to study the genetics of their production in appropriate strains,

Shaw & Hopwood (1976) found the enzyme chloramphenicol acetyltransferase in several streptomycetes and its genetic determination is being studied in one of them, *Streptomyces acrimycini* (D. A. Hopwood & H. M. Wright, unpublished results). *Streptomyces coelicolor* A3(2), the strain in which extensive genetical investigations have been carried out (Hopwood *et al.* 1973), does not have the acetylating enzyme. However, it was the most chloramphenicol-resistant strain studied by Shaw & Hopwood (1976), except for *Streptomyces venezuelae*, the chloramphenicol producer. Thus the two approaches converged on *S. coelicolor* A3(2). The present paper shows that chloramphenicol resistance is indeed influenced by some kind of extra-chromosomal element, perhaps of a novel kind, in this strain. A preliminary report of these results has been made (Freeman, Bibb & Hopwood, 1976) and we have also become aware of an independent investigation which seems to be in part similar to our own (G. Sermoni, personal communication).

METHODS

Strains. The cultures were derivatives of *S. coelicolor* A3(2) and *S. lividans* 66 (Lomovskaya *et al.*, 1972); they are listed in Table 1.

Cultural and genetic methods. Minimal (MM) and complete (CM) media and general cultural and genetic techniques were those described by Hopwood (1967). Ultraviolet (u.v.) irradiation was done as described by Harold & Hopwood (1970); survival was about 1 %.

Isolation of chloramphenicol-sensitive strains. *Streptomyces coelicolor* A3(2) colonies grew almost without inhibition when replicated to supplemented MM containing 16 µg chloramphenicol ml⁻¹ (Cambrian Chemicals, Croydon). *Streptomyces lividans* 66 replicated satisfactorily on to media containing 8 µg chloramphenicol ml⁻¹. Sensitive isolates (Cml^S) were obtained, both spontaneously and after u.v. irradiation, by plating on suitably supplemented MM at a dilution to give well separated colonies. These were replicated when they were well sporulated to supplemented MM plates containing the appropriate concentration of chloramphenicol. Colonies that failed to replicate were streaked and re-tested; most were confirmed as Cml^S.

Selection for chloramphenicol-resistant isolates. Chloramphenicol-resistant (Cml^R) spores were selected from an excess of Cml^S by plating on supplemented MM containing 8 µg chloramphenicol ml⁻¹. Cml^R spores produced discrete colonies after 3 to 4 days incubation, whereas the Cml^S spores were largely inhibited.

Antibiotic-sensitivity spectrum. Single colonies were used to inoculate 25 ml medium B (Okanishi, Ohta & Umezawa, 1970) in 250 ml conical flasks, each containing a coiled stainless-steel spring to aid growth dispersal and aeration. The cultures were grown on an orbital shaker at 30 °C for 40 h. The mycelium was allowed to settle and 1.5 ml was cooled in ice and sonicated for 15 min (in 15 s bursts) at setting 3 on a Soniprobe with a microtip (type 7530A; Dawe Instruments, London). The supernatant from this preparation (0.1 ml) was spread on plates of supplemented MM (25 ml), in which glycerol (1 %, v/v) was substituted for glucose as carbon source, and dried in a horizontal laminar-flow cabinet (Slee, London) to remove surface moisture. Each plate then received three antibiotic test discs (Mast Laboratories, Liverpool), spaced equidistantly, and was incubated for 2 days before the diameters of the inhibition zones were measured.

Isolation and characterization of covalently closed circular (c.c.c.) DNA. ³H-labelled c.c.c. DNA was isolated by dye-buoyant centrifugation from mycelium grown in a Casamino acids/glycine medium supplemented with [³H]thymidine essentially by the method of Schrepf *et al.* (1975) and Bibb, Freeman & Hopwood (unpublished). The labelled plasmid

Table 1. *Strains of S. coelicolor* A3(2) and *S. lividans* 66

Strain no.	Genotype	Fertility type	Cml phenotype
<i>S. coelicolor</i>			
A3(2)	Wild type	SCP1 ⁺	R
A3(2)/1*	Wild type	SCP1 ⁺	S
104	<i>hisA1 uraA1 strA1</i>	SCP1 ⁺	R
A332	<i>pheA1</i>	NF	R
A332/1*	<i>pheA1</i>	NF	S
A332/3*	<i>pheA1</i>	NF	S
A332/5*	<i>pheA1</i>	NF	S
A700	<i>proA1 argA1 cysD18</i>	SCP1 ⁻	R
A700/6*	<i>proA1 argA1 cysD18</i>	SCP1 ⁻	S
1098	<i>pheA1</i>	SCP1 ⁻	R
1098/1*	<i>pheA1</i>	SCP1 ⁻	S
1098/3*	<i>pheA1</i>	SCP1 ⁻	S
1190	<i>hisA1 uraA1 strA1</i>	SCP1 ⁻	R
1190/1*	<i>hisA1 uraA1 strA1</i>	SCP1 ⁻	S
1190/2*	<i>hisA1 uraA1 strA1</i>	SCP1 ⁻	S
1190/4*	<i>hisA1 uraA1 strA1</i>	SCP1 ⁻	S
1258	<i>proA1 argA1 hisC9</i>	NF	R
	<i>cysD18 uraA1 strA1</i>		
2552†	<i>cysD18 strA1</i>	NF	S
<i>S. lividans</i> 66			
1697	<i>cys-3</i>	—	R
1697/1*	<i>cys-3</i>	—	S

* Cml^s isolates obtained in this study after u.v. irradiation.

† Recombinant from cross 1258 × A332/5.

DNA was characterized by sedimentation in 5 to 20 % neutral and alkaline sucrose gradients, five-drop fractions being collected from the bottom of the tube and assayed for trichloroacetic acid precipitable radioactivity. Labelled ColE1 DNA was used as a standard, having sedimentation values of 23S and 48S in neutral and alkaline conditions respectively. Unlabelled c.c.c. DNA was prepared by a large-scale method (unpublished) using PEG 6000 precipitation (Humphreys, Willshaw & Anderson, 1975). The unlabelled plasmid DNA was characterized by digestion with SalI restriction endonuclease prepared by the method of Chater & Wilde (1976) and the digestion products were analysed by 1.4 % agarose gel electrophoresis (unpublished). A HindIII digest of phage λ DNA was used as a standard. The SalI endonuclease and phage λ DNA were kindly donated by Dr K. F. Chater.

RESULTS

Isolation and reversion of Cml^s clones

Frequency of occurrence of Cml^s isolates. *Streptomyces coelicolor* A3(2) and *S. lividans* 66 tolerate up to about 20 and 10 µg chloramphenicol ml⁻¹ respectively in MM (Shaw & Hopwood, 1976). We chose 16 and 8 µg ml⁻¹ respectively as concentrations on which to seek sensitive derivatives. Both strains yielded derivatives at least 10-fold more sensitive (Cml^s) than the parent. In the case of *S. coelicolor* A3(2), similar frequencies were observed for SCP1⁺, NF and SCP1⁻ derivatives. The spontaneous frequency was usually about 0.5 %, and this usually increased to up to 5 % after u.v. irradiation (Table 2a). Many of the Cml^s variants resembled the Cml^s parent cultures in appearance, but some sporulated less well than the parent.

Table 2. *Isolation of chloramphenicol-sensitive (Cml^S) and chloramphenicol-resistant (Cml^R) clones*(a) *Isolation of Cml^S strains from Cml^R parents*

Strain	Treatment	Total no. of colonies	Cml ^S isolates (%)
<i>S. coelicolor</i>			
A3(2)	Control	687	0.4
	u.v.	3000*	0.2
A332	Control	711	2.1
	u.v.	1721	4.4
1098	Control	2066	0.5
	u.v.	3475	2.6
1190	Control	40	< 0.4
	u.v.	287	4.5
<i>S. lividans</i>			
1697	Control	169	< 0.6
	u.v.	638	5.2
	Control	507	0.4
	u.v.	902	1.4

* Crowded plates.

(b) *Isolation of Cml^R revertants from Cml^S strains*

Strain	Viable count ml ⁻¹		Cml ^R /Cml ^S
	Total	With chloramphenicol	
<i>S. coelicolor</i>			
1190/1	7.5 × 10 ⁶	9 × 10 ¹	1.2 × 10 ⁻⁵
1190/1	6.9 × 10 ⁶	2.5 × 10 ³	3.5 × 10 ⁻⁴
1190/2	6.6 × 10 ⁶	1.2 × 10 ²	1.8 × 10 ⁻⁵
1190/4	1.7 × 10 ⁶	1 × 10 ¹	6 × 10 ⁻⁶
A332/I	8.8 × 10 ⁵	< 1 × 10 ¹	< 1 × 10 ⁻⁵
A332/I	1.2 × 10 ⁸	5.7 × 10 ²	4.7 × 10 ⁻⁶
<i>S. lividans</i>			
1697/I	8.0 × 10 ⁶	1 × 10 ¹	1 × 10 ⁻⁶
	3.0 × 10 ⁶	< 1 × 10 ¹	< 3 × 10 ⁻⁶
	6.0 × 10 ⁷	5 × 10 ¹	8 × 10 ⁻⁷

(c) *Isolation of Cml^S strains from Cml^R revertants*

Strain	Treatment	Total no. of colonies	Cml ^S isolates (%)
<i>S. coelicolor</i>			
1190/1-R6	Control	612	3.6
	u.v.	650	3.7
1190/1-R8	Control	521	5.0
	u.v.	941	3.1

Isolation of Cml^R revertants from Cml^S strains. Cml^S isolates reverted spontaneously to their original Cml^R phenotype, but at a lower frequency than that of the origin of Cml^R from Cml^R—between one and three orders of magnitude lower for *S. coelicolor* and three to four orders of magnitude lower for *S. lividans* (Table 2b).

Re-isolation of Cml^S strains from Cml^R revertants. *Streptomyces coelicolor* Cml^R revertants, obtained spontaneously from Cml^S isolates, were tested for their ability to give rise again to the Cml^S phenotype. Three Cml^R revertants from two SCP1⁻ Cml^S isolates were

Table 3. Sensitivity of *S. coelicolor* Cml^R and Cml^S strains to various antibiotics and antimicrobial drugs

Results are given as diameters (mm) of zones of inhibition after 2 days incubation

Antimicrobial agent	Strain ...	Cml phenotype ...	SCP1 ⁺			NF			SCP1 ⁻		
			A3(2)	A3(2)/1		A332	A332/1	A332/3	1098	1098/1	1098/3
			R	S		R	S	S	R	S	S
	Concn (µg/disc)										
Streptomycin	10		40	c. 40		38	34	34	40	c. 44	40
Gentamicin	2		20	c. 28		21	22	22	23	45	26
Kanamycin	30		46	c. 50		44	40	40	44	44	41
Erythromycin	5		18	c. 15		c. 14	16	16	17	15	NT
Neomycin	10		20	26		20	19	23	22	23	26
Novobiocin	5		R	R		R	R	R	R	R	R
Penicillin G	4		R	R		R	R	R	R	R	R
Ampicillin	10		R	R		R	R	R	R	R	R
Cephaloridine	25		R	R		R	R	R	R	R	R
Tetracycline	25		R	R		R	R	R	R	R	R
Sulphafurazole	200		c. 44	c. 45		45	c. 44	45	c. 40	c. 44	c. 40
Bacitracin	8		c. 13	c. 15		NT	16	16	c. 11	14	c. 12
Colistin SO ₄	25		15	18		17	18	16	19	16	16
Nalidixic acid	30		R	R		R	R	R	R	R	R
Trimethoprim	2.5		R	R		R	R	R	R	R	R
Polymyxin B	100		14	14		12	14	13	14	15	13
Rifamycin	30		R	R		R	R	R	R	R	R
Chloramphenicol	25		c. 11	c. 29		c. 9	31	31	c. 10	c. 20	c. 20

R, Resistant: no discernible zone. NT, Not tested.

studied (Table 2c). All three revertants produced Cml^S isolates at a frequency of up to 5% of the total population. The morphology of these Cml^S strains was indistinguishable from that of Cml^S strains obtained from the original Cml^R parents.

Test for occurrence of additional nutritional requirements in Cml^R revertants

To test the possibility that mutations elsewhere in the genome might have been induced on reversion of Cml^S to Cml^R as in the case of excision and re-insertion of known transposable elements (Kleckner *et al.*, 1975), auxotrophic mutations were sought by plating spores from strain 1190/1 on CM containing 8 µg chloramphenicol ml⁻¹ and replicating the resulting Cml^R revertants to MM supplemented with histidine and uracil, the nutritional requirements of strain 1190/1. Of the 1186 Cml^R revertants tested, none displayed any additional nutritional requirement.

*Sensitivity spectrum of *S. coelicolor* Cml^S and Cml^R phenotypes to antibiotics and other antimicrobial agents*

The sensitivity spectra of one Cml^S isolate from SCP1⁺ strain A3(2), two from NF strain A332 and two from SCP1⁻ strain 1098 were examined. There were no significant differences in sensitivity of Cml^R and Cml^S strains to any of the antimicrobial agents tested, except for chloramphenicol (Table 3).

Transfer of the Cml^R character, and fertility of crosses involving Cml^S variants

Crosses were made between Cml^S SCP1⁻ strains and either Cml^R SCP1⁻ or Cml^R SCP1⁺ strains to measure the transfer frequency of the Cml^R character into Cml^S genotypes and

Table 4. *Transfer of the Cml^R character and fertility of crosses of different Cml status*

Parents		(1) Cml ^R 'recipients'*	(2) Cml ^R 'recipients'*	(3)	(4)
		Cml ^S parentals	Recombinants	Recombinants Cml ^S parentals	Recombinants Cml ^R parentals
Cml ^R SCP1 ⁻ × Cml ^S SCP1 ⁻					
1190	A700/6	9.5×10^{-4}	2.7×10^3	3.7×10^{-7}	5.9×10^{-7}
1190	1098/3	7.3×10^{-5}	4.4×10^0	1.7×10^{-5}	1.3×10^{-7}
A700	1190/1	5.6×10^{-6}	2.0×10^{-1}	2.8×10^{-5}	9.1×10^{-7}
Cml ^R SCP1 ⁻ × Cml ^R SCP1 ⁻					
1190	A700	—	—	—	6.3×10^{-8} 8.3×10^{-8}
1190	A700	—	—	—	1.3×10^{-5} 5.0×10^{-8}
1190	1098	—	—	—	9.5×10^{-6} 2.9×10^{-7}
Cml ^S SCP1 ⁻ × Cml ^S SCP1 ⁻					
1190/4	A700/6	9.0×10^{-6}	3.9×10^1	2.5×10^{-7} 2.1×10^{-7}	—
1190/1	1098/3	2.0×10^{-6}	2.0×10^{-1}	1.0×10^{-5} 5.1×10^{-4}	—
Cml ^R SCP1 ⁺ × Cml ^S SCP1 ⁻					
104	1098/1	4.4×10^{-2}	2.7×10^0	1.7×10^{-2}	9.2×10^{-6}
104	1098/3	3.5×10^{-3}	8.6×10^{-1}	4.1×10^{-3}	2.0×10^{-5}

* Or 'revertants' in the case of Cml^S × Cml^S crosses.

to compare it with the chromosomal recombination frequency (fertility) in order to detect any 'infectious' transfer of Cml^R independent of chromosomal markers. The fertility of such crosses was also compared with those of Cml^S SCP1⁻ × Cml^S SCP1⁻ and Cml^R SCP1⁻ × Cml^R SCP1⁻ crosses to detect any influence on fertility of Cml^R versus Cml^S status. The crosses were plated on selective media to recover each parental phenotype and on one or two media selecting recombinants. In addition, crosses involving Cml^S strains were plated to recover a Cml^R version of the Cml^S parental phenotype(s): Cml^R 'recipients', or 'revertants' in the case of Cml^S × Cml^S crosses. Four ratios were calculated (Table 4): (1) Cml^R 'recipients' or 'revertants'/Cml^S parentals (an average of the parents when both were Cml^S); (2) Cml^R 'recipients' or 'revertants'/chromosomal recombinants; (3) chromosomal recombinants/Cml^S parentals; (4) chromosomal recombinants/Cml^R parentals. Calculation (1) measured transfer of Cml^R, (2) compared it with fertility and (3) and (4) measured fertility. The recombination frequencies for different selections in the same cross differed by a factor of only two or three and a single selection, for His⁺ Str^R throughout, is reported.

The main problem in evaluating the data concerns the varying relative yields of the two parental phenotypes from the same cross. Ideally, only well 'balanced' crosses would be considered, and examples of these could be found provided that only SCP1⁻ strains were used [compare columns (3) and (4), or the two figures in one of these columns, in Table 4]. However, in Cml^R SCP1⁺ × Cml^S SCP1⁻ crosses, the Cml^S parental phenotype was always in a small minority and considerable imbalance was also often encountered in SCP1⁻ × SCP1⁻ crosses, even between pairs of Cml^R strains.

It is apparent from column (1) in Table 4 that there is no large-scale 'infection' of the Cml^S parent by Cml^R in SCP1⁻ × SCP1⁻ crosses: transfer frequencies of Cml^R were in the order of 10^{-3} to 10^{-5} . In the unbalanced SCP1⁺ × SCP1⁻ crosses, they were higher: up to 4 %. As seen in column (2), the ratio of Cml^R transfer to recombination was highly variable, ranging from 2700 to 0.2. The fertility of crosses was also variable [columns (3) and (4)] but careful consideration of fertility in relation to the balance of the parental phenotypes suggest that in SCP1⁻ × SCP1⁻ crosses the Cml status had no significant influence on fertility, Cml^R × Cml^S, Cml^R × Cml^R and Cml^S × Cml^S crosses having similar ranges of

Fig. 1. Results of a cross between Cml^R strain 1258 (outer circle) and Cml^S strain A3(2)/1 (inner circle). Recombinants inheriting *hisC*⁺ and *strA*1 (indicated by triangles) were selected and classified for non-selected markers. Allele ratios indicate possible map positions for Cml between *cysD* and *strA* or between *strA* and *uraA*. Tests of independence between the segregation of Cml and either *ura* or *cys* exclude both map positions.

fertility values. The highest apparent fertility was seen in unbalanced crosses when recombinants were expressed as a proportion of the minority parent.

Attempts to map the Cml^S character

Crosses were made between multiply marked NF strain 1258 (*proA*1 *hisC*9 *argA*1 *cysD*18 *uraA*1 *strA*1) and a series of Cml^S derivatives of A3(2) and A332, with selection for the diametrically opposed markers *hisC*⁺ and *strA*1. This experimental design is efficient for preliminary mapping (Hopwood & Chater, 1974). The allele ratio for the unknown marker (in this case Cml) is considered in relation to those of the known non-selected markers, to give two alternative map positions for Cml. These are evaluated by tabulating the segregation of Cml^R versus Cml^S in relation to that of the non-selected marker that would be closest to Cml in each of its possible positions. For all chromosomal markers that have been studied to date in *S. coelicolor* this procedure has yielded a map position which was compatible with the data and which was confirmed in subsequent crosses. In the case of the Cml^S variants, however, the segregation data were incompatible with a map location.

An example from a series of crosses of strain 1258 with four different Cml^S derivatives of A3(2) is shown in Fig. 1. On allele ratios, Cml could have mapped in either of the intervals defined by *cysD*, *strA* and *uraA*. With a location in the interval *strA*–*cysD* the Cml^S *cysD*18 class should have been rare, as a multiple crossover class; for the interval *strA*–*uraA*, Cml^S *uraA*1 should have been rare. In fact, half the Cml^S segregants carried *cysD*18 and the same proportion carried *uraA*1. Thus neither map position was compatible with the data.

Five different Cml^S derivatives of A332 were crossed with strain 1258; an example is shown in Fig. 2(a). In each cross, only 1 to 5 % of recombinants were Cml^S; this result alone

Fig. 2. Results of crosses between: (a), (b) strain 1258 (*Cml^R*) and strain A332/5 (*Cml^S*); (c) strain A332 (*Cml^R*) and strain 2552 (*Cml^S*). See legend to Fig. 1 for explanation.

could have indicated a map location close to *strA1*, and the small proportion of *Cml^S* progeny made it difficult to exclude a position in either of the two intervals defined by *cysD*, *strA* and *uraA*. Therefore further selections from the same crosses, and further crosses involving *Cml^S* recombinants from the first crosses, were made. Pairs of crosses with *pheA⁺ cysD⁺* selection and reversed coupling of *Cml^S*/*Cml^R* excluded a unique map location since each cross was incompatible with the location indicated by the other: the data of Fig. 2(b) indicate close linkage with *pheA* and exclude close linkage with *cysD*, while in Fig. 2(c) the indications are reversed.

Fig. 3. Sedimentation profiles of [^3H]thymidine-labelled DNA isolated from strains 1098/3 (*a, b*) and 1098 (*c, d*) in 5 to 20 % sucrose gradients. The neutral gradients (*a, c*) contained 0.03 M-Tris, 0.005 M-EDTA and 0.05 M-NaCl, and were centrifuged for 45 min at 45 000 rev. min $^{-1}$ at 20 °C in an SW50.1 rotor in a Beckman L2-65B centrifuge. The alkaline gradients (*b, d*) contained in addition 0.2 M-NaOH and 0.7 M-NaCl and were centrifuged for 38 min.

The conclusion is clear that none of the nine Cml^R variants studied could be assigned to a unique chromosomal map location.

Comparison of c.c.c. DNA from Cml^R and Cml^S cultures

The major species of c.c.c. DNA isolated from a number of Cml^R *S. coelicolor* A3(2) strains of NF, SCPI⁺ and SCPI⁻ fertility type has previously been found to have sedimentation values of 43S and 90S in neutral and alkaline conditions respectively, with a minor species of 58S and 121S respectively (Schrempf *et al.*, 1975; and unpublished results). These values were determined by comparison with ColE1, which has equivalent sedimentation values of 23S and 48S respectively. A species corresponding to the open circular form of the

Fig. 4. Agarose (1.4 %, w/v) gel electrophoresis of *Sal*I restriction endonuclease digests of SCP3 from strains 1098 (a) and A332/1 (b). *Hind*III digests of phage λ DNA (c), used as standards, had fragments with the following sizes (Mdaltons): 14.5, 6.2, 4.2, 2.8, 1.6, 1.4 and 0.3. Electrophoresis was from left to right as printed.

smaller (43S) plasmid has also been observed with a sedimentation value at neutral pH of 32S. The smaller species of c.c.c. DNA corresponds to a plasmid with a molecular weight of 18.5×10^6 (Kupersztock-Portnoy, Miklos & Helinski, 1974) which has been referred to as SCP3 (unpublished results) or as pHS1 (H. Schrempf, personal communication). The larger species appears to represent a dimer of SCP3; endonuclease digestion of c.c.c. DNA containing both species yields an identical pattern to that produced by digestion of the smaller species alone.

The transition from *Cml*^R to *Cml*^S might have been due to some change in SCP3, perhaps involving detectable loss or addition of DNA to the autonomous plasmid. To investigate this possibility ³H-labelled c.c.c. DNA was isolated from strains 1098, A332, 1098/1, 1098/3, A332/1 and A332/3 and characterized by sedimentation in sucrose gradients. All strains exhibited identical sedimentation profiles (see Fig. 3 for examples). The radioactive material at the top of the gradients was residual chromosomal DNA. The 43S, 58S and 32S species were observed in all neutral gradients. Thus the change from chloramphenicol resistance to sensitivity did not appear to result from loss of SCP3 or to any gross change in its size. However, our methods would not have detected changes in its molecular weight of less than about 1×10^6 and therefore they did not eliminate the possibility that an insertion sequence might control expression of SCP3-borne chloramphenicol resistance genes in a similar way to the control of tetracycline resistance by IS3 in the plasmid R6/R6-5 (Cohen & Kopecko, 1976).

Endonuclease digestion could reveal a much smaller difference than sedimentation analysis. *Sal*I was chosen since SCP3 contains many sites of cleavage by this enzyme (unpublished results) and *Sal*I digests of SCP3 would allow detection of any changes in molecular weight from 200000 (for a change in the largest fragment produced by *Sal*I digestion) down to as little as 20000 (for a change in one of the smaller fragments). Since the smallest well characterized insertion sequence has about 700 base pairs (about 500000 daltons) (Ohtsubo & Ohtsubo, 1976) it should be possible to detect the presence or absence of a typical insertion sequence in the transition from chloramphenicol resistance to sensitivity by comparing *Sal*I digests of SCP3 isolated from *Cml*^R and *Cml*^S strains. Unlabelled SCP3 prepared from strains 1098 and A332/1 by the large-scale method was digested with *Sal*I and the digestion products were analysed by agarose gel electrophoresis. *Hind*III digests of phage λ were used to provide standard markers. Identical fragment patterns were obtained for SCP3 isolated from the two strains (Fig. 4); microdensitometer tracings (not shown) confirmed their identity. It thus seems unlikely that the transition from *Cml*^R to *Cml*^S involves a change in SCP3.

DISCUSSION

Streptomyces lividans 66 and an SCP1⁺ derivative (strain 104) of *S. coelicolor* A3(2) were found by Shaw & Hopwood (1976) to lack chloramphenicol acetyltransferase, the enzyme responsible for chloramphenicol resistance determined by R factors in enteric bacteria and staphylococci and also by chromosomal genes in other bacteria (Shaw, 1974). We tested other A3(2) derivatives, including the Cml^R strains referred to in this paper, and found they all lacked the acetylating enzyme. Moreover they failed to destroy the antimicrobial activity of chloramphenicol in the growth medium as determined by a microbiological test (Shaw & Hopwood, 1976). Thus the chloramphenicol resistance that distinguishes the Cml^R strains from their Cml^S derivatives must depend on some mechanism other than destruction of the antibiotic in the growth medium. A test for cross-sensitivity of Cml^S strains to 17 other antibiotics and synthetic antimicrobial agents, representing several chemical classes and modes of action, gave no clues, since no cross-sensitivity was revealed.

The frequent origin of Cml^S variants from Cml^R strains of *S. coelicolor* A3(2), particularly after u.v. irradiation, suggests there might be loss of an extra-chromosomal genetic element. However, reversion of the Cml^S variants to strains with a phenotype indistinguishable from that of the original Cml^R strains, including the ability to give rise to Cml^S variants again, is incompatible with such a permanent loss of genetic information. Conventional mutation of a chromosomal gene or genes is also excluded by the failure to map any Cml^S variant to the chromosomal linkage group and by the tendency of the Cml^R character to be transferred in crosses independently of chromosomal markers. The question of whether there was a consistent difference in chromosomal recombination frequency between crosses in which both, one or neither parent was Cml^S is complicated by the effects of the 'balance' of the two parental phenotypes coming from a cross, unbalanced crosses having very high apparent recombination frequencies when expressed as a proportion of the minority parental count. Certainly Cml^S × Cml^S crosses were not reduced in fertility, and any tendency for Cml^R × Cml^S to be more fertile than Cml^R × Cml^R crosses was at most marginal. Therefore the Cml^R/Cml^S difference does not appear to be associated with a change in the activity of a sex factor.

The Cml status of a strain did not depend on the presence or absence of the SCP1 sex factor since strains of SCP1⁺, SCP1⁻ or NF (integrated SCP1) type (Hopwood *et al.*, 1973; Hopwood & Wright, 1976) had identical chloramphenicol resistance and gave rise to Cml^S variants at a similar frequency. The SCP3 (pHS1) plasmid, which is so far of unknown function, might have been concerned in the Cml character. This possibility was made less likely by the finding that *S. lividans* 66, which gave rise to Cml^S variants at a similar frequency to *S. coelicolor* A3(2), did not yield this plasmid DNA. The finding that Cml^R and Cml^S derivatives of *S. coelicolor* A3(2) had c.c.c. DNA of indistinguishable molecular weight and apparently identical restriction endonuclease pattern makes it very unlikely that SCP3 is involved in the Cml phenotype; any change in it associated with the Cml^R to Cml^S transition would have had to be very small.

Recently a sex factor, SCP2 was postulated (unpublished results), which was identified by the origin of variants of SCP1⁻ strains with an enhanced fertility towards normal SCP1⁻ strains. The increased fertility shown by such variants is 'infectious' in crosses with SCP1⁻ strains. It is not yet known whether SCP2 is responsible for all of the recombination normally found in SCP1⁻ × SCP1⁻ crosses. In crosses involving such variants, and of differing Cml status, the variant fertility character was transferred independently of Cml (Bibb, unpublished results). Thus SCP2, like SCP1 and SCP3, does not appear to be directly involved in the Cml phenotype.

The frequency of Cml^R progeny in crosses of pairs of Cml^S variants of independent origin was not higher than the reversion rate of individual Cml^S variants. This finding is compatible with the notion that Cml^S variants represent a single genetic class, although the number of pairwise crosses between different Cml^S strains was not large.

The reversibility of the change between Cml^R and Cml^S and the failure to define a unique chromosomal map location for the Cml^R/Cml^S difference are suggestive of the involvement of some kind of transposition event, with certain features in common with recently described phenomena in the Enterobacteriaceae (Cohen & Kopecko, 1976). Excision and re-insertion elsewhere in the genome of an element involved in the Cml phenotype might have given rise to frequent auxotrophic mutations, as in *Salmonella typhimurium* (Kleckner *et al.*, 1975), but none were found in a limited study. In any case it seems unlikely that our results could be explained by the existence of one or more chromosomal genes subject to insertional inactivation and of fixed position, or even of variable but exclusively chromosomal location, since insertional and transpositional events occurring at frequencies of a few per cent should not have obscured a map location for any particular Cml^S variant. Unless such events were much more frequent in Cml^R × Cml^S crosses than the origin of Cml^S variants in single Cml^R cultures, it seems that some genetic element capable of continued extra-chromosomal existence may be involved in chloramphenicol resistance in *S. coelicolor*.

Note added in proof. Recent examination of genetically defined SCP2⁻ isolates (see Discussion) by dye-buoyant centrifugation has failed to reveal c.c.c. DNA corresponding to SCP3. Re-infection of the SCP2⁻ isolates with SCP2 or its increased fertility variant is correlated with the reappearance of SCP3 c.c.c. DNA. We therefore postulate that the two plasmids, SCP2 and SCP3, defined by independent genetical and physical criteria respectively, are one and the same entity. We propose to use the designation SCP2 for this sex factor in future.

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