Genetic Determination of Methylenomycin Synthesis by the SCP1 Plasmid of *Streptomyces coelicolor* A3(2)

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SUMMARY

Evidence is presented that genes determining the pathway of methylenomycin A synthesis are carried on the SCP1 plasmid. All 16 mutations (*mmy*) leading to lack of antibiotic synthesis were SCP1-linked. Phenotypic classification, by co-synthesis and other criteria, suggested that they fell into at least five classes. When the wild-type SCP1 plasmid was transferred to *Streptomyces lividans* or *Streptomyces parvulus*, material that was chromatographically and biologically indistinguishable from methylenomycin A was produced. Recombination between some pairs of *mmy* mutations was detected. In crosses of *mmy* mutants of NF (integrated SCP1 donor) strains with SCP1⁻, a very high frequency of chromosomal recombination occurred; thus methylenomycin production appears not to be an important cause of the ultra-fertility normally associated with NF × SCP1⁻ crosses.

INTRODUCTION

Vivian (1971) discovered that the SCP1 plasmid of *Streptomyces coelicolor* A3(2) controls the synthesis of a substance which inhibits SCP1⁻ strains and that the plasmid also confers resistance to this material. It was found to be an antibiotic (Kirby, Wright & Hopwood, 1975) and was later identified (Wright & Hopwood, 1976*a*) as methylenomycin A, a compound characterized in another streptomycete (Haneishi *et al.*, 1974).

Kirby *et al.* (1975) reported the isolation of plasmid-linked mutations abolishing antibiotic production. We here describe in detail the isolation and properties of the mutants. These results, and other evidence, suggest that biosynthetic genes for the whole of the pathway leading uniquely to methylenomycin A may be carried on the SCP1 plasmid.

METHODS

Media, strains and standard genetical procedures. Minimal (MM) and complete (CM) media and supplementation of MM with growth requirements or streptomycin were as described previously (Hopwood, 1967); nutrient agar (NA) was Difco Bacto-nutrient agar. Methylenomycin A (85 % pure), kindly supplied by Dr M. Arai, Sankyo Co. Ltd, Tokyo, Japan, was added to CM at a concentration of 200 µg ml⁻¹. Derivatives of three *Streptomyces* spp. (Table 1) were used: *S. coelicolor* A3(2), *S. lividans* 66 (our strain 1326), described by Lomovskaya *et al.* (1972) and kindly supplied by Dr N. D. Lomovskaya, and *S. parvulus* ATCC12434 (our strain 2266), kindly supplied by Dr H. Prauser. Eubacterial indicator strains were as listed by Wright & Hopwood (1976*a*). Standard cultural and crossing procedures were those described by Hopwood (1967) and Hopwood *et al.* (1973). *Streptomyces* cultures were incubated at 30 °C, *Erwinia carotovora* at 27 °C, and *Bacillus cereus* at 36 °C.

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Table 1. *Streptomyces* strains

Strain	Markers	Plasmid status
<i>S. coelicolor</i> A3(2)		
104	<i>hisA1 uraA1 strA1</i>	SCP1 ⁺
417	<i>hisC9</i>	SCP1 ⁺
1190	<i>hisA1 uraA1 strA1</i>	SCP1 ⁻
1546	<i>hisC9</i>	SCP1 ^{-orange}
1953	<i>cysB6</i>	SCP1 ⁻
1956	<i>hisD3 cysB6 strA1</i>	SCP1 ⁻
1984	<i>hisD3 cysB6 strA1</i>	SCP1 ^{-cysB⁺}
2455	<i>proA1 argA1 cysD18</i>	SCP1 ^{-orange}
A200	<i>adeCv10 pheA1 strA5</i>	SCP1 ⁻
A303	<i>hisA1 uraA1 strA1</i>	NF
A700	<i>proA1 argA1 cysD18</i>	SCP1 ⁻
<i>S. lividans</i> 66		
1326	Wild-type	SCP1 ⁻
1671	Wild-type	SCP1 ⁺
<i>S. parvulus</i> ATCC12434		
2266	Wild-type	SCP1 ⁻
2283	<i>str-I</i>	SCP1 ⁻
2296	<i>str-I</i>	SCP1 ⁺
2297	<i>cysB1</i>	SCP1 ⁻

Mutagenesis and mutant isolation. Mutagenesis by the following agents was by standard procedures: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (Delić, Hopwood & Friend, 1970), 8-methoxypsoralen (MOP) in the presence of 364 nm light (Townsend, Wright & Hopwood, 1971) and 254 nm ultraviolet light (u.v.) (Harold & Hopwood, 1970).

Various strains were used as parents for the isolation of antibiotic non-producing (*mmv*) mutants. An SCP1⁺ strain had the disadvantage that plasmid loss occurred more frequently than mutation (Vivian & Hopwood, 1970), so that the majority of Mmy⁻ derivatives of SCP1⁺ were SCP1⁻ strains. Use of an NF (integrated SCP1) strain avoided this difficulty, since SCP1⁻ derivatives of NF are very rare (Vivian & Hopwood, 1970), but subsequent study of the mutations were handicapped by the difficulty of establishing their plasmid-linkage unambiguously. When SCP1⁺ plasmids were isolated (Hopwood & Wright, 1973*b*), a heterozygous strain (1984) carrying SCP1⁺-*cysB*⁺, with *cysB6* on the chromosome, was used exclusively for mutant isolation; plating on MM lacking cystine ensured retention of the plasmid by all except a small minority of the colonies, since integration of *cysB*⁺ into the chromosome followed by plasmid loss is rare (Hopwood & Wright, 1976*b*). Mutants of this plasmid could be selected into further A3(2) derivatives carrying *cysB6*, or into *cysB* mutants of *S. lividans* or *S. parvulus*.

Mutagenized spore suspensions were plated on supplemented MM to yield 60 to 90 colonies per plate; these were replica-plated to CM spread with dense spore suspensions of SCP1⁻ strain 1190. After 3 to 4 days the replicas were examined for the presence of colonies that failed to inhibit growth and pigmentation (due to the pigmented antibiotic actinorhodin: Wright & Hopwood, 1976*b*) of the indicator strain. Presumptive mutants were re-tested for the Mmy⁻ phenotype.

Resistance or sensitivity to methylenomycin was tested by inoculating SCP1⁺ strain 104 at right angles to streaks of each *mmv* mutant on CM; inhibition of sensitive mutants was apparent after incubation for 3 to 4 days.

To test if the Mmy⁻ phenotypes of the mutants were due exclusively to changes in the

plasmid, each putative mutant plasmid was transferred to a new genetic background; *mmv* derivatives of 104, 1984 and A303 were transferred to 1956, 1953 and A700 respectively.

Chromatography and detection of methylenomycin and its possible precursors. Cultures were grown, extracts were prepared and analysed by thin-layer chromatography in solvent 2, and u.v.-absorbing materials were detected by the methods described by Wright & Hopwood (1976a).

Measurement of plasmid 'instability', apparent transfer frequency and 'entry disadvantage'. Plasmid loss ('instability') of derivatives of SCP1'-*cysB*⁺ was measured by plating a slant culture derived from a single colony on non-selective medium and estimating the proportion of Cys⁻ colonies by replica-plating to medium lacking cystine. Transfer frequencies of such mutants were measured by crossing with strain 1953, plating the crosses on MM containing cystine and estimating the proportion of Cys⁺ colonies by replica-plating to medium lacking cystine. Transfer frequencies of the SCP1⁺ mutant (R27), SCP1-*orange* and the NF mutants (R11 and R33) were measured by crossing non-selectively with SCP1⁻ strains and classifying samples of the genotype corresponding to the SCP1⁻ parent for methylenomycin resistance. 'Entry disadvantage' of the mutant plasmids was defined and measured as described by Kirby (1976).

Tests of reversion on transfer, and recombination, of mmy mutations. For reversion tests, a strain carrying each *mmv* mutation of SCP1-*cysB*⁺ was crossed with strain 1953 (*cysB6* SCP1⁻) and Cys⁺ progeny were selected on MM; *hisD3* served as a counterselected donor marker. The resulting colonies were tested for antibiotic production by replica-plating to lawns of strain 1190. For recombination tests, triparental crosses of pairs of *mmv* mutations with strain 1953 were analysed in the same way.

RESULTS

Isolation and general characteristics of mmy mutants

Table 2 summarizes some of the properties of the *mmv* mutations. Each plasmid was transferred to a new genetic background (see Methods) and the properties recorded in Table 2 are those of the new recipient. No important difference was found between an original and a derived strain. The frequencies of mutations were 9/21 400 (0.04 %) for u.v. mutagenesis and 6/19 000 (0.03 %) for MOP. In one experiment with NTG no mutants occurred among 800 colonies.

Several mutants differed from the parent in other characters besides antibiotic production. These were: frequency of plasmid loss ('instability'), frequency of apparent plasmid transfer to an SCP1⁻ strain, 'entry disadvantage', and sensitivity to antibiotic produced by an SCP1⁺ strain.

There was a tendency (Table 2) for instability and transfer proficiency of the SCP1'-*cysB*⁺ mutants to be inversely correlated, mutants showing instability of 10 % or more being significantly impaired in apparent plasmid transfer; this is not unexpected since a high instability would have been likely to reduce apparent transfer by causing plasmid loss from newly infected recipients.

The term 'entry disadvantage' was used by Kirby (1976) to describe the failure of SCP1 or marked derivatives of it to recombine freely with chromosomal genotypes in crosses. It is measured in crosses between strains of different chromosomal genotype, each carrying a different SCP1 derivative. The 'entry disadvantage' of the plasmid, as defined, rises in proportion to an increase in the plasmid's ability to remain in association with its original chromosomal genotype and to prevent replacement by another plasmid within the cross.

Table 2. *Properties of mmy mutants*

Parent strain	Plasmid*	Methyl- enomyacin resistance	Pigmenta- tion (see text)	Apparent frequency of plasmid transfer per recipient (%)†	Entry disadvan- tage (%)	Frequency of plasmid loss (‘instabil- ity’) (%)	Mutagen
104	SCP1	+	—	c. 99	98	0.2	.
	SCP1 (R27)	+	+	c. 98	> 92	ND	u.v.
1984	SCP1'	+	—	85–90	88	6	.
	SCP1' (R39)	+	+	32–100	87	9	MOP
	SCP1' (R40)	+	+	10–39	81	7	MOP
	SCP1' (R48)	+	+	1–21	65	14	MOP
	SCP1' (R53)	+	+	30–100	80	7	MOP
	SCP1' (R56)	—	+	0.002–0.06	0	5	MOP
	SCP1' (R274)	+	‡	low	low	high	MOP
	SCP1' (R284)	+	+	6–30	100	10	u.v.
	SCP1' (R289)	+	+	43–65	88	5	u.v.
	SCP1' (R316)	+	+	42–71	85	6	u.v.
	SCP1' (R331)	+	+	48–64	73	7	u.v.
	SCP1' (R332)	+	+	4–6	77	highly variable	u.v.
	SCP1' (R333)	—	—	39–45	3	16	u.v.
A303	NF	+	—	c. 99	100	ND	.
	NF (R11)	+	—	c. 99	100	ND	u.v.
	NF (R33)	+	—	91	100	ND	u.v.
417	SCP1						.
	SCP1- <i>orange</i>	+	orange	54–99	80	4	u.v.

ND, Not done.

* Brackets indicate origin from the same spore suspension.

† Ranges represent results of two, three or four crosses.

‡ Variable, possibly temperature-sensitive response.

Thus NF has an ‘entry disadvantage’ of 100 %. Operational difficulties associated with the mycelial nature of streptomycetes prevented an attempt to partition this effect between phenomena that might be analogous to ‘entry exclusion’ and ‘incompatibility’ in enteric bacteria. For most of the SCP1'-*cysB*⁺ mutants, ‘entry disadvantage’ did not differ significantly from its value for the parent SCP1'-*cysB*⁺ plasmid (Table 2). Exceptions were R274, whose low ‘entry disadvantage’ may have been explicable by its high instability, and R56 and R333 which showed very low ‘entry disadvantage’ but, particularly for R56, no correspondingly high instability.

Most of the mutants were resistant to antibiotic produced by the 104 tester strain, but two (R56 and R333) were apparently no more resistant than typical SCP1⁻ strains. Four other mutants (R48, R284, R316 and R332) showed a partial sensitivity, but it was uncertain whether only pigmentation was affected or whether there was also a partial inhibition of growth.

*The SCP1-*orange* mutation.* Strain 1546 was selected after u.v. treatment from strain 417 (*hisC9* SCP1⁺) for its enhanced capacity to donate *pheA*⁺ to an SCP1⁻ culture (D. A. Hopwood & H. M. Wright, unpublished results). The strain was found to be Mmy⁻ and to produce a diffusible orange pigment on CM, but to a negligible extent on supplemented MM. Under conditions of good production of this pigment on CM, little or no actinorhodin was formed; however, actinorhodin production on CM was regained on plasmid loss and was shown on MM even in the presence of the plasmid. The mutant phenotype was expressed on

Fig. 1. Interpretation of a cross between strains 1546 (*hisC9* *SCP1-orange*; outer circle) and A200 (*adeCv10 pheA1 strA5* *SCP1-*; inner circle). Figures are allele frequencies amongst total non-parental progeny. The frequencies of individual recombinant genotypes are in Table 3.

Table 3. *Non-selective analysis of a cross between strains 1546 (*hisC9* *SCP1-orange*) and A200 (*adeCv10 pheA1 strA5* *SCP1-*)*

Genotype		No.	Description	Percentage of total progeny
Chromosomal markers	Plasmid			
<i>his</i>	<i>SCP1-orange</i>	38	Donor parental	22
<i>ade phe str</i>	<i>SCP1-</i>	48	Recipient parental	28
<i>ade phe str</i>	<i>SCP1-orange</i>	56	Non-parental for plasmid	32
<i>str</i>	<i>SCP1-orange</i>	11	Non-parental for markers	18
+	<i>SCP1-orange</i>	9		
<i>ade str</i>	<i>SCP1-orange</i>	6		
<i>his str</i>	<i>SCP1-orange</i>	3		
<i>phe str</i>	<i>SCP1-orange</i>	2		
<i>his ade phe str</i>	<i>SCP1-orange</i>	1		

transfer of the plasmid to various *SCP1-* strains, indicating plasmid-linkage, and the plasmid was called *SCP1-orange*.

The donor properties of strains carrying *SCP1-orange* in crosses with *SCP1-* strains were analysed non-selectively. The frequency of recombination was much higher than in *SCP1+ × SCP1-* crosses, in which recombinants rarely represent more than 10^{-4} of the total progeny of a mixed culture (Vivian & Hopwood, 1970; Hopwood *et al.*, 1973), but the frequency and pattern of marker donation showed some change during the course of these studies. An early cross (Table 3; Fig. 1) had a high frequency of chromosomal recombinants (18%) and a clear bidirectional gradient of allele ratios resembling that in *NF × SCP1-* crosses, but the proportion of total non-parental progeny was only some 50% instead of almost 100% characteristic of *NF × SCP1-* crosses (Hopwood *et al.*, 1973). Since *SCP1-orange* was demonstrably unstable (Table 2), it was presumably autonomous, and so donation of chromosomal markers must have occurred through a transient rather than a permanent association of *SCP1-orange* with the chromosome, as in the unstable unidirectional donors described by Vivian & Hopwood (1973). In later *SCP1-orange × SCP1-* crosses the frequencies of

chromosomal recombinants were lower (1 to 5 %) and the gradients of allele ratios among recombinants were less marked. If the event that gave rise to the SCP1-*orange* plasmid was an addition (or substitution) of chromosomal DNA to SCP1, subsequent changes may have involved a loss of part of this DNA.

Phenotypes of S. lividans and S. parvulus carrying SCP1 and its derivatives

In studies of inter-specific transfer of SCP1 (Hopwood & Wright, 1973*a, b*; 1976*a*; unpublished results), *S. lividans* 66 and *S. parvulus* ATCC12434 have been used extensively. Their SCP1⁺ derivatives were previously shown to inhibit the growth of SCP1⁻ but not SCP1⁺ strains of *S. coelicolor*. We analysed extracts of liquid cultures of SCP1⁻ and SCP1⁺ strains of both species (1326, 1671, 2283, 2296) chromatographically and found the same pattern of u.v.-absorbing spots as in *S. coelicolor* (Wright & Hopwood, 1976*a*): spot A alone in SCP1⁻ strains, and spots A, B and C in SCP1⁺ strains. Spot B was shown to be methylenomycin A by Wright & Hopwood (1976*a*). Spot C, which in our hands always contained more material than spot B irrespective of which of the three species was involved, is probably structurally related to methylenomycin A and may be the compound identified in preparative chromatography and differing from methylenomycin A by 16 mass units (Wright & Hopwood, 1976*a*). Spot B from *S. lividans* and *S. parvulus* was shown to inhibit *Bacillus cereus* by the method of Kirby *et al.* (1975). Thus the SCP1 plasmid apparently confers on these two species the capacity to produce the same antibiotic as in *S. coelicolor*.

Transfer of SCP1-*orange* to *S. lividans* and *S. parvulus* was important to discriminate between two hypotheses on the nature of the orange pigment: (i) that it was a precursor or side product of methylenomycin synthesis, or (ii) that it was an intermediate or side product of the actinorhodin pathway caused to accumulate by interference with that pathway by a compound accumulating as a result of interruption of the methylenomycin pathway. *Streptomyces parvulus* does not produce actinorhodin or related pigments; *S. lividans* usually does not, although occasionally some colonies give rise to a pigment that is blue in alkaline conditions and may be actinorhodin (B. A. M. Rudd, personal communication). SCP1-*orange* was transferred to these species by crossing strain 2455 (*S. coelicolor* SCP1-*orange*) with streptomycin-resistant mutants of *S. lividans* or *S. parvulus* and selecting colonies resistant to methylenomycin and streptomycin on CM containing both antibiotics. All such colonies produced orange pigment on CM. Thus this pigment is probably directly associated with the methylenomycin pathway rather than the actinorhodin pathway and SCP1-*orange* represents a unique class of *mmv* mutation.

Some representative *mmv* mutants of SCP1'-*cysB*⁺ (R39, R40 and R48) were crossed with *S. parvulus cysB1* (strain 2297) and Cys⁺ *S. parvulus* progeny were tested for antibiotic production against strain 1190. None was detected; thus the phenotype of the mutants remained Mmy⁻ in this species.

Classification of mmv mutations

Chromatography. Extracts of liquid cultures of each mutant were compared chromatographically with those of SCP1⁺ and SCP1⁻ cultures. Each mutant extract yielded the u.v.-absorbing spot A, which is probably unrelated to methylenomycin (see above). However, spots B and C, characteristic of SCP1⁺ cultures, were not seen in extracts of any of the mutants, with the exception of a very small amount of u.v.-absorbing material sometimes seen in the spot C position in R274. Faint u.v.-absorbing spots with other *R_f* values were occasionally seen in particular mutant extracts, but their presence was not completely reproducible.

Fig. 2. Tests of antibiotic co-synthesis between pairs of *mmy* mutants. Each dish shows a pair of mutants, inoculated vertically and horizontally, on a background of SCP1⁻ strain 1190. Top: R39 (vertical), R284 (horizontal); antibiotic co-synthesis, with R284 acting as secretor and R39 as converter. Left: R48 (vertical), R39 (horizontal); no co-synthesis. Right: R11 (vertical), R33 (horizontal); no co-synthesis. Note that R39, R48 and R284 are pigmented; R11 and R33 are non-pigmented.

Co-synthesis. Ideally, complementation tests would reveal allelism or otherwise of the *mmy* mutations. However, these are not yet available for SCP1-linked mutations since co-habitation of hyphae by a pair of SCP1 derivatives is very limited. In initial tests for pairwise interactions between mutants, they were inoculated at right angles on plates of CM carrying the 1190 indicator strain, by successive replication from plates carrying a streak of each mutant separately (Fig. 2). Clear evidence of antibiotic production was seen in most pairs involving R39 and the zone of inhibition was markedly asymmetrical, extending from the area of contact of the two mutants along the R39 streak (Fig. 2, top). We were evidently observing a co-synthesis reaction (McCormick *et al.*, 1960) with R39 acting as converter of material secreted by the other (later blocked) mutant. All the mutants, except R48, R56 and R332 but including SCP1-*orange*, acted as secretors to R39 in these tests. No strong and unambiguously asymmetric reactions were seen in pairs not involving R39, although a very weak reaction was sometimes seen between R48 and R284.

Erwinia carotovora is a useful specific indicator for methylenomycin since it is totally insensitive to cultures of SCP1⁻ *S. coelicolor* derivatives, being resistant to the actinorhodin antibiotic (Wright & Hopwood, 1976*a, b*). Tests of co-synthesis between each pair of *mmy* mutants were therefore repeated using the method of Deli, Pigac & Sermonti (1969) and *E. carotovora* as indicator (Fig. 3). This test might have been more sensitive than tests with strain 1190, but there was complete agreement between the results of the two methods.

Fig. 3

Fig. 4

Fig. 3. Antibiotic co-synthesis by R40 (secretor, top) and R39 (converter, bottom) tested against *Erwinia carotovora*.

Fig. 4. Antibiotic co-synthesis between R39 and R40. The left-hand half of the dish is blank agar into which antibiotic precursor from R40 had been allowed to diffuse from a culture in the right-hand half of the dish. This was later replaced by a narrow strip of an R39 culture and a segment of agar carrying strain 1190. Note inhibition of 1190 in a band adjacent to R39 (arrows).

To prove that a positive result involving strain R39 was due to an extracellular (co-synthetic) reaction rather than to intracellular complementation, a putative secretor mutant (R40) was inoculated on half the surface of a CM plate and after 3 days' incubation this section was completely removed. A 2 cm wide strip of a 3-day-old R39 culture was placed beside the remaining uninoculated agar, and the rest of the dish was filled by a segment of CM carrying a 1-day-old lawn of strain 1190. Inhibition of this test lawn on further incubation confirmed true co-synthesis (Fig. 4). Controls consisted of plates with the secretor and converter reversed.

To confirm that the inhibition produced in co-synthetic reactions was due to methylenomycin production, extracts of mixed shake flask cultures of R39 with R33, R40, R48 or R274 were analysed chromatographically. The combinations shown earlier to co-synthesize on agar (R39 with R33, R40 or R274) produced spots A, B and C, while the R39 plus R48 combination, which failed to co-synthesize on agar, revealed spot A only.

In tests in which strain 104 (SCP1⁺), each *mmy* mutant and 1190 were juxtaposed (Fig. 5), it was found that R39, but no other mutant, was able to convert material secreted by the SCP1⁺ culture to antibiotic.

Pigmentation. The mutants differed reproducibly in their pigmentation on CM. On this medium, a typical SCP1⁻ strain, such as 1190, produces large quantities of reddish-purple pigment, the main component of which is the pH indicator antibiotic actinorhodin (Wright & Hopwood, 1976b) although other pigments are also present (Z. Hořálek, personal communication; B. A. M. Rudd, unpublished results). Pigmentation of strain 104, isogenic with 1190 except for the presence of SCP1, was considerably delayed on CM; it was straw-coloured when 1190 was deeply pigmented. On supplemented MM, on which methylenomycin is barely produced, both strains were strongly pigmented. Thus it is likely that inhibition of pigmentation of SCP1⁺ cultures on CM is related to the production of methylenomycin

Fig. 5. Co-synthesis of antibiotic by *mmv*⁺ strain 104 (left-hand half of dish) and R39 (bottom right-hand quadrant) detected by inhibition of strain 1190 (top right-hand quadrant). Note inhibition of 1190 by 104, and further inhibition due to antibiotic synthesized by R39 in proximity to 104.

or a precursor of it. It was therefore significant that the *mmv* mutants fell clearly into two classes on CM; some were as deeply pigmented as SCP1⁻ strains of the same age, while others were as pale as SCP1⁺ strains (Fig. 2; Table 2). It is possible that pigmentation may be inhibited by a precursor of methylenomycin A and that the pale mutants represent later blocks in methylenomycin synthesis than the pigmented mutants.

The mutant R274 was difficult to classify; while often causing appreciable inhibition of pigment production by strain 1190, it caused little, if any, inhibition of its growth; moreover it failed to inhibit *E. carotovora*, and only traces of spot C could be seen in chromatographic analysis (see above). It is possible that R274 accumulated a material, different from methylenomycin A, which inhibited pigment production; however, a slight leakiness of the mutation, at least at temperatures slightly below 30 °C, might have explained the results.

Grouping of mmv mutations into phenotypic classes. Two of the SCP1-linked mutations conferred unique phenotypes on their host strain and each therefore defines a phenotypic class: R39 was the only mutant unambiguously to act as converter in co-synthesis; while SCP1-*orange* caused the production of an orange pigment. Two mutants (R56 and R332) failed to co-synthesize with any other mutant and therefore define a third class, which also contains R48 if its weak co-synthetic reaction with R284 is disregarded. The remaining large group of mutants that co-synthesized as secretors with R39 is sub-divisible into two further classes on the basis of pigmentation. The distribution of the mutations into five classes on these criteria is shown in Table 4. It should be noted that this represents a minimum subdivision of the mutations; further classes would result if the weak co-synthetic reaction involving R48 and R284 were taken into account; moreover, R274, with its dubious and possibly temperature-sensitive phenotype, has been disregarded; it might possibly represent an additional class. The only mutations in the same class which might be clonal are R284 with R289 and R316 with R331 (see Table 2).

Table 4. *Classification of mmy mutants*

Class	Characteristics	No. in class	Members
R39	Pigmented; co-synthesizes (as converter) with R40, R11 and SCP1- <i>orange</i>	1	R39
R40	Pigmented; co-synthesizes (as secretor) with R39	7	R27, R40, R53, R284, R289, R316, R331
R11	Non-pigmented; co-synthesizes (as secretor) with R39	3	R11, R33, R333
SCP1- <i>orange</i>	Orange-pigmented; co-synthesizes (as secretor) with R39	1	SCP1- <i>orange</i>
R56	Pigmented; no co-synthesis	3	R56, R332, and possibly R48

Table 5. *Recombination tests between pairs of mmy mutants of SCP1'-cysB⁺ in the R40 group*

Frequencies are those of *mmy*⁺ colonies amongst Cys⁺ recombinants of triparental crosses between two *mmy* mutants of strain 1984 (SCP1'-*cysB*⁺) and strain 1953. Percentage frequencies are in parentheses.

	R40	R53	R284	R289	R316	R331
R40		0/3339 (<0.03)	7/6450 (0.1)	2/4500 (0.05)	2/5800 (0.04)	0/2712 (<0.04)
R53			0/4550 (<0.02)	0/3760 (<0.03)	0/3780 (<0.03)	0/5100 (<0.02)
R284				0/6350 (<0.02)	1/5320 (0.02)	0/4010 (<0.03)
R289					2/4600 (0.05)	0/5400 (<0.02)
R316						0/4950 (<0.02)
Reversion frequency	0/3960 (<0.03)	0/5390 (<0.02)	0/4850 (<0.02)	0/5710 (<0.02)	0/3250 (<0.03)	0/3400 (<0.03)

Reversion of mmy mutants

Reversion tests were done on some of the *mmy* mutants, Mmy⁺ colonies being sought among samples of 3000 to 8000 colonies. No spontaneous revertants were found, but some of the mutants (R39, R48, R53, R316) reverted after mutagenesis by NTG or MOP at frequencies in the order of 0.05 to 0.1 %. It was not feasible to examine larger populations of colonies, and negative results obtained with R40, R284 and R289 were therefore not evidence of non-revertibility. For those mutants that reverted, three revertants were tested and all showed plasmid linkage of the Mmy⁺ phenotype.

Recombination of mmy mutants

Recombinants arose from crosses between pairs of mutants in different phenotypic classes (Table 4) with the following frequencies: R39 × R48, 7/1860 (0.4 %); R40 × R48, 8/474 (2 %); R48 × R53, 8/635 (1 %). In a further series of crosses between all possible pairs of a sample of mutants in the same class, recombination frequencies in the range 0.02 to 0.1 % were observed in some crosses, but large enough samples of progeny could not be examined to make the negative results significant (Table 5). Although no individual positive result was significantly above the detection limit for spontaneous revertants (Table 5), controls yielded no revertants, and it is therefore very unlikely that all the apparent recom-

Table 6. Crosses of SCP1⁻ with *mmy* mutants of an NF strain

The *mmy* × SCP1⁻ data are combined results from crosses of R11 and R33 (*hisA1 uraA1 strA1* NF) with A700 (*proA1 argA1 cysD18* SCP1⁻); the *mmy*⁺ × SCP1⁻ cross was A303 (*hisA1 uraA1 strA1* NF) × A700

Genotype		Number		Percentage of total progeny		Description
		<i>mmy</i> (NF) × SCP1 ⁻	<i>mmy</i> ⁺ (NF) × SCP1 ⁻	<i>mmy</i> (NF) × SCP1 ⁻	<i>mmy</i> ⁺ (NF) × SCP1 ⁻	
Chromosomal markers	Plasmid					
<i>his ura str</i>	NF	125	19	32	10	Donor parental
<i>pro arg cys</i>	SCP1 ⁻	17	0	4	0	Recipient parental
<i>pro arg cys</i>	NF	136	81	64	90	Recombinants
<i>pro arg cys ura</i>	NF	67	77			
<i>arg cys</i>	NF	18	6			
<i>arg cys ura</i>	NF	17	6			
<i>his arg cys</i>	NF	1	1			
<i>his arg cys ura</i>	NF	1	1			
<i>his cys</i>	NF	1	1			
<i>his cys ura</i>	NF	1	0			
<i>pro arg cys ura str</i>	NF	1	0			
<i>pro arg ura str</i>	NF	1	0			
<i>pro arg cys str</i>	NF	1	0			
<i>his pro cys ura</i>	NF	0	1			
<i>pro arg</i>	NF	0	1			
<i>cys ura</i>	NF	1	0			
<i>pro cys</i>	NF	1	0			

ination frequencies in Table 5 are spurious, if indeed any of them are. In any case, it is noteworthy that the apparent within-class recombination frequencies (0.02 to 0.1 %) were an order of magnitude below the between-class values of 0.4 to 2 %.

*Ultra-fertility in crosses of SCP1⁻ with *mmy* mutants of an NF strain*

Crosses of NF and SCP1⁻ (UF) strains have been described as 'ultra-fertile' (Hopwood *et al.*, 1969) since they yield very low proportions of either parental genotype. The explanation of the almost total absence of the donor (NF) parental type from the progeny is a mystery. However, the finding of inhibition of SCP1⁻ strains by those carrying SCP1, including NF, provided a possible explanation for the absence of the SCP1⁻ genotype (Vivian, 1971; Hopwood *et al.*, 1973).

To test this idea we analysed crosses of the NF *mmy* mutants (R11 and R33) with an SCP1⁻ strain (A700). All progeny were classified in respect of chromosomal markers and tested for antibiotic production against strain 1190. The progeny from the *mmy* × SCP1⁻ crosses were tested for methylenomycin resistance by replica-plating to plates containing methylenomycin A. Such crosses gave results (Table 6) similar to those of the control NF (*mmy*⁺) × SCP1⁻ cross. There was, however, a larger proportion of the NF parental type amongst the progeny (over 30 % compared with 10 % in the control, itself a rather high value for an NF × SCP1⁻ cross) and a small proportion (4 %) of the SCP1⁻ recipient genotype.

None of the progeny of the crosses involving R11 or R33 was *Mmy*⁺. Although not providing complete proof of SCP1-linkage, these results strongly suggest it. If the *mmy* mutations in R11 or R33 were in chromosomal genes, these would have had to be very close to the site of plasmid integration.

DISCUSSION

There is evidence for plasmid involvement in the synthesis of several streptomycete antibiotics (Akagawa, Okanishi & Umezawa, 1975; Boronin, Borisoglebskaya & Sadovnikova, 1974; Kähler & Noack, 1974; Okanishi, Ohta & Umezawa, 1970). However, uniquely for methylenomycin synthesis by *S. coelicolor* A3(2), there is now extensive genetical characterization of the plasmid concerned and substantial evidence that the plasmid carries the structural genes for the biosynthetic pathway of the antibiotic. This evidence is described in the present paper and is of two kinds. Firstly, when SCP1 was transferred to two other species that do not normally produce the antibiotic, *S. lividans* and *S. parvulus*, material that was biologically and chromatographically identical with methylenomycin A was synthesized. If SCP1 acted only in a regulatory role, one would have to postulate 'silent' pathway genes in these two species. Secondly, all 16 of the *mmv* mutations so far known are SCP1-linked. Chromosomal mutations abolishing methylenomycin synthesis have been isolated in other studies, but these are the *bald* mutations that interfere with the development of aerial mycelium (Chater & Merrick, 1976; Merrick, 1976); they also lead to lack of actinorhodin. Wide pleiotropic effects undoubtedly result from these mutations and it is most unlikely that the genes defined by them are primarily concerned with methylenomycin.

Classification of the *mmv* mutations is complicated by the possibility that some may not be point mutations. One of the mutants, SCP1-*orange*, probably owes its increased capacity to donate chromosomal markers to a chromosomal insertion and it is also suggestive that one of the SCP1-*argA*, *uraB* strains (1937) described by Hopwood & Wright (1976*a*) had an Mmy⁻ phenotype; it was too unstable to include in the present study. Furthermore, several of the mutants selected on the basis of the Mmy⁻ phenotype were altered in other ways: stability, transfer proficiency, 'entry disadvantage' and methylenomycin sensitivity. We cannot yet say whether these resulted from single pleiotropic mutations or from more complex genetic changes. However, reversions of mutations in three of the five phenotypic classes (R39, R40 and R56) were easily found, suggesting that they, at least, were not multi-site events. Since two of the classes have only one member, other classes will presumably be revealed by a larger sample of mutations, especially if a reliable method of studying complementation between the mutations becomes available.

It is premature to speculate at length on the sequence of pathway interruptions represented by the mutations, until biochemical studies can be used to define the biosynthetic steps involved. Since co-synthesis was rare amongst the 16 mutations, only one of which acted as a converter, this criterion was much less useful than in the case of the tetracyclines (Delić *et al.*, 1969; Boronin & Mindlin, 1971) or platenomycins (Furumai & Suzuki, 1975). Possibly many of the intermediates of methylenomycin A biosynthesis are highly labile, unable to cross permeability barriers, or are enzyme-bound.

The *mmv* mutations are relevant to the mechanism of ultra-fertility of NF × SCP1⁻ crosses and also to the role, if any, of the plasmid-determined antibiotic in the retention of autonomous SCP1 and its derivatives by the mycelium. The finding that *mmv* mutants of NF were highly fertile in crosses with SCP1⁻ eliminates the simple possibility that the almost total absence of SCP1⁻ progeny in a normal NF × SCP1⁻ cross is due to selection against them by methylenomycin produced during growth of the cross. Similarly, it could have been argued that the wild-type autonomous plasmid is potentially lost from a significant proportion of the hyphal units in a culture but that these are selected against by the antibiotic. However, the finding that many of the *mmv* mutants were lost no more often than the parent *mmv*⁺ plasmid argues against this idea.

Kirby (1974) described recombination between SCP1 derivatives, selection being for transfer proficiency when the parent plasmids were defective in transfer. In the present study, recombination was detected by the production of *mmy*⁺ progeny in crosses between pairs of *mmy* mutations. Crosses between mutations of the same phenotypic class yielded recombinants at frequencies that could not be unambiguously distinguished from those of reverse mutation, although reversion was very unlikely to have explained the results. However, in crosses between phenotypically different mutations, significant levels of recombination certainly occurred. Whether recombination can be used as a means of mapping plasmid-borne genes remains to be seen.

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REFERENCES

- AKAGAWA, H., OKANISHI, M. & UMEZAWA, H. (1975). A plasmid involved in chloramphenicol production in *Streptomyces venezuelae*: evidence from genetic mapping. *Journal of General Microbiology* **90**, 336–346.
- BORONIN, A. M. & MINDLIN, S. Z. (1971). Genetic analysis of *Actinomyces rimosus* mutants with impaired synthesis of antibiotic. *Genetika* **7**, 125–131 (in Russian).
- BORONIN, A. M., BORISOGLEBSKAYA, A. N. & SADOVNIKOVA, L. G. (1974). Oxytetracycline-sensitive mutants of *Streptomyces rimosus*, the producer of oxytetracycline. *Abstracts of the Second International Symposium on the Genetics of Industrial Microorganisms*, p. 103. London and New York: Academic Press.
- CHATER, K. F. & MERRICK, M. J. (1976). Approaches to the study of differentiation in *Streptomyces coelicolor* A3(2). In *Second International Symposium on the Genetics of Industrial Microorganisms*, pp. 583–593. Edited by K. D. Macdonald. London and New York: Academic Press.
- DELIĆ, V., HOPWOOD, D. A. & FRIEND, E. J. (1970). Mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) in *Streptomyces coelicolor*. *Mutation Research* **9**, 167–182.
- DELIĆ, V., PIGAC, J. & SERMONTI, G. (1969). Detection and study of cosynthesis of tetracycline antibiotics by an agar method. *Journal of General Microbiology* **55**, 103–108.
- FURUMAI, T. & SUZUKI, M. (1975). Studies on the biosynthesis of basic 16-membered macrolide antibiotics, platenomycins. I. Selection of and cosynthesis by non-platenomycin-producing mutants. *Journal of Antibiotics (Japan)* **28**, 770–774.
- HANEISHI, T., KITAHARA, N., TAKIGUCHI, Y., ARAI, M. & SUGAWARA, S. (1974). New antibiotics, methylenomycins A and B. I. Producing organism, fermentation and isolation, biological activities and physical and chemical properties. *Journal of Antibiotics (Japan)* **27**, 386–392.
- HAROLD, R. J. & HOPWOOD, D. A. (1970). Ultraviolet-sensitive mutants of *Streptomyces coelicolor*. I. Phenotypic characterisation. *Mutation Research* **10**, 427–438.
- HOPWOOD, D. A. (1967). Genetic analysis and genome structure in *Streptomyces coelicolor*. *Bacteriological Reviews* **31**, 373–403.
- HOPWOOD, D. A. & WRIGHT, H. M. (1973*a*). Transfer of a plasmid between streptomyces species. *Journal of General Microbiology* **77**, 187–195.
- HOPWOOD, D. A. & WRIGHT, H. M. (1973*b*). A plasmid of *Streptomyces coelicolor* carrying a chromosomal locus and its inter-specific transfer. *Journal of General Microbiology* **79**, 331–342.
- HOPWOOD, D. A. & WRIGHT, H. M. (1976*a*). Interactions of the plasmid SCP1 with the chromosome of *Streptomyces coelicolor* A3(2). In *Second International Symposium on the Genetics of Industrial Microorganisms*, pp. 607–619. Edited by K. D. Macdonald. London and New York: Academic Press.
- HOPWOOD, D. A. & WRIGHT, H. M. (1976*b*). Genetic studies on SCP1-prime strains of *Streptomyces coelicolor* A3(2). *Journal of General Microbiology* **95**, 107–120.
- HOPWOOD, D. A., HAROLD, R. J., VIVIAN, A. & FERGUSON, H. M. (1969). A new kind of fertility variant in *Streptomyces coelicolor*. *Genetics* **62**, 461–477.
- HOPWOOD, D. A., CHATER, K. F., DOWDING, J. E. & VIVIAN, A. (1973). Advances in *Streptomyces coelicolor* genetics. *Bacteriological Reviews* **37**, 371–405.
- KÄHLER, R. & NOACK, D. (1974). Action of acridine orange and ethidium bromide on growth and antibiotic activity of *Streptomyces hygroscopicus* JA6599. *Zeitschrift für allgemeine Mikrobiologie* **14**, 529–533.
- KIRBY, R. (1974). Mutants of the SCP1 plasmid of *Streptomyces coelicolor* A3(2). *Abstracts of the Second International Symposium on the Genetics of Industrial Microorganisms*, p. 100. London and New York: Academic Press.
- KIRBY, R. (1976). *Genetic studies on Streptomyces coelicolor Plasmid One*. Ph.D. thesis, University of East Anglia, Norwich.
- KIRBY, R., WRIGHT, L. F. & HOPWOOD, D. A. (1975). Plasmid-determined antibiotic synthesis and resistance in *Streptomyces coelicolor*. *Nature, London* **254**, 265–267.

- LOMOVSKAYA, N. D., MKRTUMIAN, N. M., GOSTIMSKAYA, N. L. & DANILENKO, V. N. (1972). Characterization of temperate actinophage ϕ C31 isolated from *Streptomyces coelicolor* A3(2). *Journal of Virology* **9**, 258–262.
- MCCORMICK, J. R. D., HIRSCH, U., SJOLANDER, N. O. & DOERSCHUK, A. P. (1960). Cosynthesis of tetracyclines by pairs of *Streptomyces aureofaciens* mutants. *Journal of the American Chemical Society* **82**, 5006–5007.
- MERRICK, M. J. (1976). A morphological and genetic mapping study of bald colony mutants of *Streptomyces coelicolor*. *Journal of General Microbiology* **96**, 299–315.
- OKANISHI, M., OHTA, T. & UMEZAWA, H. (1970). Possible control of formation of aerial mycelium and antibiotic production in *Streptomyces* by episomic factors. *Journal of Antibiotics (Japan)* **23**, 45–47.
- TOWNSEND, M. E., WRIGHT, H. M. & HOPWOOD, D. A. (1971). Efficient mutagenesis by near ultraviolet light in the presence of 8-methoxypsoralen in streptomyces. *Journal of Applied Microbiology* **34**, 799–801.
- VIVIAN, A. (1971). Genetic control of fertility in *Streptomyces coelicolor* A3(2): plasmid involvement in the interconversion of UF and IF strains. *Journal of General Microbiology* **69**, 353–364.
- VIVIAN, A. & HOPWOOD, D. A. (1970). Genetic control of fertility in *Streptomyces coelicolor*: the IF fertility type. *Journal of General Microbiology* **64**, 101–117.
- VIVIAN, A. & HOPWOOD, D. A. (1973). Genetic control of fertility in *Streptomyces coelicolor* A3(2): new kinds of donor strains. *Journal of General Microbiology* **76**, 147–162.
- WRIGHT, L. F. & HOPWOOD, D. A. (1976*a*). Identification of the antibiotic determined by the SCP1 plasmid of *Streptomyces coelicolor* A3(2). *Journal of General Microbiology* **95**, 96–106.
- WRIGHT, L. F. & HOPWOOD, D. A. (1976*b*). Actinorhodin is a chromosomally-determined antibiotic in *Streptomyces coelicolor* A3(2). *Journal of General Microbiology* **96**, 289–297.