A Pigmented Mycelial Antibiotic in *Streptomyces coelicolor*: Control by a Chromosomal Gene Cluster

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Streptomyces coelicolor was found to produce a third secondary metabolite, in addition to the antibiotics methylenomycin A and actinorhodin previously described. This is a red pigmented, highly non-polar compound with antibiotic activity against certain Gram-positive bacteria. Mutants lacking the red compound fell into five cosynthetic classes. Representatives of each of the five classes were mapped to the chromosome of the producing organism, in a closely linked cluster. Genetic studies provided evidence that this new metabolite is distinct from actinorhodin and indicated that the two pigments do not share parts of the same biosynthetic pathway.

INTRODUCTION

Streptomyces coelicolor A3(2) produces the antibiotics methylenomycin A (Wright & Hopwood, 1976a; Kirby & Hopwood, 1977) and actinorhodin (Wright & Hopwood, 1976b). During a genetic study of actinorhodin biosynthesis (Rudd & Hopwood, 1979), the organism was found to produce a third secondary metabolite, a red mycelial pigment with a limited antibiotic activity. We report here the isolation of various classes of mutants defective in production of this pigment, mapping of the mutations to a short segment of the chromosomal linkage map, and the demonstration by genetic tests that biosynthesis of this pigment is unrelated to that of actinorhodin.

METHODS

General. Minimal medium (MM), complete medium (CM) and general cultural and genetic techniques were those described by Hopwood (1967). Nutrient agar (NA) was Oxoid nutrient agar. R2 agar was that of Okanishi *et al.* (1974). Ultraviolet mutagenesis of spore suspensions was carried out using a 254 nm germicidal lamp at a dose rate of approximately $4.4 \text{ J m}^{-2} \text{ s}^{-1}$. The usual dose was 704 J m^{-2} , giving a survival of approximately 1 %.

Strains. All *Streptomyces coelicolor* strains were mutational or recombinational derivatives of strain A3(2) (Hopwood *et al.*, 1973) (Table 1). The locations of markers on the circular linkage map are shown in Fig. 1. Micro-organisms used in testing for sensitivity to the red pigment were those listed by Wright & Hopwood (1976a).

Cosynthesis tests of red mutants. Plates carrying a series of red mutants were replica-plated to lawns of other red mutants on plates of CM or R2 agar in order to detect production of red pigment in pairwise combinations. Those pairs which gave a positive result were re-examined in tests in which mutants were inoculated on CM a few millimeters apart in order to characterize the mutants as secretor or converter. In some combinations there was cosynthesis of red pigment in the first test but not in the second in which the two mutants were not in contact with each other.

Tests of antibacterial activity. Plugs (7 mm diam.) were cut from 7 d old confluent CM plate cultures of

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Table 1. *Derivatives of Streptomyces coelicolor* A3(2)

All strains were from the John Innes Institute culture collection.

Strain no.	Genetic markers	Plasmid status
A700	<i>proA1 argA1 cysD18</i>	SCP1 ⁻ SCP2 ⁺
B18	<i>hisA1 uraA1 strA1 act-118</i>	SCP1 ⁻ SCP2 ⁺
B229†	<i>hisA1 uraA1 strA1 act-118 red-60</i>	SCP1 ⁻ SCP2 ⁺
B244	<i>proA1 argA1 cysD18 guaA1 act-118</i>	SCP1 ⁻ SCP2 [*]
B269	<i>argA1 guaA1 act-117</i>	SCP1 ⁻ SCP2 [*]
B306	<i>argA1 guaA1 act-177</i>	SCP1 ⁻ SCP2 [*]
B307	<i>argA1 guaA1 act-178</i>	SCP1 ⁻ SCP2 [*]
B309	<i>argA1 guaA1 act-105</i>	SCP1 ⁻ SCP2 [*]
B314	<i>hisA1 guaA1 act-118 red-60</i>	SCP1 ⁻ SCP2 [*]
B317	<i>argA1 cysD18 guaA1 act-118 red-60</i>	SCP1 ⁻ SCP2 [*]
B369	<i>leuB5 mthB2 guaA1 act-118</i>	SCP1 ⁻ SCP2 [*]
B395	<i>proA1 argA1 cysD18 strA1 act-118 red-1</i>	SCP1 ⁻ SCP2 [*]
B396	<i>proA1 argA1 cysD18 strA1 act-118 red-7</i>	SCP1 ⁻ SCP2 [*]
B397	<i>proA1 argA1 cysD18 strA1 act-118 red-13</i>	SCP1 ⁻ SCP2 [*]
B398	<i>proA1 argA1 cysD18 strA1 act-118 red-17</i>	SCP1 ⁻ SCP2 [*]
B701†	<i>hisA1 uraA1 strA1 act-118 red-1</i>	SCP1 ⁻ SCP2 ⁺
B707†	<i>hisA1 uraA1 strA1 act-118 red-7</i>	SCP1 ⁻ SCP2 ⁺
B713†	<i>hisA1 uraA1 strA1 act-118 red-13</i>	SCP1 ⁻ SCP2 ⁺
B717†	<i>hisA1 uraA1 strA1 act-118 red-17</i>	SCP1 ⁻ SCP2 ⁺

† Mutant derivatives of strain B18 isolated in the course of this work.

Fig. 1. Linkage map of *S. coelicolor* A3(2) showing locations of the markers referred to in this paper.

red mutants and other strains, embedded in NA seeded with indicator organisms, and incubated overnight at the optimal growth temperature of the indicator.

Extraction of pigments from mycelium. Strains were grown on 60 mm diam. circles of dialysis membrane on the surface of CM agar. The mycelium was scraped off and extracted once with 10 to 20 ml methanol. The filtered extract was concentrated to about one-tenth volume by rotary evaporation.

Thin-layer chromatography (t.l.c.). Crude methanol extracts from mycelium were analysed on pre-coated silica gel plates (5 × 20 cm, 0.25 mm thick, silica gel 60, no. 5724; Merck, Darmstadt, Germany). Solvent A was chloroform/methanol (70:30, v/v); solvent B was chloroform/methanol (99:1, v/v).

RESULTS

Preliminary evidence for a new pigment

In a study of mutants blocked in actinorhodin biosynthesis (*act* mutants), seven phenotypic classes were recognized (Rudd & Hopwood, 1979). Five of these classes (III to VII)

Fig. 2. Examples of cosynthesis between *red* mutants. The central plate, with four *red* mutants of different classes, was replicated to lawns of the same four mutants on R2 agar. A, B707 (class A); B, B717 (class B); C, B701 (class C); D, B713 (class D). Cosynthesis is shown by dark patches of pigment synthesis.

produced diffusible pigments, presumably precursors or shunt products of actinorhodin, which is a pigmented antibiotic with acid-base indicator properties. Mutants of all classes except one (class VI) possessed a red pigment in the mycelium when grown on CM or MM. During growth the pigment remained associated with the mycelium rather than diffusing into the medium. On t.l.c. this pigment had an R_f of 0.84 in solvent A and 0.59 in solvent B. In mutants of *act* classes I and II, which produced no diffusible pigment, this red compound was the major pigment in crude methanol extracts of mycelium.

Members of the one class of *act* mutants (class VI) in which t.l.c. did not reveal any red mycelial pigment in extracts of cultures grown on CM could be shown to produce red mycelial pigment when grown on NA with added calcium salts (e.g. 4 mM); on this medium wild-type (*act*⁺) strains produced little actinorhodin and *act* mutants of classes III to VII made little or none of their characteristic diffusible pigment.

Thus, under appropriate conditions, all *act* mutants could be shown to accumulate a red pigment in their mycelium.

Table 2. *Classes of red mutants and their cosynthetic reactions*

<i>red</i> mutant class	No. of mutants isolated	Type strain	<i>red</i> allele of type strain	Cosynthetic reactions
A	20	B707	<i>red-7</i>	Converter to classes B, C, E
B	5	B717	<i>red-17</i>	Secretor to A; cosynthesizes with C*
C	5	B701	<i>red-1</i>	Secretor to A; cosynthesizes with B*
D	4	B713	<i>red-13</i>	None
E	3	B229	<i>red-60</i>	Secretor to A

* Polarity of cosynthesis not determined (see text).

Table 3. *Numbers and classes of the red mutants isolated from various act parents*

Parental strain	<i>act</i> class	No. of <i>red</i> mutants in each class					Total
		A	B	C	D	E	
B18	I	8	3	4	3	2	20
B307	I	2	0	0	0	0	2
B309	II	8	2	0	0	1	11
B306	II	0	0	1	1	0	2
B269	IV	2	0	0	0	0	2
Total		20	5	5	4	3	37

Isolation of mutants blocked in red pigment biosynthesis

Mutants (*red*) lacking the red mycelial pigment were isolated from five *act* mutants, belonging to classes I, II and IV, at a frequency of approximately 0.2 % after u.v.-irradiation. Only *act* mutants producing little (class IV) or no diffusible pigment (classes I and II) were suitable as parents since actinorhodin or related compounds obscured red pigment accumulation in the mycelium. The mutants were recognized as yellowish colonies lacking the normal red mycelial colour on CM plates carrying 500 to 1000 colonies.

Some pairs of *red* mutants cosynthesized red pigment when grown in mixed culture (Fig. 2); on the basis of these cosynthetic reactions the 37 *red* mutants fell into five classes, A to E (Table 2). The numbers of mutants of each class isolated from each parental strain are shown in Table 3. The polarity of some, but not all, of the cosynthetic reactions could be recognized: mutants of class A acted as converters with mutants of classes B, C and E, which acted as secretor strains. Intimate mixtures of mutants of classes B and C cosynthesized red pigment, but a polarity in this reaction could not be detected since no pigment was produced when the two strains were separated by a short distance on the agar surface.

The *red* mutants from the five *act* parents showed no alteration in *act* phenotype compared with the parental strains (Rudd & Hopwood, 1979): the *red act* strains showed precisely the same cosynthetic reactions with respect to actinorhodin as their *red*⁺ *act* parental strains; and the *red* mutants isolated in *act* class IV strains still produced small quantities of reddish-brown diffusible pigment characteristic of class IV mutants.

Genetic analysis of red mutants

The type strain of each class of *red* mutant was selected for genetic mapping. All strains used in these experiments carried *act-118* in order to avoid confusion of the *red*⁺/*red* classification by the presence of actinorhodin. One or both parents in all the crosses contained the variant SCP2* of the SCP2 sex plasmid, resulting in rather fertile, non-polarized crosses (Bibb *et al.*, 1977; Bibb, 1978) which were very suitable for mapping. Type strains of classes A, B, C and D (strains B707, B717, B701 and B713, respectively, all carrying markers *hisA1 uraA1 strA1*) were crossed with strain B244 (*proA1 argA1 cysD18 guaA1*) and recombinants inheriting *hisA*⁺ and *strA1* were selected. The results (data not shown) clearly indicated a map location for each of the four *red* alleles on the chromosome between *cysD* and *guaA*.

Fig. 3. Allele frequencies in crosses (Table 4) of strain B369 (*leuB5 mthB2 guaA1 act-118*) with recombinant strains carrying type mutations of *red* classes A to D (with markers *proA1 argA1 cysD18 strA1 act-118*). Capital letters indicate the class of the *red* mutation introduced into the cross in the following parent strains: A, B396; B, B398; C, B395; D, B397. Selection was made for *cysD*⁺ and *strA1* (indicated by triangles). The *proA1* and *act-118* markers have been ignored.

Table 4. Results of crosses of strain B369 with recombinant strains carrying the type mutations of *red* classes A to D (see Fig. 3)

Recombinant genotypes		Crossovers† required	No. of progeny from crosses involving each <i>red</i> allele (class)			
			<i>red-7</i> (A)	<i>red-17</i> (B)	<i>red-1</i> (C)	<i>red-13</i> (D)
<i>str mth leu</i> + <i>arg</i>		2, 6	0	0	0	0
<i>str mth leu</i> + +		2, 7	2	0	0	0
<i>str</i> + <i>leu</i> + <i>arg</i>		3, 6	3	1	5	2
<i>str</i> + <i>leu</i> + +		3, 7	7	0	4	7
<i>str</i> + + + <i>arg</i>		4, 6	21	16	24	17
<i>str</i> + + + +		4, 7	20	54	31	23
<i>str</i> + + <i>red arg</i>		5, 6	12	6	6	30
† <i>str</i> + + <i>red</i> +		5, 7	15	17	22	13
* <i>str</i> + <i>leu red arg</i>		3, 4, 5, 6	0	1	0	1
* Multiple crossovers if <i>red</i> is between <i>leuB</i> and <i>cysD</i>			0	1	0	1
† Multiple crossovers if <i>red</i> were between <i>cysD</i> and <i>argA</i>			15	17	22	13

‡ See map intervals in Fig. 3, assuming *red* is between *leuB* and *cysD*.

For more precise mapping, four recombinant strains bearing the markers *proA1 argA1 cysD18 strA1* and each carrying a different *red* allele (*red-7*, *red-17*, *red-1* or *red-13*: strains B396, B398, B395 and B397, respectively) were crossed with strain B369 (*leuB5 mthB2 guaA1*). The allele ratio for *red*⁺/*red* (Fig. 3) was compatible with two alternative locations of the *red* mutations, between *cysD* and *leuB* or between *cysD* and *argA*. The latter location was excluded by the data since it would have required relatively high frequencies of multiple crossover recombinants (Table 4).

Several crosses were made to map the type mutation of class E, *red-60*. As in the cases of the other *red* alleles, early crosses gave a location for *red-60* between *cysD* and *guaA*.

Fig. 4. Analysis of a cross between strains B317 [*argA1 guaA1 cysD18 act-118 red-60* (class E)] and B369 [*leuB5 mthB2 guaA1 act-118*] with selection for *cysD*⁺ and *mthB*⁺ (indicated by triangles). The two alternative locations for *red-60*, compatible with the observed allele frequencies, are discriminated by the tabulations of the segregations of *red*⁺/*red* with respect to *leu*⁺/*leu* and *arg*⁺/*arg*; the data indicate a position for *red-60* between *cysD* and *leuB* since segregation at the *red* locus is strongly correlated with that at the *leuB* locus but independent of segregation at the *argA* locus.

A further cross, of a recombinant (B317) bearing *red-60* and markers *argA1 cysD18 guaA1*, with B369, gave data which located *red-60* between *cysD* and *leuB* (Fig. 4).

Thus the type mutations of all five *red* classes were unambiguously located in the relatively short chromosomal region between *cysD* and *leuB*, somewhat removed from the actinorhodin cluster which is between *hisD* and *guaA* (Rudd & Hopwood, 1979).

Phenotypes of red-60 in combination with other act mutations and act⁺

To examine further the apparent independence of the red pigment and actinorhodin, double mutant strains carrying *red-60* with *act* mutations of classes II, III, IV, V and VI were prepared. This was achieved by crossing *act* strains carrying *cysD* (which is closely linked to the *red* alleles) with the *red-60* strain B314 bearing the *guaA* allele, which is closely linked to the *act* cluster. Selection of *cysD*⁺*guaA*⁺ yielded recombinants carrying *red-60* together with each *act* mutation at high frequency. Similarly, a cross between the *act*⁺ strain A700 (*proA1 argA1 cysD18*) and B314 gave an *act*⁺ *red-60* recombinant on selection for *cysD*⁺*guaA*⁺. The absence of red pigment production in these strains was demonstrated by t.l.c., and also by growing them on NA with 4 mM-Ca(NO₃)₂, in order to depress the production of actinorhodin and pigments related to it but to enhance any production of red pigment that might have occurred.

The phenotypes, in respect of the actinorhodin pathway, of strains of *act* classes II, III, IV, V and VI carrying *red-60* were unchanged when compared with the parent *red*⁺*act* strains: their diffusible pigments (where relevant) and cosynthetic reactions were those expected (Rudd & Hopwood, 1979). Similarly, an *act*⁺*red-60* strain produced normal levels of actinorhodin while having no red mycelial pigment.

Nature and antibiotic properties of the red pigment

The structure of the red pigment has not been elucidated. It appears to be highly non-polar, being almost insoluble in water but soluble in most organic solvents. In dilute alkaline solutions the pigment gives a yellow colour, turning to red in acidic solutions. In tests for antimicrobial activity of *red*⁺ and *red* strains, *red*⁺ strains gave minute (1 mm or less) zones

of inhibition of certain *Bacillus* spp., including *B. mycoides* and *B. cereus*. No red mutant showed any inhibition of these strains. No activity was noted against any of the other micro-organisms tested. The small zone size noted in the antibiotic assay used may well reflect the poor diffusibility of the red pigment in agar.

Recent work on the chemistry of the red mycelial pigment (S. W. Lee, C.-J. Chang, B. A. M. Rudd & H. G. Floss, unpublished data) indicates that the pigment is similar to prodigiosin-like pigments produced by other *Streptomyces* strains, including an actinorhodin-producing strain of *S. coelicolor* (see, for example, Gerber & Le Chevalier, 1976). Prodigiosin is a red pigment produced by *Serratia marcescens*. Prodigiosin and related (prodiginine) compounds produced by *Streptomyces* spp. and other micro-organisms have been reported to possess activity against Gram-positive bacteria (Gerber, 1975). Structural elucidation of the red pigment is in progress.

DISCUSSION

On the evidence of its genetics, the red mycelial pigment appears to be a new secondary metabolite of *S. coelicolor* A3(2). Genetically it is distinct from actinorhodin on the basis of several criteria: mutations abolishing red pigment production did not affect the *act* phenotype of parent strains; a red allele (*red-60*) could be crossed into *act* mutants of most classes and into *act*⁺ strains, with no effect on the *act* phenotype; all red mutants so far investigated mapped to a short segment of the chromosome between *cysD* and *leuB*; and no class of mutant was discovered in the search for *act* mutations (Rudd & Hopwood, 1979) that was both fully Act⁻ and Red⁻, apart from strains which can reasonably be assumed to be pleiotropically defective in secondary metabolite production, such as *bld* mutants which have no aerial mycelium (Merrick, 1976). Thus, it is unlikely that the red pigment is a precursor of actinorhodin, or that actinorhodin and the red pigment are end-products of a branched pathway. If there is a common precursor it must be a very early one.

Of the five classes of mutants blocked in red pigment production isolated in this study, one (class D) was not implicated in cosynthesis with other mutants. A similar situation exists with the classes of *act* mutants of *S. coelicolor*, in which mutants of one class (class II) showed no cosynthetic reactions with mutants of other classes (Rudd & Hopwood, 1979). This class may represent mutations in a regulatory gene. The order in which the genes represented by red mutants of classes B, C and E acted in the biosynthesis of the red pigment could not be elucidated, since although mutants of classes B and C cosynthesized red pigment in mixed culture, it was not possible to demonstrate which mutants acted as secretor and which as converter. Mutants of class A acted as converter to mutants of classes B, C and E and were thus revealed to be early-blocked mutants.

The finding that genes controlling the biosynthesis of this new antibiotic in *S. coelicolor*, like those for actinorhodin (Rudd & Hopwood, 1979), appear to be closely linked in a single chromosomal cluster strengthens the idea that genes for antibiotic synthesis in streptomycetes may generally be organized in clusters, perhaps corresponding to operons. This generalization, if confirmed, has obvious implications in attempts to transfer the genes for complete antibiotic pathways, or parts of them, from one species to another for analytical purposes or with the objective of developing strains capable of making new antibiotics.

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