Genetics of Actinorhodin Biosynthesis by *Streptomyces coelicolor* A3(2)

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A series of 76 mutants of *Streptomyces coelicolor* A3(2) specifically blocked in the synthesis of the binaphthoquinone antibiotic actinorhodin were classified into seven phenotypic classes on the basis of antibiotic activity, accumulation of pigmented precursors or shunt products of actinorhodin biosynthesis, and cosynthesis of actinorhodin in pairwise combinations of mutants. The polarity of cosynthetic reactions, and other phenotypic properties, allowed six of the mutant classes to be arranged in the most probable linear sequence of biosynthetic blocks. One member of each mutant class was mapped unambiguously to the chromosomal linkage map in the short segment between the *hisD* and *guaA* loci, suggesting that structural genes for actinorhodin biosynthesis may form an uninterrupted cluster of chromosomal genes.

INTRODUCTION

Streptomyces coelicolor A3(2) produces two characterized antibiotics: methylenomycin A, the genes for the synthesis of which appear to be carried by the SCP1 plasmid (Kirby *et al.*, 1975; Wright & Hopwood, 1976*a*; Kirby & Hopwood, 1977; Hopwood, 1978); and the pigmented antibiotic actinorhodin (Wright & Hopwood, 1976*b*).

Actinorhodin is an acid–base indicator; it is blue and very soluble in polar solvents at pH values above 7 and red and poorly soluble below pH 7. Its structure (Fig. 1) was elucidated by Brockmann *et al.* (1966). Actinorhodin is now considered to be a member of a chemically related group of antibiotics called isochromanequinones (Zeeck *et al.*, 1974), which also includes kalafungin (Hoeksema & Krueger, 1976), nanaomycins (Tanaka *et al.*, 1975; Ōmura *et al.*, 1976), the griseusins (Tsuji *et al.*, 1976), granaticin (Carbaz *et al.*, 1957) and the naphthocyclinones (Zeeck & Mardin, 1974; Zeeck *et al.*, 1974).

In a preliminary study of the genetic determination of actinorhodin biosynthesis, five mutations blocking its production (*act*) were mapped to the *S. coelicolor* chromosome (Wright & Hopwood, 1976*b*). Considering actinorhodin as a representative member of the isochromanequinones – and indeed of the very large and important class of polyketide streptomycete antibiotics, all of which are made by strains in which there is little or no genetic analysis (Hopwood & Merrick, 1977) – a more detailed study of its genetic determination was undertaken. We here report the isolation and properties of 75 *act* mutants which fall into a minimum of seven distinct phenotypic classes. At least one member of each class was mapped to the same short segment of the chromosome, suggesting that most, if not all, of the genes specifically involved in actinorhodin biosynthesis are closely linked.

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Fig. 1. The actinorhodin molecule according to Zeeck *et al.* (1974).

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

All strains were from the John Innes Institute culture collection.

Strain no.	Genetic markers	Plasmid status
1190	<i>hisA1 uraA1 strA1</i>	SCP1 ⁻ SCP2 ⁺
2377*	<i>hisA1 uraA1 strA1 act-3</i>	SCP1 ⁻ SCP2 ⁺
B1†	<i>hisA1 uraA1 strA1 act-101</i>	SCP1 ⁻ SCP2 ⁺
B17†	<i>hisA1 uraA1 strA1 act-117</i>	SCP1 ⁻ SCP2 ⁺
B18†	<i>hisA1 uraA1 strA1 act-118</i>	SCP1 ⁻ SCP2 ⁺
B22†	<i>hisA1 uraA1 strA1 act-122</i>	SCP1 ⁻ SCP2 ⁺
B40†	<i>hisA1 uraA1 strA1 act-140</i>	SCP1 ⁻ SCP2 ⁺
B41†	<i>hisA1 uraA1 strA1 act-141</i>	SCP1 ⁻ SCP2 ⁺
B201	<i>proA1 argA1 cysD18 uraA1 strA1 act-3</i>	SCP1 ⁻ SCP2*
B204	<i>proA1 argA1 cysD18 uraA1 strA1 act-101</i>	SCP1 ⁻ SCP2*
B210	<i>proA1 argA1 cysD18 uraA1 strA1 act-117</i>	SCP1 ⁻ SCP2*
B211	<i>proA1 argA1 cysD18 uraA1 strA1 act-118</i>	SCP1 ⁻ SCP2*
B212	<i>argA1 cysD18 uraA1 strA1 act-122</i>	SCP1 ⁻ SCP2*
B241	<i>argA1 guaA1 hisD3</i>	SCP1 ⁻ SCP2*
B400	<i>argA1 cysD18 uraA1 strA1 act-141</i>	SCP1 ⁻ SCP2*
2808	<i>argA1 cysD18 uraA1 strA1 act-140</i>	SCP1 ⁻ SCP2*

* An *act* mutant, derived from strain 1190 and described by Wright & Hopwood (1976*b*), adopted during the course of this work as the type strain of *act* class II.

† Mutant derivatives of strain 1190 isolated in the course of this work.

METHODS

General. Complete medium (CM), minimal medium (MM) and general cultural and genetic techniques were those described by Hopwood (1967). Nutrient agar was Oxoid nutrient agar (ONA). For u.v.-mutagenesis, spore suspensions were irradiated at a dose rate of approximately 4.4 W m² for 160 s, giving a survival of about 1%.

Strains. *Streptomyces coelicolor* strains (Table 1) were mutational and recombinational derivatives of strain A3(2) (Hopwood *et al.*, 1973). This is a strain of *Streptomyces violaceoruber* according to Kutzner & Waksman (1959). The locations on the circular linkage map of the markers used in genetic analysis are shown in Fig. 2. Micro-organisms used in testing for sensitivity to actinorhodin or related compounds were those listed by Wright & Hopwood (1976*a*).

Isolation of *act* mutants. The parent strain was that chosen by Wright & Hopwood (1976*b*), the SCP1⁻ strain 1190. Irradiated spores of many separate clones were plated to yield 500 to 1000 colonies per plate on CM. On this medium, actinorhodin accumulates in the mycelium in the red acidic form (proto-actinorhodin) which is relatively insoluble in water; thus *act* mutants are not obscured by nearby *act*⁺ colonies even on crowded plates. Exposure of mature colonies to ammonia fumes, by inverting the plates over 5 ml of 35% ammonia solution in a Petri dish lid, caused conversion of proto-actinorhodin to the blue, water-soluble form; blue droplets appeared on the colony surface and haloes of blue diffusing pigment developed around the colonies. Presumptive *act* colonies, recognized by lack of this response after a short period (Fig. 3), were picked and purified.

Cosynthesis tests. Patches of mutants, a few millimetres apart, were made on CM plates. Cosynthesis of actinorhodin was revealed by ammonia fuming or by overlaying the plates with *Staphylococcus aureus* in ONA (Fig. 4).

Tests of antibacterial activity. Plugs (7 mm diam.) were cut from 5 d-old confluent CM plate cultures of *act* mutants and other strains, embedded in ONA seeded with the indicator organism, and incubated overnight at the optimal growth temperature of the indicator.

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

Fig. 3. Isolation of *act* mutants. Colonies of strain 1190 grown on CM agar for 5 d (a) were exposed to ammonia fumes (b). Presumptive *act* mutants (one is seen at the bottom of b) showed no blue droplets or the halo of blue diffusing pigment characteristic of the starting strain.

RESULTS

Isolation and phenotypic classification of act mutants

Of 129 presumptive *act* mutants isolated, 75 were interpreted as having lesions specifically affecting actinorhodin synthesis since they appeared normal in other respects; they were 'true' *act* mutants. A few of these mutants produced small quantities of actinorhodin; by other phenotypic criteria (see below) they were interpreted as carrying leaky *act* mutations of particular classes. The remaining 54 strains were defective, to varying degrees, in differentiation, some being completely devoid of aerial mycelium (*bld* mutants; Merrick, 1976) and most of them produced limited quantities of actinorhodin; these strains were not studied further. The frequency of occurrence of 'true' *act* mutants after u.v.-irradiation was about 0.2% (for example, 21 out of 10700 colonies in one experiment).

Fig. 4. Cosynthesis tests between *act* mutants of classes I, II and IV. Patches of three mutants were made on two CM plates, in mirror image orientation. In (a), seen from above, the surface mycelial growth was killed by chloroform after 5 d and overlaid with *Staph. aureus* in nutrient agar. After overnight incubation, cosynthesis of actinorhodin is revealed by inhibition of the overlay. In (b), seen from below, cosynthesis was revealed by exposing the plate to ammonia fumes: a dark band of (blue) pigment has developed in that part of the converter class I mutant closest to the secretor class IV mutant. The class II mutant is not involved in cosynthesis. Note the corresponding location of the blue pigment (b) and the inhibition of *Staph. aureus* (a).

The 75 new *act* mutants and one of those isolated previously (Wright & Hopwood, 1976b) were classified phenotypically by three criteria: colour of any diffusible pigment(s) produced; antibiotic activity against *Staph. aureus*; and cosynthesis of actinorhodin with other mutants. Cosynthesis, where it occurred, was always non-reciprocal, one mutant (of one class) acting as secretor to another mutant (of a different class) which acted as converter (Fig. 4). The secretor, which supplied the converter with a precursor or cofactor required for actinorhodin biosynthesis, presumably carried a later block in the biosynthetic sequence than the converter mutant. The classes of mutant are summarized in Table 2.

Members of five of the seven mutant classes produced diffusible pigments, which were apparently different for each cosynthetic class. Most mutants appeared to produce a mixture of pigments at least some of which were acid-base indicators, and resembled actinorhodin in being less soluble at low pH values.

Apart from the two members of class VII, which produced small quantities of a red/blue acid-base indicator pigment assumed to be actinorhodin, only mutants of class V had any antibacterial activity and this showed a somewhat different specificity from that of the wild-type (*act*⁺): class V mutants were less active than the parent strain 1190 against some Gram-positive bacteria, notably *Bacillus cereus*, *Micrococcus lysodeikticus*, *Streptococcus faecalis* and *Corynebacterium glutamicum*, but there was little or no difference between class V mutants and the wild-type in their activity against *Bacillus subtilis* and *Staph. aureus*.

The mycelium of mutants of classes I and II, which produced no diffusible pigment, contained a red, non-diffusible pigment which was apparently genetically and structurally unrelated to actinorhodin (Rudd, 1978). This pigment was also present in *act*⁺ strains and in mutants of other *act* classes where it tended to be masked by diffusible pigments.

Genetic analysis of act mutations

Data from preliminary experiments to map a selection of 12 *act* mutations, in which each mutant was crossed with strains carrying a complementary set of standard markers, were consistent with a position for each *act* mutation between *cysD* and *strA* on the linkage map (Fig. 2). The mutants included the type member of each of the seven phenotypic classes

Table 2. *Classes of act mutants*

Mutant class	Type strain	Type mutation	No. of mutants in class	Diffusible pigment on CM	Antibiotic activity against <i>Staph. aureus</i>	Cosynthetic reactions	
						Converter from classes	Secretor to classes
II	2377	<i>act-3</i>	26	—	—	—	—
I	B18	<i>act-118</i>	13	—	—	IV, V, VI, VII	—
III	B41	<i>act-141</i>	7	Red	—	IV, V, VI, VII	—
VII*	B40	<i>act-140</i>	2	Light golden brown	(+)	?IV, V, VI	I, III
IV	B17	<i>act-117</i>	5	Reddish brown	—	V, VI	I, III, (?VII)
VI	B22	<i>act-122</i>	2	Light brown	—	V	I, III, IV, (?VII)
V	B1	<i>act-101</i>	21	Brown	+	—	I, III, IV, VI, (?VII)

* Both mutants of class VII produced small quantities of actinorhodin, presumed to be responsible for the limited inhibition of *Staph. aureus*. This also made the polarity of some cosynthetic reactions doubtful, but class VII mutants probably acted as converter with mutants of classes IV, V and VI.

Table 3. Analysis of crosses (Fig. 5) between strain B241 and strains carrying the type mutation of each *act* class (*argA1* and *proA1* ignored)

Recombinant genotypes	Crossovers† required	No. of progeny from crosses involving each <i>act</i> allele (class)						
		<i>act-118</i> (I)	<i>act-3</i> (II)	<i>act-141</i> (III)	<i>act-117</i> (IV)	<i>act-101</i> (V)	<i>act-122</i> (VI)	<i>act-140</i> (VII)
† <i>ura gua</i> + <i>his</i>	1, 5	0 } 2	0 } 5	1 } 3	2 } 11	0 } 3	2 } 4	8 } 13
+ <i>gua</i> + <i>his</i>	1, 6	2 } 2	5 } 5	2 } 2	9 } 9	3 } 3	2 } 2	5 } 5
† <i>ura</i> + + <i>his</i>	2, 5	9 } 14	16 } 50	13 } 13	16 } 53	9 } 69	5 } 7	15 } 22
+ + + <i>his</i>	2, 6	5 } 5	34 } 34	0 } 0	37 } 37	60 } 60	2 } 2	7 } 7
<i>ura</i> + <i>act his</i>	3, 5	20 } 24	6 } 26	10 } 10	3 } 11	7 } 11	8 } 10	3 } 12
+ + <i>act his</i>	3, 6	4 } 4	20 } 20	0 } 0	8 } 8	4 } 4	2 } 2	9 } 9
<i>ura</i> + + <i>act</i> +	4, 5	245 } 254	149 } 215	241 } 256	162 } 213	95 } 209	209 } 224	223 } 250
+ + + <i>act</i> +	4, 6	9 } 9	66 } 66	15 } 15	51 } 51	114 } 114	15 } 15	27 } 27
* † <i>ura</i> + + +	2, 3, 4, 5	1 } 2	1 } 1	1 } 1	0 } 1	0 } 0	3 } 3	1 } 1
* + + + +	2, 3, 4, 6	1 } 1	0 } 0	0 } 0	1 } 1	0 } 0	0 } 0	0 } 0
* † + <i>gua act</i> +	1, 2, 4, 6	0	0	0	0	0	1	0
Totals		296	297	283	289	292	249	298
* Multiple crossovers if <i>act</i> is between <i>hisD</i> and <i>guaA</i>		2	1	1	1	0	4	1
† Multiple crossovers if <i>act</i> were between <i>uraA</i> and <i>strA</i>		10	17	15	18	9	11	24

‡ See map intervals in Fig. 5, assuming *act* is between *hisD* and *guaA*.

Fig. 5. Allele frequencies in crosses of strain B241 with strains carrying the type mutation of each *act* class, calculated from the data in Table 3. Roman numerals indicate the class of the *act* mutant introduced into the cross in the following parent strains: I, B211; II, B201; III, B400; IV, B210; V, B204; VI, B212; VII, 2808.

listed in Table 2, together with one additional member of each of classes I and II and three of class V.

More precise analysis was carried out for each of the seven type mutations by making crosses between recombinant derivatives of the original mutant strains and strain B241, with selection for *strA1* and *cysD*⁺. All the parents lacked the SCP1 plasmid but carried the second recognized sex factor, SCP2, in its variant form SCP2* (Bibb *et al.*, 1977); such crosses are convenient for genetic analysis since they have an enhanced recombination frequency compared with SCP2⁺ × SCP2⁺ crosses and are typically non-polarized, each parent apparently functioning as donor or recipient with about equal probability (Bibb, 1978). In each cross, the allele ratio for *act*⁺/*act* was consistent with a map location either between *uraA* and *strA* or, as indicated by the preliminary crosses, between *cysD* and *strA*. The former location was excluded by the data, since it required relatively large proportions of recombinants to have arisen by multiple crossing-over (Table 3). The position of each *act* mutation in the *cysD*–*strA* segment was therefore narrowed down to the short interval between *hisD* and *guaA* since only this location was compatible with the gradient of allele frequencies at the *hisD*, *act* and *guaA* loci (Fig. 5).

DISCUSSION

Mutations leading specifically to non-production of actinorhodin fell into seven distinct phenotypic classes. Two of the classes each contained only two members so the possibility exists that other classes of mutants remain to be isolated. The cosynthetic reactions of most of the mutant classes are compatible with sequential blocks in a linear biosynthetic sequence:

Since their cosynthetic reactions were identical, the relative order of the blocks in mutants of classes I and III cannot be determined; however, since class III mutants secreted a diffusible pigment, they are perhaps more likely to represent the later block. The position of class VII mutants in the sequence is somewhat equivocal since both the mutants of this class were leaky; production of small quantities of actinorhodin by these mutants cast some doubt on reactions in which class VII mutants appeared to act as converter. However, their converter reaction with class IV mutants, at least, was reasonably certain, serving to establish their position in the sequence. Interestingly, class V mutants, which were the only mutants to have antibiotic activity against *Staph. aureus* not attributable to leaky actinorhodin production, represent blocks in the final step in the sequence defined by this set of mutants. Perhaps an immediate precursor of actinorhodin, or a shunt product of it, is antibiologically active.

Class II mutants, which showed no cosynthesis with any other mutant, cannot be included in the biosynthetic sequence. Regulatory mutations may be included in this class, but its large size (26 out of the 76 mutants) may make it unlikely that all members of the class are of this type. Another possibility is that this class includes polar mutations within an operon.

It is possible that mutants in classes IV to VII, which can act alternatively as secretor or converter, carry defects in genes determining single function enzymes involved in modifications of the molecule subsequent to the formation of the basic structure from a polyketide chain, while mutations in classes I and III, which act only as converter, are more likely to represent defects in a multifunctional aromatic synthetase which may assemble the chain. That the polyketide route (Birch, 1967) is indeed involved in actinorhodin biosynthesis, as is likely *a priori*, is strongly suggested by the finding that the nanaomycins, which closely resemble the actinorhodin half-molecule, are appropriately labelled by [1-¹³C]acetate (Tanaka *et al.*, 1975). ¹³C-labelling experiments with the related compounds granaticin (Snipes *et al.*, 1979) and α -naphthocyclinone (Schröder & Floss, 1978) also support this mode of derivation of the basic isochromane naphthoquinone unit.

At least one mutation of each of the seven classes of *act* mutations was mapped between the closely linked chromosomal loci *hisD* and *guaA*, suggesting that the genes directly involved in actinorhodin biosynthesis may form an uninterrupted cluster on the chromosome. This raises the interesting possibility that an operon may be involved. Recent developments in the study of plasmids in *S. coelicolor* (Bibb *et al.*, 1977) and of conditions for the efficient transformation of protoplasts by plasmid DNA (Bibb *et al.*, 1978) open the possibility of gene cloning in this organism. Analysis of the physical organization of the actinorhodin biosynthetic genes and of their regulation by *in vitro* studies are therefore exciting possibilities for the future.

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