Actinorhodin is a Chromosomally-determined Antibiotic in *Streptomyces coelicolor* A3(2)

By L. F. WRIGHT* AND D. A. HOPWOOD

John Innes Institute, Colney Lane, Norwich NR4 7UH

(Received 23 February 1976)

SUMMARY

Streptomyces coelicolor A3(2) synthesizes a second antibiotic, in addition to the plasmid-determined methylenomycin A. It was identified, primarily on the evidence of mass spectroscopy of its diethyl ester, as actinorhodin, which has been described previously in other strains. It inhibited most Gram-positive bacteria tested, but only at a comparatively high concentration. Five independent mutations leading to lack of actinorhodin synthesis were located between *cysD* and *strA* on the chromosome.

INTRODUCTION

Brockmann & Pini (1947) first noted the inhibitory activity of the pH indicator pigment of *Streptomyces coelicolor* against *Staphylococcus aureus*. The antibiotic was named actinorhodin, and its chemical structure was subsequently elucidated (Brockmann *et al.*, 1966).

Antibiotic synthesis by *S. coelicolor* A3(2) has recently been described (Kirby, Wright & Hopwood, 1975; Wright & Hopwood, 1976), and biosynthetic genes for methylenomycin A were shown to be carried on the SCP1 plasmid. We present evidence that *S. coelicolor* A3(2) produces at least one other major antibiotic component, namely actinorhodin, and that there are probably several closely linked chromosomal genes controlling its synthesis.

METHODS

General. Minimal medium (MM), complete medium (CM) and general techniques were those described by Hopwood (1967). Growth of cultures in liquid CM was as described by Wright & Hopwood (1976). Nutrient agar was Difco Bacto-nutrient agar. Ultraviolet-mutagenesis for the isolation of actinorhodin non-producing (*act*) mutants was by the method of Harold & Hopwood (1970).

Strains. *Streptomyces coelicolor* strains (Table 1) were mutational and recombinational derivatives of strain A3(2) (Hopwood, 1959). The locations on the circular linkage map of the markers used in genetic analysis are shown in Fig. 1. *Streptomyces coelicolor* (*violaceoruber*) strain 199 (Bradley, 1962) was used in preliminary comparative experiments on actinorhodin production. The strains of micro-organisms used in testing for sensitivity to actinorhodin were the same as those used for methylenomycin (Wright & Hopwood, 1976). *Staphylococcus aureus* NCTC8532 was used routinely in bio-assays of actinorhodin.

Extraction of actinorhodin from liquid cultures of strain 1190. The extraction procedure was that described by Brockmann *et al.* (1966). Mycelium (66 g), filtered from 3 l of culture

* Present address: Biological Laboratory, The University, Canterbury, Kent CT2 7NH.

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

All strains were from the John Innes Institute culture collection.

Strain no.	Genetic markers	Fertility type
8	<i>hisD3</i>	SCP ⁺ ₁
250	<i>hisA1 guaA1</i>	SCP ⁺ ₁
1190	<i>hisA1 uraA1 strA1</i>	SCP ⁻ ₁
2377*	<i>hisA1 uraA1 strA1 act-3</i>	SCP ⁻ ₁
2379*	<i>hisA1 uraA1 strA1 act-19</i>	SCP ⁻ ₁
2380*	<i>hisA1 uraA1 strA1 act-24</i>	SCP ⁻ ₁
2381*	<i>hisA1 uraA1 strA1 act-27</i>	SCP ⁻ ₁
2382	<i>argA1 uraA1 strA1 act-3</i>	SCP ⁻ ₁
2388	<i>hisD4 act-1</i> †	SCP ⁻ ₁
A700	<i>proA1 argA1 cysD18</i>	SCP ⁻ ₁

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

† The *act-1* mutation was previously referred to as *redA1*. Fig. 1. Linkage map of *Streptomyces coelicolor* A3(2) showing locations of the markers referred to in this paper.

medium, was washed with 6 l 0.1 M-HCl and successively extracted for 2 h with 330 ml 2 M-HCl and for 30 min with 330 ml acetone. After drying *in vacuo* for 4 to 5 h, the powdered mycelium was ground with 2 vols sand and actinorhodin was extracted in 330 ml 2 M-NaOH. The supernatant after centrifugation was adjusted to pH 3, and the red precipitate of crude actinorhodin which formed was collected, dried and ground to a powder. Exhaustive extraction with acetone in a Soxhlet extractor for 10 h left actinorhodin in the thimble. Pure actinorhodin was recovered from this material by extraction and recrystallization using dry 1,4-dioxan. The yield was 1.3 g actinorhodin.

Preparation of actinorhodin diethyl ester. This was prepared according to Brockmann *et al.* (1966). The presumptive actinorhodin (0.75 g) was suspended in 20 ml dry 1,4-dioxan and heated with 10 ml ethanol saturated with HCl gas in a sealed glass ampoule at 70 °C for 7 h with gentle agitation. After cooling, the residue was filtered off and dried. Recrystallization from benzene gave bright red crystals of the ester. A pure sample of actinorhodin diethyl

Fig. 2. Actinorhodin. A is the axis of symmetry referred to in the text.

ester was kindly donated by Professor H. Brockmann, Organisch-Chemisches Institut, University of Göttingen, Germany, for comparison with our material.

Infrared spectrometry. This was performed with the assistance of Dr J. Boulton, School of Chemical Sciences, University of East Anglia, on a Perkin-Elmer model 237 grating infrared spectrometer (Perkin-Elmer, Beaconsfield, Buckinghamshire).

Mass spectrometry. This was performed by Mr J. Eagles of the Agricultural Research Council Mass Spectrometry Service at the Food Research Institute, Colney Lane, Norwich, using an AEI MS-902 spectrometer at a source temperature of 200 °C and an ionization energy of 70 eV. Accurate masses were obtained at a resolution of 1000 (10 % valley definition), and processed by computer by the method of Johnson, Gordon & Self (1975).

Isolation of act mutants. Since the initial screening for actinorhodin production involved a test for antibiotic activity, an SCP1⁻ strain was chosen as the parent culture to avoid the complication of methylenomycin production. Another advantage of using SCP1⁻ strains was that they usually produce more actinorhodin than SCP1⁺ strains (R. Kirby & D. A. Hopwood, unpublished results). Strain 1190 was a particularly good producer of actinorhodin in solid and liquid media.

Ultraviolet-irradiated spores of several separate cultures of strain 1190 were diluted and plated on CM. The agar plug technique (Ichikawa *et al.*, 1971) was used to detect actinorhodin non-producing (*act*) mutants. Colony-centred plugs (6 mm diam.) were cut out after incubation for 48 h and arranged in rows in a 23 × 23 cm Bio-Assay dish (Nunc, Roskilde, Denmark). Moist filter paper was used to line the lid of the dish to prevent the plugs from drying during incubation for a further 48 h. Colonies were assayed for production of actinorhodin by pouring nutrient agar seeded with *Staph. aureus* into the dish.

RESULTS

Preliminary evidence for a second antibiotic

It was noted while testing various bacteria for their sensitivity to methylenomycin A, the antibiotic determined by the SCP1 plasmid, that control SCP1⁻ strains caused inhibition of some bacteria (Wright & Hopwood, 1976). Bioautography of thin-layer chromatograms of SCP1⁺ and SCP1⁻ culture filtrates also revealed a non-specific area of inhibition that was always located at the origin after running in solvents which moved methylenomycin A. These observations suggested the presence of a second antibiotic, which was produced by *S. coelicolor* A3(2) irrespective of the presence of the SCP1 plasmid.

A priori, it was likely that this antibiotic was actinorhodin in view of the resemblance of

Fig. 3. Mass spectra of actinorhodin diethyl ester: (a) sample donated by Professor H. Brockmann; (b) sample prepared from *S. coelicolor* A3(2), strain 1190.

S. coelicolor A3(2) to known actinorhodin producers, such as Bradley's strain 199 of *S. coelicolor* (*violaceoruber*), and because colonies of strain A3(2) have red undersides in acid conditions and produce a blue diffusible pigment in alkaline conditions. A comparison of the products in liquid culture of *S. coelicolor* A3(2) strain 1190 and Bradley's strain 199 supported this hypothesis. Thin-layer chromatography on silica gel (Kirby, Wright & Hopwood, 1975) in two solvents [benzene/methanol (95:5, by vol.); acetone/benzene (1:2, by vol.)] showed co-migrating pigments in the two strains.

Identification of the pigmented antibiotic of S. coelicolor A3(2)

Crystalline material from liquid-grown mycelium of strain 1190 was compared spectrophotometrically with its diethyl ester and with the diethyl ester of actinorhodin donated by Professor Brockmann. At $100 \mu\text{g ml}^{-1}$ in 0.1 M-NaOH , all three substances showed strong absorption peaks at 588 and 632 nm. The infrared spectrum of the presumptive actinorhodin was that expected for a compound with the structure shown in Fig. 2. Strong absorption peaks were located at 3200 cm^{-1} due to $-\text{OH}$ groups; at 3050 cm^{-1} due to the two $=\text{CH}$ groups; at 2850 to 2950 cm^{-1} due to $-\text{COOH}$ groups; at 1725 cm^{-1} due to carboxylic-acid carbonyl groups; and, notably, a maximum at approximately 1650 cm^{-1} due to quinone absorption. Quinone absorption is normally at slightly higher wavenumbers (1670 to 1690 cm^{-1}); the lower value was probably due to extensive hydrogen bonding within the molecule.

Comparison of the mass spectra of the diethyl esters of the presumptive actinorhodin from strain 1190 and the pure substance confirmed the identity (Fig. 3). When actinorhodin diethyl ester (mol. wt, 690) was heated on the spectrometer probe, the molecule broke in half about the axis of symmetry A shown in Fig. 2. The acquisition of three protons gave the resulting moiety a mass of 348 units and this entity was found to be the M^+ ion in further breakdown.

The spectrum of antibacterial activity of actinorhodin

Cultures of strains 1190 and Bradley's 199 were tested by the agar plug method against the bacteria previously tested for sensitivity to methylenomycin (Wright & Hopwood, 1976). Strains of the following species were inhibited weakly: *Bacillus cereus*, *B. megaterium*, *B. mycoides*, *B. subtilis*, *Micrococcus lysodeikticus* and *Staphylococcus aureus*.

Plugs of CM supplemented with actinorhodin at different concentrations were assayed against *Staph. aureus*. Extrapolation of the inhibition zones to zero radius indicated a minimum inhibitory concentration of actinorhodin against *Staph. aureus* of 25 to 30 $\mu\text{g ml}^{-1}$.

The amount of actinorhodin produced in liquid cultures of strain 1190 incubated for 72 h in the conditions described was calculated. Although the antibiotic was isolated from mycelium, a convenient way of expressing the antibiotic titre is in $\mu\text{g/ml}$ culture. It was assumed that there was no actinorhodin dissolved in the medium, since at this pH (about 6.7) the antibiotic is insoluble and remains localized in or on the mycelium. Assuming no losses during purification, the amount of actinorhodin produced as determined by chemical analysis was 430 $\mu\text{g ml}^{-1}$.

Isolation of act mutants and their phenotypes

Out of 4075 colonies tested, several produced no inhibition zones and were presumptive *act* mutants. Five of these gave rise to pure Act^- strains on subculture. They were designated *act-3*, *act-18*, *act-19*, *act-24* and *act-27*. The mutants *act-18* and *act-19* arose from the same mutagenized culture, and only *act-19* was used in genetic analysis.

Different phenotypes were observed in the mutants: *act-3* was pink on the underside of the colonies, *act-19* and *act-24* were orange and *act-27* was reddish-pink. Another mutant (*red-1*) had been isolated previously (Hopwood, 1965) on the basis of altered pigmentation. The available strain carrying *red-1* (strain 411) was SCP1^+ . An SCP1^- derivative was isolated after testing u.v.-treated spores of this strain, as described by Vivian & Hopwood (1970), and on testing against *Staph. aureus* it did not produce actinorhodin; *red-1* was therefore renamed *act-1*. Act^+ and Act^- colonies, arising through recombination in crosses described below, could be distinguished by means of the pH indicator properties of actinorhodin. Patches of spores from recombinants arising on selective media were streaked in arrays of 20 per plate on the same medium. After 3 days growth, the patches were replica plated to classify the standard markers. The *Act* character was scored by placing 5.0 ml ammonia (sp. gr. 0.88) in the lid of the inverted Petri dish. Act^+ patches rapidly changed colour from red to blue, and the blue actinorhodin soon began to diffuse; Act^- patches did not change colour. This method was compared with the bio-assay against *Staph. aureus* of agar plugs cut from the centre of patches, and a complete correlation was found when 100 colonies were tested. The ammonia test was therefore used routinely because of its simplicity and its resolution in revealing mixed patches containing Act^+ and Act^- colonies.

Table 2. Recombinant genotypes from crosses of the type *hisA1 uraA1 strA1 act SCP1⁻ × A700 (proA1 argA1 cysD18 SCP1⁻)*: selection was for *hisA⁺* and *strA1* (see Fig. 4)

Recombinant genotypes*	Number from each cross			
	2377 × A700	2379 × A700	2380 × A700	2381 × A700
<i>proA1 argA1 cysD18 uraA1 strA1</i>	5	0	0	7
<i>proA1 argA1 cysD18 uraA1 strA1 act</i>	3	0	1	0
<i>proA1 argA1 cysD18 strA1</i>	147	1	3	80
<i>proA1 argA1 cysD18 strA1 act</i>	102	2	1	0
<i>proA1 argA1 uraA1 strA1 act</i>	18	4	6	2
<i>argA1 cysD18 uraA1 strA1</i>	0	0	0	3
<i>proA1 argA1 strA1 act</i>	84	60	71	7
<i>proA1 uraA1 strA1 act</i>	0	2	1	0
<i>argA1 uraA1 strA1 act</i>	4	6	5	1
<i>proA1 strA1 act</i>	0	4	2	0
<i>proA1 argA1 strA1</i> †	0	1	0	0
<i>proA1 cysD18 strA1 act</i> †	0	1	0	0
<i>argA1 strA1 act</i> †	0	1	0	0
Totals	363	82	90	100

* Wild-type alleles omitted.

† Quadruple crossover classes on the hypothesis of *act* between *strA* and *cysD*.

Genetic analysis of *act* mutants

Crosses of the type *SCP1⁻ × SCP1⁻* were used in preliminary genetic analysis to avoid the presence of methylenomycin. These have a low fertility but are typically non-polarized, each parent acting as donor in some zygotes and as recipient in others (Hopwood *et al.*, 1973). This provides a convenient situation for preliminary mapping, just as in *NF × NF* crosses (Hopwood, 1967).

Initial mapping of *act-3*, *act-19*, *act-24* and *act-27* was done by crossing each of the strains 2377, 2379, 2380, and 2381 (*hisA1 uraA1 strA1 act*) with strain A700 (*proA1 argA1 cysD18*). The data are in Table 2. Allele frequencies (Fig. 4) gave possible locations for *act-3*, *act-19* and *act-24* between *strA* and *uraA*, or between *cysD* and *strA*. To minimize quadruple crossing-over classes, the mutations were placed in the latter region (see the tests of independence in Fig. 4). The *act-27* mutation was also located anticlockwise of *strA* by these criteria but no recombination occurred between *act-27* and *cysD*.

A recombinant from the 2377 × A700 cross (strain 2382; *argA1 uraA1 strA1 act-3*) was crossed in duplicate to strain 8 (*hisD3*). Evidence for a map location between *strA* and *hisD* came from the allele frequencies for the combined data (Fig. 5) and the finding of five recombinants of genotype *act-3 hisD* and none of the complementary *act⁺ his⁺* genotype (Table 3). The *guaA* locus lies between *hisD* and *strA* (Fig. 1). Sixty recombinants inheriting *argA⁺* and *strA* selected from a cross of strains 2382 and 250 (*hisA1 guaA1*) showed no recombination between *guaA* and *act-3*.

A previous investigation had mapped the *act-1* (*redA1*) mutation between *hisD* and *strA* (Hopwood, 1965). Mapping of the *act-1* mutation with respect to *guaA* was attempted by crossing strain 2388 (*hisD4 act-1*) with strain 250, with selection for *hisA⁺* and *hisD⁺*. Out of 96 progeny, none showed recombination between *act-1* and *guaA*, as in the cross between strain 2382 and strain 250.

Fig. 4. Interpretation of crosses designed to determine the locations of *act* mutations. Strains (a) 2377, (b) 2379, (c) 2380 and (d) 2381 (inner circles) were each crossed with strain A700 (outer circles) and the alleles *hisA*⁺ and *strA*⁺ (indicated ▲) were selected. The allele ratio for each *act* mutation is compatible with a location between *strA* and *uraA* or between *cysD* and *strA*. Analyses of the segregation of *act* in relation to that of *ura* or of *cys* (see the 2 × 2 tabulations) indicate a position between *cysD* and *strA* (or close to *cysD* in the case of cross d). The frequencies of individual recombinant genotypes are given in Table 2.

DISCUSSION

Streptomyces coelicolor A3(2) produces the pigmented antibiotic actinorhodin, previously studied chemically by Brockmann *et al.* (1966). Apart from the observation that actinorhodin inhibited *Staph. aureus*, its range of antibiotic activity and the genetic determination of its synthesis were unknown.

All the mutations leading to loss of actinorhodin synthesis mapped in the same region of the A3(2) linkage map, between *cysD* and *strA*. Since different mutants showed various types of pigmentation, it is unlikely that the mutations are all allelic. The isolation and mapping of more mutations and, if possible, the application of complementation tests would permit

Table 3. Recombinant genotypes from duplicate crosses of strain 2382 (*argA1 uraA1 strA1 act-3 SCP1⁻*) with strain 8 (*hisD3 SCP1⁺*): selection was for *argA⁺* and *strA1* (see Fig. 5)

Recombinant genotypes*	Number from each cross		Total
	A	B	
<i>hisD3 uraA1 strA1</i>	1	2	3
<i>hisD3 uraA1 strA1 act-3</i>	1	1	2
<i>hisD3 strA1</i>	13	24	37
<i>hisD3 strA1 act-3</i>	1	2	3
<i>uraA1 strA1 act-3</i>	50	62	112
<i>strA1 act-3</i>	34	40	74
Totals	100	131	231

* Wild-type alleles omitted.

Fig. 5. Interpretation of a cross designed to locate *act-3* with respect to *hisD*. Strain 2382 (inner circle) was crossed with strain 8 (outer circle) and the alleles *argA⁺* and *strA1* (indicated ▲) were selected. The allele ratio for *act-3* indicates a location between *strA* and *hisD*. The frequencies of individual recombinant genotypes are given in Table 3.

exploration of the possibility that a gene cluster is involved in actinorhodin synthesis. Remarkably little is yet known about the organization of genes controlling antibiotic synthesis, and actinorhodin in *S. coelicolor* A3(2) may provide an illuminating model system for such a study, particularly since the important polyketide route is likely to be involved in actinorhodin synthesis (Brockmann *et al.*, 1966).

Actinorhodin inhibited the growth of some Gram-positive bacteria although its activity was low. The minimum inhibitory concentration of actinorhodin against *Staph. aureus* was 25 to 30 $\mu\text{g ml}^{-1}$.

Perhaps it is significant that actinorhodin, an antibiotic of low activity, is produced in large quantities by a wild-type strain of *S. coelicolor*. The calculated figure was at least 430 $\mu\text{g ml}^{-1}$, which contrasts with 10 to 50 $\mu\text{g penicillin ml}^{-1}$ for *Penicillium notatum* wild-types (Alikhanian, 1962) and 50 to 100 $\mu\text{g streptomycin ml}^{-1}$ for *Streptomyces griseus* (Schatz & Waksman, 1945). If antibiotic production is adaptive in increasing the competitive advantage of microbes in areas of high nutrient concentration (Garrett, 1956; Brian, 1957), the

producing organism might have evolved a high level of production to compensate for the weak activity of the antibiotic.

It is not unusual to find that a single *Streptomyces* strain produces more than one antibiotic. A novel situation exists, however, in *S. coelicolor* A3(2) where, of the two antibiotics so far discovered, one, methylenomycin, is plasmid determined (Kirby, Wright & Hopwood, 1975; Wright & Hopwood, 1976) and the other, actinorhodin, is chromosomally determined.

One of us (L.F.W.) is grateful to the Agricultural Research Council for a Postgraduate Studentship.

REFERENCES

- ALIKHANIAN, S. I. (1962). Induced mutagenesis in the selection of microorganisms. *Advances in Applied Microbiology* **4**, 1-50.
- BRADLEY, S. G. (1962). Biosynthesis of an actinorhodin-like pigment by *Streptomyces violaceoruber*. *Developments in Industrial Microbiology* **3**, 362-369.
- BRIAN, P. W. (1957). The ecological significance of antibiotic production. In *Microbial Ecology*, pp. 168-188. Edited by R. E. O. Williams and L. C. Spicer. Cambridge University Press.
- BROCKMANN, H. & PINI, H. (1947). Actinorhodin, ein roter Farbstoff aus Actinomyceten. *Naturwissenschaften* **34**, 190.
- BROCKMANN, H., ZEECK, A., VAN DER MERWE, K. & MÜLLER, W. (1966). Die Konstitution des Actinorhodins. *Justus Liebigs Annalen der Chemie* **698**, 209-229.
- GARRETT, S. D. (1956). *The Biology of Root-infecting Fungi*. Cambridge University Press.
- HAROLD, R. J. & HOPWOOD, D. A. (1970). Ultraviolet-sensitive mutants of *Streptomyces coelicolor*. 1. Phenotypic characterization. *Mutation Research* **10**, 427-438.
- HOPWOOD, D. A. (1959). Linkage and the mechanism of recombination in *Streptomyces coelicolor*. *Annals of the New York Academy of Sciences* **81**, 887-898.
- HOPWOOD, D. A. (1965). New data on the linkage map of *Streptomyces coelicolor*. *Genetical Research, Cambridge* **6**, 248-262.
- HOPWOOD, D. A. (1967). Genetic analysis and genome structure in *Streptomyces coelicolor*. *Bacteriological Reviews* **31**, 373-403.
- HOPWOOD, D. A., CHATER, K. F., DOWDING, J. E. & VIVIAN, A. (1973). Advances in *Streptomyces coelicolor* genetics. *Bacteriological Reviews* **37**, 371-405.
- ICHIKAWA, T., DATE, M., ISHIKURA, T. & OZAKI, A. (1971). Improvement of kasugamycin-producing strain by the agar piece and the prototroph method. *Folia microbiologica* **16**, 218-224.
- JOHNSON, M. W., GORDON, B. J. & SELF, R. (1975). Mass-spectral data processing using a general purpose computer. *Laboratory Practice*, 267-271.
- KIRBY, R., WRIGHT, L. F. & HOPWOOD, D. A. (1975). Plasmid determined antibiotic synthesis and resistance in *Streptomyces coelicolor*. *Nature, London* **254**, 265-267.
- SCHATZ, A. & WAKSMAN, S. A. (1945). Strain specificity and production of antibiotic substances. IV. Variations among actinomycetes, with special reference to *Streptomyces griseus*. *Proceedings of the National Academy of Sciences of the United States of America* **31**, 129-137.
- VIVIAN, A. & HOPWOOD, D. A. (1970). Genetic control of fertility in *Streptomyces coelicolor* A3(2): the IF fertility type. *Journal of General Microbiology* **64**, 101-117.
- WRIGHT, L. F. & HOPWOOD, D. A. (1976). Identification of the antibiotic determined by the SCP1 plasmid of *Streptomyces coelicolor* A3(2). *Journal of General Microbiology* **95**, 96-106.