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

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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 SCPI 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

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

All strains were from the John Innes Institute culture collection.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Genetic markers</th>
<th>Fertility type</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>hisD₃</td>
<td>SCP₁⁺</td>
</tr>
<tr>
<td>250</td>
<td>hisA₁ guaA₁</td>
<td>SCP₁⁺</td>
</tr>
<tr>
<td>1190</td>
<td>hisA₁ uraA₁ strA₁</td>
<td>SCP₁⁻</td>
</tr>
<tr>
<td>2377*</td>
<td>hisA₁ uraA₁ strA₁ act-3</td>
<td>SCP₁⁻</td>
</tr>
<tr>
<td>2379*</td>
<td>hisA₁ uraA₁ strA₁ act-19</td>
<td>SCP₁⁻</td>
</tr>
<tr>
<td>2380*</td>
<td>hisA₁ uraA₁ strA₁ act-24</td>
<td>SCP₁⁻</td>
</tr>
<tr>
<td>2381*</td>
<td>hisA₁ uraA₁ strA₁ act-27</td>
<td>SCP₁⁻</td>
</tr>
<tr>
<td>2382</td>
<td>argA₁ uraA₁ strA₁ act-3</td>
<td>SCP₁⁻</td>
</tr>
<tr>
<td>2388</td>
<td>hisD₄ act-1†</td>
<td>SCP₁⁻</td>
</tr>
<tr>
<td>A700</td>
<td>proA₁ argA₁ cysD₁₈</td>
<td>SCP₁⁻</td>
</tr>
</tbody>
</table>

* 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 610·1 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
The antibiotic actinorhodin in S. coelicolor A3(2)

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

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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 x 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
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 µg 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 μg 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 μg/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 μg 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 hisAI uraAI strAI act SCP1− × A700 (proAI argAI cysD18 SCP1−): selection was for hisA+ and strAI (see Fig. 4)

<table>
<thead>
<tr>
<th>Recombinant genotypes*</th>
<th>2377 × A700</th>
<th>2379 × A700</th>
<th>2380 × A700</th>
<th>2381 × A700</th>
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<tr>
<td>proAI argAI cysD18 uraAI strAI act</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>proAI argAI cysD18 uraAI strAI act</td>
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<td>0</td>
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<td>0</td>
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<td>proAI argAI cysD18 strAI act</td>
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<td>1</td>
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<td>80</td>
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<td>0</td>
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<td>2</td>
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<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
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<td>0</td>
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<tr>
<td>argAI strAI act</td>
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<tr>
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<td>363</td>
<td>82</td>
<td>90</td>
<td>100</td>
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</table>

* Wild-type alleles omitted.
† Quadruple crossover classes on the hypothesis of act between strA and cysD.

Genetic analysis of act mutants

Croses 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 (hisAI uraAI strAI act) with strain A700 (proAI argAI 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-overs, 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-I (redA1) mutation between hisD and strA (Hopwood, 1965). Mapping of the act-I mutation with respect to guaA was attempted by crossing strain 2388 (hisD4 act-I) with strain 250, with selection for hisA+ and hisD+. Out of 96 progeny, none showed recombination between act-I and guaA, as in the cross between strain 2382 and strain 250.
The antibiotic actinorhodin in *S. 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)

<table>
<thead>
<tr>
<th>Recombinant genotypes*</th>
<th>A</th>
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<th>Total</th>
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<td>3</td>
</tr>
<tr>
<td>hisD3 uraA1 strA1 act-3</td>
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<td>24</td>
<td>37</td>
</tr>
<tr>
<td>hisD3 strA1</td>
<td>50</td>
<td>62</td>
<td>112</td>
</tr>
<tr>
<td>strA1 act-3</td>
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<td>40</td>
<td>74</td>
</tr>
<tr>
<td>Totals</td>
<td>100</td>
<td>131</td>
<td>231</td>
</tr>
</tbody>
</table>

* 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 μg 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 μg ml⁻¹, which contrasts with 10 to 50 μg penicillin ml⁻¹ for Penicillium notatum wild-types (Alikhanian, 1962) and 50 to 100 μg 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


