Genetic and Biochemical Characterization of the red Gene Cluster of Streptomyces coelicolor A3(2)

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Production of the red antibiotic, undecylprodigiosin, by Streptomyces coelicolor A3(2) was studied by DNA cloning and biochemical analysis. Over 21 kb of genomic DNA were cloned, in several segments, into plasmid vectors. The cloned DNA 'complemented' several specific mutations in the red gene cluster. Four red genes (redA, B, E, and F) were mapped to different regions within the cloned DNA. Screening with redE probes for DNA homologies among various streptomycetes revealed hybridizing DNA in three strains, one of them not known to synthesize prodigiosin pigments. Biochemical studies using protoplasted cells revised our interpretation of the nature of redE and redF mutations. Two forms of undecynornprodigiosin: S-adenosylmethionine O-methyltransferase activity on gel filtration columns were detected: a very high molecular mass peak (> 5 MDal) and a 49 kDal peak. Analyses of extracts from red mutants suggested that these two forms are related, and that at least the redE and redF gene products are necessary for O-methyltransferase activity in vivo. Lack of activity of the redE gene in a heterologous host, S. glaucescens, is consistent with the necessity for a biosynthetic complex involving several red gene products for efficient expression. Experiments in liquid antibiotic production medium indicated that prodigiosin compounds in S. coelicolor are examples of 'secondary metabolites' whose synthesis lags behind that of cell mass. The peak of specific activity of O-methyltransferase coincided with the 'late exponential' phase of growth. Thus, understanding the genetic regulation of undecylprodigiosin biosynthesis in S. coelicolor may be relevant to other antibiotic production pathways, and perhaps to 'secondary' metabolism in general.

INTRODUCTION

Understanding the regulation of gene expression during secondary metabolism is of widespread interest for both academic and industrial reasons. Antibiotic production by Streptomyces is particularly suitable for the study of these questions because of recent advances in recombinant DNA technology in members of this genus (Chater et al., 1982; Hopwood & Chater, 1982; Bibbet al., 1983; Hopwood et al., 1983, 1984). Previous work (Feitelson & Hopwood, 1983) had resulted in the isolation of two clones involved in the production of an O-methyltransferase leading to the synthesis of undecylprodigiosin, the red antibiotic of Streptomyces coelicolor A3(2). Here we describe further physical and genetic mapping of the red gene cluster, a search for red sequences among diverse streptomycetes, partial purification of the O-methyltransferase from protoplasts of the wild-type and red mutants, and initial characterization of the time course of production of undecylprodigiosin in liquid medium.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in, or derived from, this study are listed in Tables 1 and 2.

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### Table 1. Bacterial strains

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<tr>
<th>Organism</th>
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<th>Genetic markers</th>
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<td>TK16</td>
<td>argAl guaAl act(V)117 redA59</td>
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<td></td>
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<td>JF5</td>
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<td></td>
</tr>
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<td></td>
<td>B257</td>
<td>argAl guaAl act(VII) red⁺</td>
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<tr>
<td></td>
<td>M145</td>
<td>prototroph</td>
<td>Williams (1973)</td>
</tr>
<tr>
<td><em>Serratia marcescens</em> HY</td>
<td>W5445</td>
<td>C600 leuB pro thrB rpsL lac tonA supE44</td>
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<td><em>Escherichia coli</em></td>
<td>ED8767</td>
<td>recA54 met supE4 supF hsd s⁺ r⁺ m⁺</td>
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Table 2. Plasmids

<table>
<thead>
<tr>
<th>DNA Construction</th>
<th>Reference or figure</th>
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<tr>
<td>pIJ255 E. coli cosmid vector; pJB8 with a 0.89 kb SphI insert for vph</td>
<td>T. Kieser (pers. comm.)</td>
</tr>
<tr>
<td>pIJ350 Streptomyces multicopy vector derived from pIJ101, carrying tsr</td>
<td>Kieser et al. (1982)</td>
</tr>
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<td>pIJ702 Streptomyces multicopy vector derived from pIJ350, carrying tsr + mel⁺</td>
<td>Katz et al. (1983)</td>
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<td>pIJ750 pIJ702 with a 4.72 kb BciI insert complementing redE</td>
<td>Feitelson &amp; Hopwood (1983)</td>
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<tr>
<td>pIJ751 pIJ702 with a 2.00 kb BclI-SstI insert complementing redE</td>
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<td>pIJ752 pIJ702 with a 9.65 kb SstI cosmid-derived insert complementing redB,E,F</td>
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<td>pIJ754 pIJ255 with a 9.65 kb cosmid-derived insert (redB,E,F probe)</td>
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<td>pIJ757 pIJ702 with a 13.03 kb MboI partial insert complementing redB,E,F</td>
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<td>pIJ759 pIJ922 with a 20.7 kb MboI partial insert complementing redA,B,E,F</td>
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The red gene cluster of *S. coelicolor*

**Media.** Solid media used for *Streptomyces* strains were R2YE (Thompson et al., 1982) or minimal medium (MM: Hopwood, 1967). Liquid cultures were grown in YE + 34% (w/v) sucrose + 5 mM-MgCl₂ (Chater et al., 1982) or aureothricin medium (AM: Okanishi et al., 1970) with the following composition (per l): Difco beef extract, 5 g; Difco Bacto-Peptone, 5 g; NaCl, 5 g; glycerol, 10 ml (added after autoclaving). Selection for thioestrepton resistance was made at 50 μg ml⁻¹ in plates or 5 μg ml⁻¹ in liquid. Thioestrepton was kindly provided by Mr S. J. Lucania of E. J. Squibb and Sons, New Brunswick, NJ, USA.

*Escherichia coli* was grown in Luria broth or Luria agar (Miller, 1972). Transformants were selected and grown with a final concentration of 50 μg ampicillin ml⁻¹.

**Transformation.** *S. coelicolor* strains were transformed by a rapid method, modified from the procedure described by Thompson et al. (1982). Frozen protoplasts (1 ml of 4 × 10⁶ ml⁻¹ stored at −70 °C), were quickly thawed at 37 °C, then dispensed into a number of sterile 1-5 ml Eppendorf centrifuge tubes. The protoplasts were pelleted in an Eppendorf microtube for 10 s. The supernatant was discarded and the cell pellet gently resuspended in the residual buffer. Plasmid DNA was added in less than 10 μl TE buffer (10 mM-Tris/HCl, pH 8.0; 1 mM-EDTA), 0-1 ml 25% (w/v) PEG 1000 (Koch-Light) in T buffer (Thompson et al., 1982) was immediately added and mixed, and the entire mixture was spread gently on pre-dried R2YE agar. After allowing 16–24 h at 30 °C for regeneration, the plates were overlaid with 3 ml Difco soft nutrient agar containing 500 μg thioestrepton ml⁻¹ (Kieser et al., 1982). Transformed protoplasts formed sporulating and Red⁺ colonies 2–3 d after overlay selection.

*E. coli* strains were transformed by the CaCl₂-heat shock method of Lederberg & Cohen (1972).

**DNA purification.** Total DNA was isolated from *Streptomyces* strains as described by Chater et al. (1982). *Streptomyces* plasmid DNA was purified from mycelium grown in liquid, or on the surface of sterile dialysis membranes laid over agar medium, by the method of Kieser (1984b). *E. coli* plasmid DNA was isolated from 100 ml liquid cultures by scaling up the Birnboim & Doly (1979) method.

**Cloning, restriction digests and electrophoresis.** In general, DNA fragments were cloned in linearized, calf-intestine alkaline phosphatase-treated (Boehringer-Mannheim, grade I) plasmid vectors using insert-to-plasmid molar ratios of about 5 to 1, at a final DNA concentration of 40–100 μg ml⁻¹ (Feitelson & Hopwood, 1983). Restriction enzymes were used under conditions recommended by the supplier (BRL); T4 DNA ligase, prepared by C. J. Bruton, was used as described by Sugino et al. (1977). Reactions were halted by heating at 70 °C for 10 min, followed by 2-propanol precipitation and resuspension in sterile TE buffer.

Agarose gel electrophoresis was performed in 20 × 20 cm submerged horizontal slab gels (0.7%, or 1%) in TEB (89 mM-Tris/HCl, pH 8.3; 2.5 mM-EDTA; 89 mM-boric acid). The gels were stained for 15 min in ethidium bromide (0.5 μg ml⁻¹), destained for 10 min in distilled water, and photographed with UV (310 nm) light. Molecular weight standards were phage λ DNA digested with HindIII and pBR322 digested with HaeIII (Southern, 1979). Fragment sizes were measured with a Houston digitizer linked to an Apple IIe computer by the method of Kieser (1984a).

**Nick-translation and Southern hybridization.** Nick-translation of pIJ755 and pIJ761, and hybridization to BamHI-digested chromosomal DNA from various *Streptomyces* strains, were as described by Rigby et al. (1977) and Wahl et al. (1979).

**Other biochemical procedures.** *Streptomyces* mycelium grown on the surface of sterile dialysis membranes on R2YE (with thioestrepton for plasmid-containing strains) was scraped off after 3 d growth into four times its weight (80 ml liquid cultures by scaling up the Birnboim & Doly, 1979) with the following composition (per l): Difco beef extract, 70 g; NaCl, 5 g; MgCl₂, 5 g; CaCl₂, 25 g; TES buffer, pH 7.2: 2 mg lysozyme ml⁻¹; H. Ikeda, personal communication). Incubation was at 30 °C for 30 min to form protoplasts. After trituration, the protoplasts were filtered through cotton wool, centrifuged at 3000 g for 8 min, and the supernatant containing the enzyme activity was transferred to a fresh tube. All further operations were carried out at 0–4 °C.

Phenylmethylsulphonyl fluoride (PMSF) was added to 0.1 mM to inhibit proteolysis. Solid ammonium sulphate was slowly added to 60% saturation (390 mg ml⁻¹); the solution was stirred for 30 min, and the precipitate was recovered from the pellet by gentle resuspension into column buffer [CB: 5 mM-phosphate buffer, pH 6.5; 5 mM-EDTA; 1 mM-dithiothreitol (DTT); 10% v/v, glycerol; 0.1 mM-PMSF]. Protein concentration was determined by the dye-containing 172-5 ml fraction, 24 ml absorbance at 280 nm

The red gene cluster of *S. coelicolor* JF3 extracted with methanol, partitioned into chloroform, concentrated by rotary evaporation, and resuspended in methanol to *A₄₀₀ = 72*. The reactions were worked up as previously described (Feitelson & Hopwood, 1983).
Physical and genetic mapping of the red gene cluster

Feitelson and Hopwood (1983) described the cloning of two DNA fragments which restored the activity of an O-methyltransferase involved in methylation of the yellow undecylprodigiosin precursor to the red undecylprodigiosin final product to redE mutants defective in this activity. Subsequent shotgun cloning of S. coelicolor M145 (red+) DNA into various plasmid vectors has now resulted in the isolation of several further overlapping cloned red+ DNA inserts. Primary selection was done in the redE60 mutant (JF4); redE+ plasmid DNA was then purified and introduced by retransformation into protoplasts of this and other red mutants, with selection for thiostrepton resistance, in order to assay the genetic content of the plasmid inserts.

Fig. 1 shows the series of clones and their genetic content. Note that redF is a new gene represented by a mutation (red-57) previously grouped with other redE mutants (Rudd & Hopwood, 1980) but now recognized as a separate gene (see below). pIJ750 and pIJ752 were described previously. pIJ755 was obtained from one Red+ colony among approximately 2000 thiostrepton-resistant JF4 transformants resulting from a shotgun cloning of PstI fragments of M145 DNA ligated into pIJ350 digested with the same enzyme. pIJ755 DNA complemented the redA and redE mutations, but failed to complement the redF or redD mutations.

In a similar manner, pIJ757 was obtained from a ligation between M145 DNA partially digested with MboI (to an average size of 15–20 kb) and pIJ702 cleaved with BglII. The recombinant plasmid came from one of two Red+ colonies among about 4000 Mel- Thior transformants of JF4; the other plasmid, pIJ758, had only a 4.2 kb insert and was not extensively mapped. Although the insert in pIJ757 extends 1.5 kb to the left and 1.6 kb to the right of the insert in pIJ752, it has the same complementing activity for redE and redF mutations.

Finally, pIJ759 was obtained by ligating partially MboI-cleaved and size-fractionated (15–20 kb) M145 DNA into pIJ922; this was from the same experiment which resulted in the isolation of two actinorhodin clones (Malpartida & Hopwood, 1984). The 20.7 kb insert in pIJ759 complemented all four classes of red mutations previously cloned; it extends 0.9 kb to the left of the leftmost site of the insert in pIJ757 and 4.74 kb to the right of the rightmost site of the insert in pIJ755. The low copy number of pIJ759 (1–2 per chromosome) compared to the other clones in the high copy number pIJ101-based vectors (several tens to hundreds of copies per chromosome) seemed to have little effect on the Red+ phenotypes for the four red genes cloned; e.g. the level of pigmentation was restored to roughly equal levels by both types of clones.

In order to assign genes to particular restriction fragments, regions of pIJ752, pIJ755 and pIJ759 were subcloned into pIJ702, and tested for complementation of each of the four red mutations as before. The results are summarized at the bottom of Fig. 1. All clones and subclones carrying the 3.30 kb SstI-BamHI fragment contained in pIJ764 complemented redB and redF mutations; clones lacking this fragment failed to complement these mutations. The two genes have not been physically separated because of the lack of internally mapped restriction sites.

To the right of the redB+F fragment is a 4.67 kb gap of unknown function. This region may well contain genetic information involved in undecylprodigiosin production or resistance, but there is no mutation available with which to identify it. Nevertheless, the physical distance between redF and redE tends to confirm the tentative conclusion based on cosynthetic and complementation differences (Feitelson & Hopwood, 1983) that these mutations identify two different genes.

The 1.57 kb BclI–SstI fragment to the right of the gap and present in pIJ751 is sufficient to complement redE mutations, but on the basis of phage subcloning experiments (not shown) does not contain the entire functional redE gene. The 3.89 kb SstI–PstI fragment on the right side of pIJ755 and pIJ759, carried by pIJ766 and included in pIJ768 and pIJ769, complements redA mutations. A few unexplained rearrangements were apparent between the clones (e.g. a missing PstI site in pIJ752, and probably a large deletion in pIJ750). However, Southern hybridizations to the M145 chromosome and to plasmids pIJ752, pIJ755, pIJ757 and pIJ759 digested with SstI.
The red gene cluster of *S. coelicolor*

Fig. 1. *S. coelicolor* Red+ clones: restriction maps and gene assignments. Five primary clones which complement various red mutations are shown, along with subclones propagated in pIJ702 (solid lines underneath primary clones) or pIJ725 (dashed lines underneath primary clones). Restriction maps were derived from the appropriate single and double digests with the enzymes indicated. The sizes of defined regions which complement redB+E, redE, and redA are shown in kb. (*) indicates a missing PstI site in pIJ752; [ ] indicates the probable location of a large deletion in pIJ750.

+ BamHI and SstI + XhoI gave precisely the same signals when probed with nick-translated pIJ761. This indicates that the physical maps quite accurately reflect the genome organization.

Transformations of a heterologous host

*S. glaucescens* GLA-0 has no detectable homology to pIJ702 or to the DNA inserts in pIJ750 and pIJ752 (Fig. 2), removing the possibility of integrational repair; thus, introduction of the clones into this host resembles the use of a Rec- mutation in the homologous species. The cloned insert in pIJ752 extends far to the left of that in pIJ750 (Fig. 1), and crude extracts of redE mutants containing pIJ752 have elevated levels of O-methyltransferase activity compared to Red+ and redE(pIJ750) (Feitelson & Hopwood, 1983, and below). Therefore, if pIJ752 contained all the information necessary for O-methyltransferase expression in trans, we would expect that thiostrepton-resistant transformants of GLA-0 would have O-methyltransferase activity. There was no enzyme activity detectable in sonicated extracts of two independent transformants of GLA-0(pIJ752), as well as a control extract of GLA-0(pIJ750) (data not shown). Furthermore, pIJ750 and pIJ752 DNAs recovered from the *S. glaucescens* transformants were able to transform *S. coelicolor* JF4 protoplasts to Red+, ruling out the possibility of DNA rearrangements causing their inability to express O-methyltransferase activity in GLA-0.

Search for red sequences among *Streptomyces* species

Preliminary results using pIJ755 as a hybridization probe against BamHI-digested chromosomal DNAs from various species showed two strongly hybridizing bands of 5.5 kb and 4.7 kb in both *S. lividans* and *S. coelicolor*, consistent with the maps of the clones. There was a band at 10 kb in DNA of *S. violaceolatus*, a species known to produce actinorhodin and suspected to produce prodigiosin pigments. This suggested a polymorphism whereby an internal BamHI site near the centre of the red gene cluster had been deleted in *S. violaceolatus* during the evolutionary divergence of these strains, or alternatively that *S. lividans* and *S. coelicolor* had
Fig. 2. Southern blot analysis of DNA of various strains for hybridization with redE DNA. Approximately 0.8 μg chromosomal DNA from each species was digested with BamHI and subjected to electrophoresis through a 0.7% agarose gel at 1.5 V cm⁻¹ for 13 h. The gel was stained, photographed, and blotted to nitrocellulose (Schleicher & Schüll BA85). After vacuum drying and prehybridization, the filter was hybridized to 2 x 10⁶ c.p.m. nick-translation pIJ761 probe at 42 °C for 20 h. The filter was washed and exposed to preflashed X-ray film (Fuji RX) at −70 °C for 20 h. Species abbreviations are as follows: achr, Streptomyces achromogenes ATCC 112767; albo, S. alboniger ATCC 12461; ambo, S. ambofuciens ATCC 15154; capr, S. capreolus NCIB 9801; eryt, S. erythreus NRRL 2338; glau, S. glaucescens ETH 22794 (GLA-0); hygr, S. hygroscopicus ATCC 27438; livi, S. lividans 66 (JII 1326); parv, S. parvulus str-1 JII 2283 (derived from ATCC 12434); rimo, S. rimosus NRRL 2234; scab, S. scabies A26 (JII 1289); vene, S. venezuelae; vina, S. vinaceus NCIB 8852; viol, S. violaceolatus ISP 5438; vior, S. violaceoruber NCIB 9622; Smar, Serratia marcescens HY. Two different strains of S. coelicolor A3(2) (coel) were loaded: JF4(pIJ702) in lane 4 and JF4 in lane 6.

Preliminary characterization of the O-methyltransferase complex
Having a sensitive and specific assay for the O-methyltransferase enzyme activity in crude extracts of S. coelicolor DNA gained the site. To eliminate potential artefacts caused by a small amount of contaminating Streptomyces chromosomal DNA in the nick-translation reaction, pIJ761 (pBR322 carrying the 1.57 kb BamHI–SsrI fragment of pIJ750: Fig. 1) was used as a hybridization probe (Fig. 2). Only one heavy 5-5 kb band appeared in the S. coelicolor and S. lividans tracks (light bands seen in the S. coelicolor DNA are due to partial digestion) and a 10 kb band in S. violaceolatus. Sonicated extracts of S. violaceolatus had high levels of O-methyltransferase activity in vitro (data not shown), indicating that this strain has a functional red enzyme complex. S. scabies also showed homology to the redE fragment and also had apparent O-methyltransferase activity in sonicated extracts, although it is not known to produce prodigiosins. The S. vinaceus signal is due to a small (0.4 kb) residual portion of the vph gene left in the pIJ255 vector (S. vinaceus produces viomycin and was the source of the vph gene: Thompson et al., 1982).

Serratia marcescens DNA failed to hybridize to the redE probe, though non-prodigiosin-producing mutants of S. marcescens can cosynthesize with Streptomyces coelicolor red mutants (Feitelson & Hopwood, 1983). Perhaps this is due to differing codon usage which follows from the extremely high G + C content of Streptomyces DNA (73%) (Bibb et al., 1983) compared with that (60%) of Serratia DNA.

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sonicated extracts, we attempted to purify the enzyme using conventional biochemical techniques. The previous results were obtained using mycelium grown on solid medium followed by sonication and assay of the cleared extract (Feitelson & Hopwood, 1983). It seemed reasonable to eliminate much of the cell wall material prior to column chromatography by the formation of protoplasts. Releasing the cell wall and associated material by lysozyme treatment in an osmotically stabilizing medium might have resulted in cleaner starting material.

Under the conditions used, virtually all of the enzyme activity was found in the supernatant of the filtered and centrifuged protoplast preparation (data not shown). This suggested that the enzyme was nearly quantitatively released during protoplast formation or that partial lysis occurred. SDS–PAGE of sonicated mycelial preparations and protoplast supernatants revealed a similar pattern of polypeptides. Thus, partial cell lysis during protoplast formation may have been responsible for the O-methyltransferase activity in the supernatant. However, the protoplast method was much gentler than sonication and was therefore used for subsequent purification.

Gel filtration column chromatography of protoplast supernatants from the red* wild-type, several red mutants, and the redE mutant harbouring the 'high-expressing' pIJ752 is shown in Fig. 3. The red* extract showed two peaks of activity: in the void volume and at approximately 49 kDal (Fig. 3b). This extract was also chromatographed through Bio-Gel A-5M, which excludes globular molecules of >5 MDal. The same two peaks were seen. These results suggested that the enzyme activity in the excluded material is complexed in an unknown fashion to very high molecular weight cell wall or cell membrane components.

The very prominent 49 kDal peak in the redE extract (Fig. 3c) demanded explanation. Previously, little or no enzyme activity was found in sonicated extracts of this strain (Feitelson & Hopwood, 1983); this confirmed the deduction that the redE mutation affected O-methyltransferase expression. However, the activity of the excluded material exactly follows
predictions from previous work: (1) the redE mutant has no activity; (2) the redE strain has moderate levels; and (3) the redE(pIJ752) extract has about five times the activity of the wild-type. There appears to be an inverse relationship between the two peaks in the chromatograms in Fig. 3(a, b and c): as the 49 kDal peak gets larger, the excluded peak gets smaller, suggesting that the two peaks may be different forms of the same enzyme.

Protoplast preparations of redE(pIJ752) mycelium were assayed for O-methyltransferase activity under several different conditions. A non-ionic detergent, NP-40, completely destroyed the high molecular weight peak but the 49 kDal peak survived; sonicated extracts had no 49 kDal peak but did have the excluded peak (data not shown). NaCl at 0.5 M had little effect on the pattern. Thus the activities in the two peaks were differentially sensitive to sonication and detergent.

Another approach to elucidating the relationship between these two forms was to chromatograph extracts of other red mutants. The redF pattern (Fig. 3d) was qualitatively similar to that of redE: no excluded peak was seen, but there was a significant 49 kDal peak. The redA pattern (Fig. 3e) was very similar to that of the wild-type, but the peaks of activity were lower, consistent with the levels of activity in sonicated extracts. However, the strongest evidence suggesting that the two forms are related is provided by the redD mutant extract (Fig. 3f), which had no detectable activity in any column fractions, demonstrating that this mutant, which has no activity in sonicated extracts and fails to cosynthesize with any other red mutants, simultaneously loses both peaks.

These data suggest that the two forms of O-methyltransferase activity seen on gel filtration columns are related and that the relevant activity in vivo is the high molecular weight form. The situation is more complex than that originally postulated: that the redE gene was the structural gene for the O-methyltransferase. It now appears that at least both the redE and redF genes must be wild-type for a functional O-methyltransferase to be present in vivo. Their gene products may be subunits of an O-methyltransferase complex. However, the lack of enzyme activity in vitro of extracts of S. glaucescens containing pIJ752 strongly suggests that genetic information additional to that found in the clone is required for heterologous expression.

Expression of red genes in liquid media

In order to study the kinetics of gene expression, it is essential to have a physiologically uniform culture. S. coelicolor red+ strains failed to produce red pigment in liquid media commonly used in the laboratory; for this reason we used mycelium scraped from the surface of agar plates for enzyme assays. To test whether it was possible for the wild-type to produce undecylprodigiosin in liquid, or whether there was some 'anchorage dependency' for the production of this antibiotic, spores of S. coelicolor B257 were inoculated into 15 different liquid media used for antibiotic fermentations. Two of the media yielded Red+ mycelium: aureothricin media (AM) and Czapek–Dox containing 0.1% yeast extract. The cells grew and became Red+ much faster in AM than in Czapek–Dox, and therefore AM was used in subsequent experiments.

Fig. 4 shows a typical liquid fermentation in AM. It displays the classic pattern of secondary metabolism (Bu'Lock, 1961), very similar to that seen for prodigiosin biosynthesis in Serratia marcescens (Williams, 1973). There is a delayed onset of antibiotic production until mid- to late-exponential phase; the specific activity of O-methyltransferase peaks at or just before the onset of antibiotic production, and a drop of specific activity occurs at the end of the fermentation coincident with a tailing off of the levels of undecylprodigiosin. In contrast to the finding with cells grown on solid media, however, there is an accumulation of the yellow precursor, undecylnorprodigiosin, with time. Apparently this conversion is not as complete in liquid as on plates; the coincidence of O-methyltransferase activity and red pigment production suggests that this may be the rate-limiting step, at least in the liquid medium.

DISCUSSION

The combined application of DNA cloning and biochemical analysis in attempting to
understand antibiotic biosynthesis in *Streptomyces* promises to yield insights into the nature of gene expression in these micro-organisms. The ‘red system’ in *S. coelicolor* A3(2) has many attractive features for studying these questions. The biosynthetic pathway is reasonably well characterized, by analogy with *Serratia marcescens*. The final and penultimate products are differently pigmented compounds, providing an easy assay for antibiotic production. The enzymology is intriguing, suggesting a membrane- or cell-wall-bound biosynthetic enzyme complex, possibly located outside the protoplasm. The gene cluster coding for the biosynthetic enzymes has been partially cloned and characterized, providing a framework within which to elucidate more detailed aspects of the red genetic architecture.

None of the clones isolated complement mutations in the redC or redD genes. Rudd & Hopwood (1980) showed that all the red loci detected by mutation are genetically closely linked; what this means in precise physical distance on a DNA molecule remains to be seen. The question of the evolution of the cluster is approachable by cloning homologous regions from related species and comparing their organization, in a manner similar to that used on the tryptophan operon in the eubacteria (Miozzari & Yanofsky, 1978). Subtle changes in regulatory strategies, e.g. attenuator and termination control, may be fruitfully studied by such comparative analyses.

However, the most interesting near-term prospect for understanding gene expression in the red system is to identify and characterize regulatory regions in the DNA. If undecylprodigiosin biosynthesis is at least partially controlled at the level of transcription, red promotors are obvious candidates for analysis, using available tools such as S1 nuclease mapping (Favarolo et al., 1980) and cloning into promoter-probe vectors (Bibb & Cohen, 1982). DNA sequencing of red promotors may reveal structural features characteristic of promotors for secondary metabolic genes, and recognized by specific sigma subunits in *Streptomyces* RNA polymerase.

Fig. 4. Fermentation of *S. coelicolor* redE60(pIJ752). Spores of JF3(pIJ752) were inoculated into 500 ml of a 1% yeast extract/1% glucose seed culture and grown at 30 °C with shaking at 150 r.p.m. for 24 h in a 2-litre flask. The culture was divided into 25 ml amounts and frozen at −20 °C, and later thawed and inoculated into 500 ml AM in a 2-litre flask. Growth conditions were as described above. Samples of 100 ml were removed at the indicated times, weighed (a), and frozen until the end of the fermentation. Then 1 g of each sample was sonicated and assayed for enzyme activity (c) and 2 g was extracted with methanol for spectrophotometric analysis of the antibiotic (b): ○, $A_{410}$ (undecylnorprodigiosin); □, $A_{430}$ (undecylprodigiosin).
(Westpheling et al., 1985) in a fashion analogous to Bacillus spore formation genes (Losick & Pero, 1981). We anticipate that such basic knowledge may have profound effects on the rational design of antibiotic discovery and strain improvement programmes.

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REFERENCES


The red gene cluster of *S. coelicolor*


