Additional copies of the *actII* regulatory gene induce actinorhodin production in pleiotropic *bld* mutants of *Streptomyces coelicolor* A3(2)

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In *S. coelicolor* there are mutants (named *bld*) which fail to form aerial mycelium and which are also pleiotropically blocked for synthesis of several antibiotics, including the blue compound actinorhodin. Using a high copy-number vector, a DNA fragment was cloned which restored actinorhodin production, but not aerial mycelium formation, to *bldA*, *D*, *F*, *G* and *H* mutants. Subcloning and Southern blotting revealed that the fragment was from the region (*actII*) that regulates actinorhodin biosynthesis. The ability to elicit actinorhodin production and to complement an *actII* mutant was localized to a 1.3 kb fragment. These results indicate that the structural genes for actinorhodin biosynthetic enzymes do not contain targets for the *bldA*, *D*, *F*, *G* and *H* gene products, and suggest that the corresponding *bld* genes control actinorhodin synthesis principally via a single gene in the *actII* regulatory region.

Introduction

In *Streptomyces coelicolor* A3(2), genetic approaches have been used extensively to analyse the regulation of morphological and physiological differentiation (the formation of a sporulating aerial mycelium and the synthesis of secondary metabolites, respectively). These two processes both take place after the period of most rapid vegetative growth. This temporal coincidence at least partially reflects common regulatory elements, since five classes of mutants (*bldA*, *B*, *D*, *G* and *H*) have been described that – on the normally used laboratory media – are simultaneously deficient in aerial mycelium formation and the synthesis of several distinct antibiotics, including the blue-coloured actinorhodin and the red undecylprodigiosin (Merrick, 1976; Champness, 1988). Although the molecular nature of most of these *bld* gene products is unknown, it has been shown that one of the genes, *bldA*, specifies the leucyl tRNA for the rare UUA codon (Lawlor *et al.*, 1987; Leskiw *et al.*, 1991). In addition, two laboratories have isolated *bld* mutants that produce undecylprodigiosin but not actinorhodin, (*bldE*: Hodgson, 1980; *bldF*: Puglia *et al.*, 1986). The work described here was initiated by attempts to clone *bldF*, but went on to incorporate experiments with four of the other relatively well characterized mutant classes; *bldA*, *D*, *G* and *H*. The fifth well characterized class, *bldB*, was not analysed because it produces spores and actinorhodin on prolonged incubation. In this study, a DNA

fragment cloned from *S. coelicolor* is shown to be capable of restoring actinorhodin production – but not sporulation – to representative mutants of the five classes. The cloned fragment is shown to correspond to *actII*-ORF4, a positive regulatory gene located among, and apparently controlling most or all of, the clustered genes for actinorhodin biosynthesis (Rudd & Hopwood, 1979; Malpartida & Hopwood, 1986; Fernández-Moreno, *et al.*, 1991). These results may help to unravel the apparently complex regulatory network in which the *bld* genes participate.

Methods

Bacterial strains. The mutant strains (Table 1) were derived from the wild-type *S. coelicolor* A3(2) (Hopwood *et al.*, 1985a). *S. lividans* 66 (John Innes Institute strain 1326; Hopwood *et al.*, 1985a) was used as cloning host and for phage propagation. *Escherichia coli* JM101 (*supE thi Δ(lac-proAB)* [F', *traD36 proAB lac⁺ lacZΔM15*] (Messing *et al.*, 1981) was used as plasmid host.

Plasmids and phages. High copy number *Streptomyces* plasmid vectors were pIJ702 (Katz *et al.*, 1983) and pIJ699 (Kieser & Melton, 1988). pIJ2925 [a pUC18 (Yanisch-Perron *et al.*, 1985) derivative with a modified polylinker flanked by *Bgl*II sites: G. H. Janssen, personal communication] was provided by M. J. Bibb. pIJ2323 and pIJ2327 [pBR325 derivatives containing segments of DNA extending from sites 2–11 and 10–13, respectively, of the *act* restriction map of Malpartida & Hopwood (1986)] were constructed by F. Malpartida and provided by D. A. Hopwood. Plasmid pUC19 (Yanisch-Perron *et al.*, 1985) was

Table 1. *S. coelicolor* strains

Strain	Genotype	Plasmid status	Source or Reference
M145	Prototroph	SCP1 ⁻ SCP2 ⁻	M. J. Bibb
JF2	<i>argA1 guaA1 actII105 redB52</i>	SCP1 ⁻ SCP2 ⁺	Feitelson & Hopwood (1983)
J1700	<i>hisA1 uraA1 strA1 pgl⁻ bldA39</i>	SCP1 ⁻ SCP2 ⁻	Lawlor <i>et al.</i> (1987)
J774	<i>cysA15 pheA1 mthB2 bldD53</i>	SCP1 ^{NF} SCP2 ⁺	Merrick (1976)
(w)C103*	<i>hisA1 uraA1 strA1 pgl⁻ bldG103</i>	SCP1 ⁻ SCP2 ⁻	Champness (1988)
(w)C109*	<i>hisA1 uraA1 strA1 pgl⁻ bldH109</i>	SCP1 ⁻ SCP2 ⁻	Champness (1988)
166†	<i>hisD3 pheA1 strA1 bldF</i>	SCP1 ⁺ SCP2 ⁺	A.-M. Puglia and coworkers (unpublished)

* These strain designations have been prefixed with (w) to avoid confusion with previously described *S. coelicolor* strains with C designations (Hopwood *et al.*, 1970; Chater, 1972).

† University of Palermo strain collection number.

purchased from Pharmacia. KC304, a derivative of the *Streptomyces* temperate phage ϕ C31 previously described by Hopwood *et al.* (1987), was used in the subcloning.

Culture conditions, transformation and transfection procedures. *Streptomyces* strains were grown in liquid YEME medium supplemented with 34% (w/v) sucrose, 5 mM-MgCl₂ and auxotrophic requirements of the individual strains, or on solid medium R2YE (Hopwood *et al.*, 1985a). Protoplasting, transformation and transfection of *S. lividans* and *S. coelicolor* strains were as in Hopwood *et al.* (1985a). *E. coli* JM101 was grown and transformed by standard procedures (Maniatis *et al.*, 1982).

Transduction. Phage suspensions were mixed with spores or mycelial fragments of the recipient strain directly on R2YE agar plates. After incubation at 30 °C for 6 d, the growth was replicated onto medium containing thiostrepton (50 µg ml⁻¹) to select for transductants.

DNA procedures. Chromosomal, plasmid and bacteriophage DNA were isolated as in Hopwood *et al.* (1985a). For shotgun cloning, DNA from strain M145 was partially digested with *Sau*3AI and size-fractionated on a sucrose gradient (Hopwood *et al.*, 1985a). Fragments in the 5 kb to 15 kb size range were ligated with *Bgl*II-digested pIJ702. Southern blotting was as in Hopwood *et al.* (1985a). Probes were radiolabelled with [α -³²P]dCTP by using the Multiprime DNA labelling kit (Amersham). Plaque lifts onto Hybond membranes (Amersham) and hybridization were carried out according to the manufacturer's recommendations.

Results

Cloning of a DNA fragment which restored actinorhodin production to a bldF mutant

Previous mutagenesis experiments identified a novel mutant (*bldF*) of *S. coelicolor* (Puglia *et al.*, 1986 and manuscript in preparation). This mutant never forms aerial hyphae and spores under any growth conditions tested, nor does it produce the antibiotics actinorhodin, 'calcium-dependent antibiotic' and methylenomycin. It produces only the mycelium-bound red antibiotic undecylprodigiosin. In an attempt to clone *bldF*, a genomic library of *S. coelicolor* DNA was constructed in the *bldF* mutant using the high copy number vector pIJ702. In spite of a high probability of having a complete library,

no colonies developing aerial mycelium were observed. However, a bald colony producing large amounts of blue pigment (presumed to be actinorhodin) was isolated. The recombinant plasmid (pSCbl-1) extracted from this colony carried a 13 kb DNA insert. Successive subcloning experiments showed that actinorhodin production could be induced by a fragment of only 1.3 kb, located at one end of the insert (pSCbl-3; Fig. 1). Removal of 0.2 kb from the righthand end of the 1.3 kb fragment, leaving a 1.1 kb *Sst*I-*Bam*HI fragment (pIJ69; Fig. 1) eliminated the effect.

Localization and identification of the 1.3 kb DNA fragment.

In *S. coelicolor*, actinorhodin synthesis is controlled by the *act* gene cluster (Rudd & Hopwood, 1979). Of eight classes of *act* mutants defined by cosynthesis tests, one (*actII*), failed to cosynthesize actinorhodin with any other mutants. This class seems to define a positively acting control gene (Rudd & Hopwood, 1979; Fernández-Moreno *et al.*). In complementation tests with an *actII* mutant, JF1, Malpartida & Hopwood (1986) localized *actII* to a ~5 kb section of the *act* cluster (Fig. 2). The right-hand end of this region has been implicated in the induction of actinorhodin overproduction in *S. coelicolor* and *S. lividans* by certain recombinant plasmids (Hopwood *et al.*, 1985b and D. A. Hopwood and H. M. Kieser, personal communication).

In the course of the analysis of the cloned DNA in pSCbl-1, it became apparent that part of its restriction map resembled that of part of the *act* cluster. Further detailed restriction map comparison (Fig. 2) showed that pSCbl-1 included 8 kb that aligned with the *actV-actII* region, and 5 kb from elsewhere in the *S. coelicolor* genome. Southern blots with cloned *act* DNA as probe (i.e. using pIJ2323 and pIJ2327) confirmed the presence of the *actV-actII* region in pSCbl-1, and showed that the 1.3 kb smallest fragment eliciting actinorhodin produc-

Fig. 1. Construction of plasmids. At each step, the DNA was cut at the sites indicated by boxes, then either self-ligated or ligated with a suitably cut vector. pIJ68 was constructed using *BglII*-digested pIJ699 as vector. White segments; vector DNA; black segments, cloned *S. coelicolor* DNA; stippled segments, fd terminator sequences (T) of pIJ699.

Fig. 2. Localization of the 8 kb and 1.3 kb fragments carried by pSCbl-1 and pSCbl-3 respectively, within the actinorhodin cluster of *S. coelicolor*. (a) Actinorhodin biosynthetic pathway. (b) Locations of mutant classes (Malpartida & Hopwood, 1986). (c) Restriction map of the actinorhodin cluster showing coordinates assigned by Malpartida & Hopwood (1986). (d) Restriction map of an 8 kb segment from one end of the insert in pSCbl-1. (e) Restriction map of 1.3 kb fragment capable of eliciting actinorhodin production in *bld* mutants and in an *actII* mutant. P, *Pst*I; B, *Bam*HI.

tion in the *bldF* mutant corresponded to a segment at the right-hand end of the *actII* region. In confirmation of this, pSCbl-3 restored actinorhodin production to the *actII* mutant strain JF2. Furthermore, the 1.3 kb sequence was probably expressed from its own promoter; this was shown by cloning the smaller *Sph*I–*Sst*I fragment of pSCbl-3, via pIJ2925 to modify its ends, between the *Bgl*II sites of pIJ699, a vector in which transcription terminators prevent readthrough from vector promoters (Kieser & Melton, 1988) (Fig. 1). Actinorhodin production was obtained when the resulting plasmid, pIJ68, was introduced into the *bldF* mutant.

Restoration of actinorhodin synthesis in *bldA*, *D*, *G* and *H* strains.

Since pIJ68 could cause the *bldF* mutant to produce actinorhodin, it was of interest to investigate its effect on other representative *bld* mutants (*bldA*, *D*, *H* and *G*). All the strains carrying pIJ68 produced actinorhodin after 2–3 d growth. To investigate whether just a single extra copy of the 1.3 kb sequence was able to restore actinorhodin synthesis, phage KC810 was constructed. KC810 carried the same *Bgl*II fragment as described above for pIJ68, ligated between the *Bam*HI sites of vector KC304. (This vector, derived from ϕ C31, carries the ϕ C31 *attP* site, and can readily integrate into the *S. coelicolor* chromosomal *attB* site, to give thiostrepton-resistant transductants.) With the exception of the *bldF* mutant, all the *bld* strains transduced with KC810 failed to produce actinorhodin. The actinorhodin synthesis in *bldF* transductants was delayed compared with the high

copy number cloning experiments; a low amount of actinorhodin production was detectable only after 7–8 d incubation at 30 °C.

Discussion

A 1.3 kb fragment of DNA from the *actII* region, capable of complementing an *actII* mutant, could elicit conspicuous actinorhodin production in *bldA*, *D*, *F*, *G* and *H* mutants when present at a high copy-number. Other aspects of the pleiotropic *bld* mutant phenotypes, such as aerial mycelium deficiency and (for *bldA*, *D*, *G* and *H*) non-production of the red compound undecylprodigiosin, were not affected. From the DNA sequence of the *actII* region (Fernández-Moreno *et al.*, 1991) it is clear that this 1.3 kb fragment contains a single complete coding sequence (*actII*-ORF4) preceded by an intergenic region and the last ~200 bp of another gene, *actII*-ORF3. The fragment also contains about 130 bp of DNA downstream of *actII*-ORF4. The simplest interpretation of these results is that the actinorhodin deficiency of each of the five classes of *bld* mutant is due to a reduction in the active product of the *actII*-ORF4 regulatory gene. This would be predicted to cause reduced transcription of the *act* genes specifying the enzymes of actinorhodin biosynthesis. In support of this, a transcriptional fusion of the *xylE* reporter gene to the *actI* transcription unit encoding early steps in the biosynthetic pathway gave strong *xylE* expression in *bldA*⁺, but not in *bldA*[–], strains (Bruton *et al.*, 1991). Probably, a low level of *actII*-ORF4 gene product is made in each of the *bld* mutants tested: thus, increasing the copy number of *actII*-ORF4 in the *bld* mutants would cause a proportionate increase in the amount of its gene product up to a level capable of eliciting expression of the *act* structural genes. (Our results make it unlikely that *bld* mutants fail to make actinorhodin because they cannot supply a precursor or cofactor for its synthesis: over-expression of *actII*-ORF4 would not be expected to suppress such a metabolic block.)

The molecular nature of most of the *bld* genes is still undetermined. However, *bldA* is known to specify a leucyl tRNA (probably the only one) for the codon UUA, a particularly rare codon in streptomycetes (Lawlor *et al.*, 1987; Leskiw *et al.*, 1991). Among several TTA-containing genes tested by Leskiw *et al.* (1991), some were completely unexpressed in *bldA* mutants (at the level of gross phenotype), whereas others showed partial expression, most probably because of a low level of translation of UUA codons by an unidentified non-cognate tRNA. DNA sequencing has shown that *actII*-ORF contains a TTA codon, providing a potential target for *bldA* action (Fernández-Moreno *et al.*, 1991). Thus,

our results are compatible with the proposal of Fernández-Moreno *et al.* (1991), supported in their paper by site-directed mutagenesis of the TTA codon, that *actII*-ORF4 expression in a *bldA* mutant is limited at the level of translation of the corresponding UUA codon. Since the effect of increasing *actII*-ORF4 copy-number appears to be very similar in *bldD*, *G* and *H* mutants to that observed in the *bldA* mutant, these mutations may also eliminate the active form of the *bldA*-specific tRNA (for example, by interfering with its transcription, processing, modification or charging). On the other hand, actinorhodin production in the *bldF* mutant is probably affected in a way that gives a less drastic reduction of *actII* expression. This could result from any of several causes including: reduced transcription of *actII*; reduced translation of its mRNA (perhaps by an imperfectly modified *bldA* gene product); or reduced stability of its protein product. Any satisfactory explanation of the *bldF* phenotype must be compatible with the following three features: first, the mutant is a strong producer of undecylprodigiosin; second, even a single extra copy of *actII* is sufficient to cause production of some actinorhodin (a circumstance that failed to elicit production in the other *bld* mutants); and third, the morphological defect of the *bldF* mutant, unlike that of *bldA*, *D*, *G* or *H* mutants, is not phenotypically revertible by changing the carbon source in minimal medium from glucose to mannitol.

The ability of the *actII* gene at high copy number to cause strong actinorhodin production in a *bldA* mutant leads to the expectation that there are either no TTA codons, or none with strong *bldA* dependence, in the genes encoding the enzymes of actinorhodin synthesis. The same expectation arises from the site-directed mutagenesis experiment of Fernández-Moreno *et al.* (1991); and available sequence information both in that paper, and from Hallam *et al.* (1988), J. L. Caballero (personal communication) and Fernández-Moreno (1990) has revealed only one further TTA codon (in a putative actinorhodin export gene: Fernández-Moreno *et al.* (1991) in more than 14 kb of *act* DNA. Undecylprodigiosin synthesis can be switched on under some conditions in *bldA* mutants, indicating that TTA codons are also absent from all the *red* genes for biosynthetic enzymes of this pathway (Guthrie & Chater, 1990). Taken together, these results support the suggestion that the primary translation-level effects of the *bldA*-specified tRNA on antibiotic synthesis are exerted principally on regulatory genes (Leskiw *et al.*, 1991).

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