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 lacZAM15]) (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 et al., 1988). pIJ2925 [a pUC18 (Yanisch-Perron et al., 1985) derivative with a modified polylinker flanked by BgII 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
myces strains were grown in liquid YEME medium supplemented with temperate phage 4C31 previously described by Hopwood et al. (1987), purchased from Pharmacia. KC304, a derivative of the Streptomyces coelicolor strains were as in Hopwood et al. (1985*). E. coli Protoplasting, transformation and transfection of S. lividans and S. coelicolor strains with C designations (Hopwood et al., 1970; Chater, 1972).

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 (pSCb1-1) 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 (pSCb1-3: Fig. 1). Removal of 0.2 kb from the right-hand end of the 1.3 kb fragment, leaving a 1.1 kb SstI-BamHI 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

Table 1. S. coelicolor strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Plasmid status</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>M145</td>
<td>Prototroph</td>
<td>SCP1- SCP2-</td>
<td>M. J. Bibb</td>
</tr>
<tr>
<td>16f</td>
<td>hisD3 pheA1 strA1 bldF</td>
<td>SCP1+ SCP2+</td>
<td>A.-M. Puglia and coworkers (unpublished)</td>
</tr>
</tbody>
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* 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.
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.
of eliciting actinorhodin production in the insert in pSCbl-1. 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 the actinorhodin cluster showing coordinates assigned by Malpartida 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, PstI; B, BamHI.

Actinorhodin production was obtained when the resulting plasmid, pIJ68, was introduced into the bldF mutant. The actinorhodin synthesis in 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 act11 mutant strain JF2. Furthermore, the 1.3 kb sequence was probably expressed from its own promoter; this was shown by cloning the smaller SphI-SstI fragment of pSCbl-3, via pIJ2925 to modify its ends, between the BglII 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 BglII fragment as described above for pIJ68, ligated between the BamHI sites of vector KC304. (This vector, derived from φC31, carries the φC31 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 reversible 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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