Physical identification of a chromosomal locus encoding biosynthetic genes for the lipopeptide calcium-dependent antibiotic (CDA) of *Streptomyces coelicolor* A3(2)

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Putative peptide-synthetase-encoding DNA fragments were isolated from the *Streptomyces coelicolor* A3(2) chromosome using a PCR-based approach and mapped to a single ~35 kb segment. In integrative transformation experiments, DNA fragments from this region disrupted production of the calcium-dependent antibiotic (CDA) and had sequences characteristic of non-ribosomal peptide synthetases, thus proving that the *cda* locus had been cloned.

Keywords: *Streptomyces coelicolor*, antibiotic biosynthesis, lipopeptide, gene disruption, peptide synthetase

INTRODUCTION

Non-ribosomally synthesized peptides constitute one of the most interesting classes of natural products from a biosynthetic point of view (Kleinkauf & von Döhren, 1996; Lipmann, 1980; Stein & Vater, 1996). Moreover, they include antibiotics such as the β -lactams, gramicidin and tyrocidine, immunosuppressants like cyclosporin, and toxins such as enniatin. The synthesis of this class of compounds is directed by large multifunctional enzymes termed peptide synthetases and the corresponding biosynthetic genes have been isolated from a variety of fungi and bacteria, including actinomycetes (e.g. Coque *et al.*, 1991; de Crécy-Lagard *et al.*, 1997; Stachelhaus *et al.*, 1996). Recently, considerable interest has developed in the manipulation of peptide-synthetase-encoding genes from *Bacillus* spp. and filamentous fungi (Stachelhaus & Marahiel, 1995; Stachelhaus *et al.*, 1995, 1996; Zuber *et al.*, 1993). The filamentous bacterium

Streptomyces coelicolor A3(2) is genetically one of the most studied and amenable micro-organisms and so the study of non-ribosomal peptide synthesis in this organism could add an additional dimension to understanding the genetic control of peptide antibiotic synthesis. A particular feature of this strain is its production of four antibiotics, all of different chemical classes, allowing analysis of the regulation of antibiotic biosynthesis at different levels. These include pathway-specific regulation, pleiotropic control of more than one antibiotic, and co-regulation of antibiotic production and morphological development (Bibb, 1996; Chater & Bibb, 1997; Hopwood *et al.*, 1995).

The structure of three of the four *S. coelicolor* antibiotics has been known for some time, but that of the fourth, the calcium-dependent antibiotic (CDA; Hopwood & Wright, 1983; Lakey *et al.*, 1983; Lakey & Ptak, 1988), was not. However, on the basis of some similarities between the action of CDA and of amphomycin and LY146032 on biological membranes (Lakey *et al.*, 1983, 1988), it was surmised that CDA might be a lipopeptide, containing a non-ribosomally derived peptide moiety (Hopwood *et al.*, 1995). An attempt to clone the

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Abbreviation: CDA, calcium-dependent antibiotic.

biosynthetic genes for CDA was therefore based on this premise. This succeeded, as described here. Meanwhile, chemical characterization of CDA has indeed shown it to be a lipopeptide antibiotic consisting of 11 amino acid residues, with a 6-carbon hydroxylated fatty acid at the N-terminus (Kempter *et al.*, 1997).

METHODS

Bacterial strains, plasmids and microbiological procedures.

The *S. coelicolor cda*⁺ strain 2377 has been described by Hopwood & Wright (1983) and was handled by procedures described by Hopwood *et al.* (1985). For *S. coelicolor* MT1109, a prototrophic strain, see Podmore (1995). The cosmid clones were from the library described by Redenbach *et al.* (1996). The *Escherichia coli* non-methylating host was ET12567 (MacNeil *et al.*, 1992). Plasmid vectors pMT3008 and pMT3009 have been described by Paget (1994) and pBluescript II SK(+) was from Stratagene. For tests of CDA production (e.g. Fig. 5), two Oxoid nutrient agar (ONA) plates supplemented with histidine and uracil were inoculated with spores from a single colony of each culture. After incubation for 48–60 h at 30 °C, the two plates were overlaid with soft ONA containing a strain of *Bacillus mycoides* as indicator (Hopwood & Wright, 1983); Ca(NO₃)₂ was added to one plate to a final concentration of 12 mM (Podmore, 1995). Plates were examined for calcium-dependent antibiosis after 16 h further incubation at 30 °C.

DNA manipulations. General procedures were as described by Hopwood *et al.* (1985) for *Streptomyces* and Sambrook *et al.* (1989) for *E. coli*. The PCR conditions were 35 cycles with *Taq* polymerase in the presence of 5% DMSO. Each cycle was 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C.

RESULTS

Use of synthetic oligonucleotide probes to isolate PCR products carrying putative peptide-synthetase-encoding genes using *S. coelicolor* DNA as template

As a strategy to clone a putative peptide synthetase gene(s) from *S. coelicolor*, two degenerate oligonucleotides were designed to encode the conserved motifs 'Core I' (KAGGA) and 'Core II' (SGTTG), revealed by aligning the amino acid sequences of a series of peptide synthetases (e.g. Borchert *et al.*, 1992; Turgay & Marahiel, 1994) using *Streptomyces* codon usage (Wright & Bibb, 1992) and incorporating an *Eco*RI site at each 5' end (Fig. 1). These oligonucleotides were used as PCR primers, with *S. coelicolor* chromosomal DNA partially digested with *Hind*III as template. This yielded an apparently doublet amplified band of approximately the expected length. DNA from this band was cloned into the *Eco*RI site of pBluescript II SK(+). The nucleotide sequences of five of the clones were determined. All had a G+C content of ~70% (characteristic of *Streptomyces* DNA). The clones fell into three non-overlapping classes, represented by fragments A, B and C. Clones A and B represented the smaller PCR-amplified band (239 and 244 bp, respectively) and encoded sequences that were 82% similar to each other, while clone C was longer (306 bp) and its product was only 60% similar to that of the other two clones. The sequences of all three clones revealed partial open

reading frames for proteins highly similar to known peptide synthetases (data not shown).

Use of PCR products to clone putative CDA biosynthetic genes from *S. coelicolor*

The *cda* locus had been mapped genetically (Hopwood & Wright, 1983) to a region of the *S. coelicolor* linkage map later deduced to correspond to the *Ase*I-E fragment of the physical map (Kieser *et al.*, 1992). Significantly, all three cloned PCR products (A, B and C) hybridized uniquely to this fragment. When the B fragment was used as a probe against an unordered library of cosmid clones corresponding to the *Ase*I-E fragment (Redenbach *et al.*, 1996), it hybridized to six cosmids, designated 2B7, 2E5, 3D10, 4C5, 6D5 and 6E4. The six cosmids and *S. coelicolor* chromosomal DNA were subjected to Southern analysis using each of the three PCR products as probes (Fig. 2). Probes A and B yielded identical hybridization patterns, whereas probe C hybridized to different fragments; cosmids 2B7, 2E5 and 4C5 contained sequences that hybridized to all three probes, whereas DNA from the other three cosmids (3D10, 6D5 and 6E4) hybridized only to the A/B probes. It is notable that all the chromosomal sequences that hybridized with the three PCR products are represented by the six cosmids (Fig. 2). Restriction mapping of these cosmids showed them to overlap extensively, together spanning 88 kb, and demonstrated that all the sequences that hybridized to one or more of the PCR products are contained within one contiguous segment of ~35 kb (Fig. 3). Use of a segment of the overlapping DNA as a probe against the ordered cosmid encyclopaedia covering the entire *S. coelicolor* A3(2) chromosome (Redenbach *et al.*, 1996) located the DNA on the overlap between cosmids E8 and E63.

Gene disruption demonstrates that the cloned peptide-synthetase-encoding sequences are required for CDA biosynthesis

The above Southern analysis identified five small *Sal*I fragments of 1.5–4.3 kb that hybridized to either the A/B peptide synthetase probes (*Sal*I fragments AB2.1, AB2.5 and AB4.3) or the C probe (*Sal*I fragments C1.5 and C2.5) (Fig. 2); their approximate map locations are shown in Fig. 3. The observed hybridization of each PCR product to more than one *Sal*I fragment is considered to be attributable to heterologous hybridization of the clones to different peptide synthetase modules; different amino acid activating modules within peptide synthetases share differing degrees of sequence similarity (e.g. Coque *et al.*, 1991; Stachelhaus & Marahiel, 1995). The simplest interpretation of the Southern analysis is that the three PCR products (A–C) collectively hybridize to five different peptide synthetase modules. The above *Sal*I fragments were chosen for targeted gene disruption experiments to investigate their involvement in CDA biosynthesis. They were each cloned into either pMT3008 or pMT3009, *E. coli* vectors based on pBR322 that contain, respectively, a hygromycin-resistance gene (*hyg*) and a thiostrepton-

Primer 1 (Core I: 'KAGGA' motif)

5'-G ^{EcoRI} GAA TTC L K A G G A Y V P
 CTC AAG GCG GGC GGC GCC TAC GTG CCC IAC CC-3'
 G A C T C G

Primer 2 (Core II: 'SGTTG' motif)

3'-AG ATG TGG AGG CCC IGC CCG GIC GGG TTC CCT TAA GG-5'
 G G C T C

Fig. 1. Degenerate oligonucleotide primers used for PCR amplification of putative peptide-synthetase-encoding DNA fragments from the *S. coelicolor* chromosome. The consensus amino acid sequences of the Core I and Core II motifs are indicated above the respective DNA sequences.

resistance gene (*tsr*) for selection in *Streptomyces*. The resulting recombinants were passaged through the non-methylating *E. coli* strain ET12567 and used to transform *S. coelicolor* 2377. This is the *cda*⁺ strain used by Hopwood & Wright (1983) as a parent for the *cda* mutants used in genetic mapping of the *cda* locus. Selection for hygromycin- or thiostrepton-resistant transformants yielded recombinants in which a single crossover resulted in integration of the plasmid into the region of the chromosome homologous with the cloned *SalI* fragment. The expected genomic structure of two independent integrants derived from each disrupting plasmid was confirmed by Southern hybridization and at least four independent transformants for each construct were tested for CDA production. Plasmid integration via four of the fragments (AB2.1, AB2.5, AB4.3 and C1.5) led to almost complete inhibition of CDA production by the respective transformants, whereas integration via C2.5 caused a lesser reduction in CDA production as judged by plate bioassays (data not shown).

These results provided presumptive evidence of the direct involvement of the cloned DNA in CDA biosynthesis. The slight 'leakiness' of the *Cda*⁻ phenotype of these single-crossover disruptants was attributed to excision of the disrupting plasmid by a reversal of the integration event in a small proportion of the chromosomes in the tested mycelial patches, an event that could not be selected against because antibiotic selection had to be omitted from the test plates so as not to inhibit the indicator bacterium *B. mycoides*. A gene replacement was therefore made by targeted double crossing-over in an attempt to generate a stable *cda*-null mutant. To do so, two of the *SalI* fragments (AB2.1 and AB2.5 in Fig. 3) were cloned on either side of the *tsr* gene, in their natural chromosomal orientation, in the pMT3008 vector to yield pMT3350 (Fig. 4). Strain 2377 was transformed with this plasmid, with selection for hygromycin resistance, to yield single-crossover integrants. Four of these were shown by Southern hybridization to have arisen by homologous recombination via either the AB2.1 or the AB2.5 fragment (two in each). As before, the recombinants had a leaky *Cda*⁻ phenotype.

Fig. 2. Southern analysis of *S. coelicolor* MT1109 chromosomal DNA (CHR) and the six putative peptide-synthetase-encoding cosmid DNA (2B7–6E4). All DNA samples were digested with *SalI*. *HindIII*-digested λ DNA was used as a size marker (indicated on the right of each autoradiograph). (a) Hybridization with radiolabelled PCR fragment B (an identical hybridization pattern was obtained using PCR fragment A). (b) Hybridization with radiolabelled PCR fragment C. The five hybridizing *SalI* fragments used in gene disruption experiments are indicated to the left (coded as in Fig. 3).

Two of the integrants generated by crossing-over in fragment AB2.5 were grown non-selectively and Hyg^S Thio^R segregants were recovered. Southern analysis of five of them showed that the expected second crossover, in fragment AB2.1, had occurred, resulting in the replacement of the 8.2 kb segment of chromosomal DNA lying between the two *SalI* fragments by the *tsr*

Fig. 3. Physical map of the putative peptide-synthetase-encoding *cda* locus. The extent of each cosmid insert is indicated below the map, and the approximate physical locations of the *Sa*I fragments (indicated by black boxes) that hybridize to the respective PCR products (see Fig. 2) are indicated. The relative positions of the two fragment-C-hybridizing *Sa*I fragments, C1.5 and C2.5, within the *Eco*RI-*Xho*I segment has not been established. The restriction map was deduced from a combination of Southern analysis using the PCR products, conventional restriction mapping of the cosmids and directional mapping of partially digested cosmids using T3 and T7 oligonucleotide probes (see Evans *et al.*, 1989); in the latter approach, cosmids were digested to completion with *As*I before partial digestion with the respective enzymes. All sites are shown for *Bam*HI (Ba), *Bgl*II (Bg), *Eco*RI (E) and *Xho*I (Xh). The seven fragments used in gene disruption experiments are indicated, as is the CDA production phenotype of the respective disruptants. The DNA segment flanked by *Sa*I fragments AB2.1 and AB2.5 has been deleted in strain MT1160 (see Fig. 4). 'ps' indicates that the DNA fragment contains an (incomplete) open reading frame for a protein homologous to known peptide synthetases; only one end of fragment C2.5 contained such an open reading frame. The direction of transcription of one *cda* segment is indicated by an arrow, and was deduced from the DNA sequences of the ends of fragments AB2.1 and AB2.5 relative to the physical map.

marker (Fig. 4). Plate tests (e.g. Fig. 5) showed that all five strains produced no detectable CDA.

To gain some insight into the length of DNA involved in CDA biosynthesis, single-crossover disruptions were made with two fragments from near the ends of the ~88 kb chromosomal region encompassed by the six overlapping cosmids; these were two 2.2 kb *Eco*RI-*Bam*HI fragments from the left extremity of cosmid 2E5 and the right extremity of cosmid 3D10 (2.2L and 2.2R, respectively; Fig. 3). In both cases the site of integration was confirmed by Southern analysis; CDA production was not affected in either class of integrant as judged from plate bioassays (data not shown).

Sequencing of *cda* DNA reveals similarities to known peptide synthetases

Short lengths of sequence were obtained from each end of the seven fragments that were used in the gene disruption experiments. The derived amino acid

sequences from both ends of the four fragments that disrupted CDA production aligned well with those of peptide synthetases from a variety of organisms, while the sequence from only one end of fragment C2.5 did so. Conversely, the deduced sequences of the flanking 2.2L and 2.2R fragments showed no similarities to peptide synthetases.

DISCUSSION

The results described here provide clear evidence for the cloning of one or more structural genes encoding a peptide synthetase for the lipopeptide antibiotic CDA. We have isolated and physically mapped a DNA segment corresponding to ~1% of the *S. coelicolor* chromosome, and all putative peptide-synthetase-encoding DNA fragments identified in this study were contained in a ~35 kb region within this segment. It should be noted that, because of the inherent variability in the extended Core I and Core II motifs, the PCR primers

Fig. 4. Construction of a stable *cda* null mutant, MT1160, containing the $\Delta cda1$ mutation. (a) Southern analysis of chromosomal DNA from three independent $\Delta cda1$ clones. *Sal*I fragment AB2.5 was used as the radiolabelled probe. λ , *Hind*III-digested λ DNA; wt, *S. coelicolor* 2377 chromosomal DNA. All chromosomal DNA was digested with *Bam*HI. (b) Schematic representation of the double-crossover gene replacement. The 8.2 kb DNA segment between the AB2.1 and AB2.5 *Sal*I fragments (Fig. 3) was replaced with the *tsr* gene and the resulting DNA segment was cloned into the delivery plasmid pMT3008 to produce pMT3350.

used in this study would not be expected to amplify the 'KAGGA/SGTTG' encoding sequences from all peptide synthetase domains. It cannot therefore be ruled out that there may be other peptide-synthetase-encoding loci elsewhere in the *S. coelicolor* chromosome.

Fig. 5. CDA plate bioassays of wild-type *S. coelicolor* 2377 and the $\Delta cda1$ null mutant, MT1160. Calcium-dependent antibiotic is only observed with strain 2377.

Assuming that the segment of the synthetase required to activate and incorporate each amino acid into the peptide would be encoded by about 3.5 kb DNA (e.g. Turgay *et al.*, 1992), ~ 39 kb would be required to synthesize the 11-residue oligopeptide moiety of CDA. Other genes must be involved in the synthesis of some of the unusual amino acid residues themselves, as well as for some other post-assembly modifications of the structure. In addition, synthesis and attachment of the fatty acid at the N-terminus of CDA presumably requires some dedicated genes. If, as expected from precedent, all the biosynthetic genes for synthesis of the antibiotic are clustered together on the chromosome, it is likely that all genes required for CDA production are contained within the 88 kb DNA segment reported here. The two flanking fragments (2.2L and 2.2R; Fig. 3) used in single-integration disruptions did not influence CDA production, indicating that they were not *internal* to transcription units required for CDA synthesis. The extent of the *cda* cluster and its detailed organization are currently under investigation.

A particularly relevant question concerns genetic control of the biosynthesis of the fatty acid moiety of CDA and whether this involves a kind of developmental cross-talk between the vegetative fatty acid synthase and the CDA peptide synthetase (Hopwood, 1997). Elucidation of this question will throw further light on the interrelationships between fatty acid biosynthesis and that of at least two polyketide metabolites of *S. coelicolor*, the antibiotic actinorhodin and the spore pigment (Revill *et al.*,

1995, 1996; Yu & Hopwood, 1995). This is a specific example of the special interest in studying the genetics of several interrelated primary and secondary metabolites in one and the same genetically amenable micro-organism (Hopwood *et al.*, 1995).

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