Genetic and biochemical characterization of the $\alpha$ and $\beta$ components of a propionyl-CoA carboxylase complex of *Streptomyces coelicolor* A3(2)

E. Rodríguez and H. Gramajo

Two genes, *accA1* and *accA2*, with nearly identical nucleotide sequences were cloned from *Streptomyces coelicolor* A3(2). The deduced amino acid sequences of the product of these two genes showed high similarity to BcpA2 of *Saccharopolyspora erythraea* and other biotin-containing proteins from different organisms assumed to be the $\alpha$ subunit of a propionyl-CoA carboxylase. A gene, *pccB*, encoding the carboxyl transferase subunit of this enzyme complex was also characterized. Strains disrupted in *accA1* did not show any change in acetyl- or propionyl-CoA carboxylase activity, whilst cell-free extracts of a *pccB* mutant strain contained a reduced level of propionyl-CoA carboxylase. No mutants in *accA2* could be isolated, suggesting that the gene may be essential. Heterologous expression of *accA1*, *accA2* and *pccB* in *Escherichia coli* and in vitro reconstitution of enzyme activity confirmed that PccB is the $\beta$ subunit of a propionyl-CoA carboxylase and that either AccA1 or AccA2 could act as the $\alpha$ component of this enzyme complex. The fact that *accA2* mutants appear to be inviable suggests that this gene encodes a biotinylated protein that might be shared with other carboxyl transferases essential for the growth of *S. coelicolor*.

**Keywords:** acyl-CoA carboxylase, primary metabolism, propionyl-CoA carboxylase, biotinylated protein

INTRODUCTION

Streptomyces produce a large number of structurally diverse polyketide antibiotics whose carbon skeletons are synthesized by multifunctional polyketide synthase enzymes. These enzymes catalyse repeated condensation cycles between acyl-CoA thioesters in a process similar to the biosynthesis of long-chain fatty acids (Hopwood & Sherman, 1990). The genes involved in the biosynthesis and in the regulation of many secondary metabolites, including polyketides, have been thoroughly studied (Martín & Liras, 1989). However, primary metabolism, which supplies the building blocks for antibiotic biosynthesis, has received much less attention.

In *Streptomyces* species, malonyl-CoA and methylmalonyl-CoA (mmCoA) are the most common chain extender units for the biosynthesis of many polyketide antibiotics (Hopwood & Sherman, 1990). Since they can both be synthesized via different primary metabolic pathways, they occupy a key position in the linkage between primary and secondary metabolism. Substrate availability for synthesis of the carbon skeletons of some polyketide antibiotics is one of the points of control for polyketide metabolism (Katz & Donadio, 1993). Therefore, knowledge of the enzymes involved in the supply of these precursors would enable the design of more rational approaches for the increased production of many useful secondary metabolites.

Several pathways leading to mmCoA have been described in bacteria (Gottschalk, 1986). In the genus *Streptomyces*, for example, mmCoA can be synthesized either from succinyl-CoA by mmCoA mutase (Birch et
al., 1993) or by carboxylation of propionyl-CoA by propionyl-CoA carboxylase (PCCase; EC 6.4.1.3) (Huanaiti & Kolattukudy, 1982; Donadio et al., 1996; Bramwell et al., 1996). It can also be derived from the catabolism of valine, methionine or threonine (Studier & Moffatt, 1986) or from n-butyryl-CoA through isobutyryl-CoA mutase (Zerbe-Burkhardt et al., 1998). Malonyl-CoA is synthesized in most species through the carboxylation of acetyl-CoA by an acetyl-CoA carboxylase (ACCCase; EC 6.4.1.2) (Bloom & Vance, 1977; Harwood, 1988). However, in some Streptomyces species, a second pathway for the biosynthesis of malonyl-CoA has been described (Behal et al., 1977; Laakel et al., 1994). This route involves the anaplerotic enzymes phosphoenolpyruvate carboxylase and oxaloacetate dehydrogenase.

In Streptomyces coelicolor A3(2), four secondary metabolites have been characterized so far (Hopwood et al., 1994). Two of them are wholly or partly polyketide-derived (Wasserman et al., 1976; Gorst-Allman et al., 1981) and malonyl-CoA is the predicted substrate for all of the condensation reactions involved in their biosynthesis. No evidence for the presence of oxaloacetate dehydrogenase has been found in S. coelicolor; therefore biosynthesis of malonyl-CoA in this organism seems to occur exclusively via ACCCase (Bramwell et al., 1993). Since in bacteria malonyl-CoA is required for all the elongation steps of fatty acid biosynthesis, the ACCCase of S. coelicolor should be a key enzyme linking primary and secondary metabolism. Interestingly, several complexes purified from actinomycetes with the ability to carboxylate acetyl-CoA also showed carboxylase activity with other substrates such as propionyl- and butyryl-CoA (Erfle, 1973; Henrikson & Allen, 1979; Huanaiti & Kolattukudy, 1982). This property has led to these enzyme complexes being called acyl-CoA carboxylases and all of them have been shown to consist of two subunits, a larger one (α chain) with the ability to carboxylate its covalently bound biotin group, and a smaller subunit (β chain) bearing the carboxyl transferase activity.

In S. coelicolor, a complex exhibiting only PCCCase activity has recently been purified (Bramwell et al., 1996) and shown to contain a biotinylated protein of 88 kDa as well as a non-biotinylated component, the carboxyl transferase, of 66 kDa. However, there is good evidence to suggest the existence of other acyl-CoA carboxylase(s) in this micro-organism. This evidence is the readily detectable levels of ACCCase activity in crude extracts of S. coelicolor (Bramwell et al., 1996) and the presence of a biotinylated protein with a molecular mass (65 kDa) similar to that of the α component of several acyl-CoA carboxylase complexes isolated from actinomycetes (Erfle, 1973; Henrikson & Allen, 1979; Huanaiti & Kolattukudy, 1982). In order to bring new insights to the characterization of the acyl-CoA carboxylases present in S. coelicolor, we set out to clone the gene(s) encoding the 65 kDa biotinylated protein and a gene encoding a carboxyl transferase (highly homologous to other known carboxyl transferases; Donadio et al., 1996; Cole et al., 1998). These studies led us to the genetic and biochemical characterization of a new PCCCase complex from S. coelicolor.

**METHODS**

**Bacterial strains, cultures and transformation conditions.** S. coelicolor strain M145 (SCP1 SCP2) was manipulated as described by Hopwood et al. (1985). Spores were prepared on SFM (2% (w/v) mannitol, 2% (w/v) soya flour and 2% (w/v) agar, in tap water) and stored as spore suspensions in 20% (v/v) glycerol at −20 °C.

*Escherichia coli* strain DH5α was used for routine subcloning and was transformed according to Sambrook et al. (1989). Transformants were selected on media supplemented with the appropriate antibiotics: ampicillin (Ap) 100 µg ml⁻¹; chloramphenicol (Cm) 25 µg ml⁻¹ or kanamycin (Km) 30 µg ml⁻¹. Strain BL21(DE3) is an *E. coli* B strain [F⁰ ompT (rbs)] (DE3]) lysogenized with λDE3, a prophage that expresses the T7 RNA polymerase downstream of the IPTG-inducible lacUV5 promoter (Studier & Moffatt, 1986). Unmodified plasmids purified from the dam dcm *E. coli* strain ET12567 (MacNeil et al., 1992) were used to transform S. coelicolor ET12567/pUZ8002 (a gift from M. Paget, John Innes Centre, Norwich, UK) was used for *E. coli*–*S. coelicolor* conjugation experiments (Bierman, 1992). For selection of Streptomyces transformants and exconjugants, media were overlaid with thioestrepton (Th) (300 µg per plate) or apramycin (Am) (1 mg per plate). Strains and recombinant plasmids are listed in Table 1.

**Growth conditions, protein expression and preparation of cell-free extracts.** *S. coelicolor* strain M145 was grown at 30 °C in shake flasks in YEME medium for 24–48 h. When necessary, 10 µg Am ml⁻¹ or 5 µg Th ml⁻¹ were added to the medium. Mycelia were harvested by centrifugation at 5000 g for 10 min at 4 °C, washed in 100 mM potassium phosphate buffer pH 8 containing 0·1 mM DTT, 1 mM EDTA, 1 mM PMSF and 10% glycerol (buffer A) and resuspended in 1 ml of the same buffer. The cells were disrupted by sonic treatment (4 or 5 s bursts) using a VibraCell Ultrasonic Processor (Sonic & Materials, Inc.). Cell debris was removed by centrifugation and the supernatant used as cell-free extract.

For the expression of heterologous proteins, *E. coli* strains harbouring the appropriate plasmids were grown at 37 °C in shake flasks in LB medium in the presence of 25 µg Cm ml⁻¹ or 100 µg Ap ml⁻¹ for plasmid maintenance. For the expression of biotinylated proteins, 10 µM n-biotin was added to the medium. Overnight cultures were diluted 1:10 in fresh medium and grown to A₅₉₀ 0·4–0·5 before the addition of IPTG to a final concentration of 0·1 mM. Induction was allowed to proceed for 2–4 h. The cells were then harvested, washed and resuspended in 1 ml buffer A. Cell-free extracts were prepared as described above.

**Protein methods.** Cell-free extracts were analysed by denaturing (SDS)-PAGE (Laemmli, 1970) using the Bio Rad mini-gel apparatus. The final acrylamide monomer concentration was 12% (w/v) for the separating gel and 5% for the stacking gel. Coomassie brilliant blue was used to stain protein bands. The biotinylated proteins were detected by a modification of the Western blotting procedure described by Nikolau et al. (1985). After electrophoretic separation, proteins were electroblotted onto nitrocellulose membranes (Bio-Rad) and probed with alkaline phosphatase–streptavidin conjugate (Bio-Rad) diluted 1:10000. Immunoblotting was
### Table 1. Strains and plasmids used

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Reference/source</th>
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<tr>
<td><strong>S. coelicolor</strong></td>
<td>Parental strain, SCP1− SCP2−</td>
<td>Hopwood <em>et al.</em> (1985)</td>
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<td>M145</td>
<td>Mutant carrying pTR36 inserted into <em>accA1</em> (Th&lt;sup&gt;R&lt;/sup&gt;)</td>
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<tr>
<td>MA4</td>
<td>Mutant carrying <em>aacC4</em> (Am&lt;sup&gt;R&lt;/sup&gt;) inserted into pccB</td>
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<td>MTC21</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; ΔlacU169 (g80lacZAM15) endA1 recA1 bsdR17 deoR supE44 thi-1 gyrA96 relA1</td>
<td>Hanahan (1983)</td>
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<td><strong>E. coli</strong></td>
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<td>F&lt;sup&gt;−&lt;/sup&gt; ompT (r&lt;sub&gt;H&lt;/sub&gt; m&lt;sub&gt;H&lt;/sub&gt;) (DE3)</td>
<td>Studier &amp; Moffatt (1986)</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; supE44 hsdS20 (r&lt;sub&gt;B&lt;/sub&gt; m&lt;sub&gt;B&lt;/sub&gt;) ara-14 proA2 lacY17 galK2 rpsL20 xyl-5 mtl-1 dam dcm hisG4 argR3 lacU169 (φ80lacZAM15) endA1 recA1 hsdR17 deoR supE44 thi-1 gyrA96 relA1</td>
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<td><strong>Plasmids</strong></td>
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<td>pBluescript SK(+)</td>
<td>Phagemid vector (Ap&lt;sup&gt;R&lt;/sup&gt; lacZ&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>Stratagene</td>
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<td>pIJ2460</td>
<td>pSK(+) with a <em>tsr</em> (Th&lt;sup&gt;R&lt;/sup&gt;) insert</td>
<td>Floriano &amp; Bibb (1996)</td>
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<td>pSU18</td>
<td>pACYC184-derived cloning vectors compatible with pBR322 and its derivatives (Cm&lt;sup&gt;R&lt;/sup&gt; lacZ&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>Bartolome &amp; Campbell (1981)</td>
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<td>pIJ2926</td>
<td>pUC18 derivative (Ap&lt;sup&gt;R&lt;/sup&gt; lacZ&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>Janssen &amp; Bibb (1993)</td>
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<td>pSET151</td>
<td>Used for the conjugal transfer of DNA from <em>E. coli</em> to <em>Streptomyces</em> spp. (Ap&lt;sup&gt;R&lt;/sup&gt; Th&lt;sup&gt;R&lt;/sup&gt; lacZ&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>Bierman <em>et al.</em> (1992)</td>
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<td>pET22b(+)</td>
<td>Phagemid vector (Ap&lt;sup&gt;R&lt;/sup&gt; lacZ&lt;sup&gt;−&lt;/sup&gt;) for expression of recombinant proteins under control of strong T7 transcription and translation signals</td>
<td>Novagen</td>
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<td>pBA11</td>
<td>Vector containing <em>E. coli birA</em> gene</td>
<td>Barker &amp; Campbell (1981)</td>
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<td>pUZ8002</td>
<td>RK2 derivative with defective ori&lt;sup&gt;T&lt;/sup&gt; (Km&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Paget <em>et al.</em> (1999)</td>
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<td>pSK(+) with a <em>Pst</em>I insert carrying <em>accA2</em></td>
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<td>pSET151 with an <em>aacC4</em> (Am&lt;sup&gt;R&lt;/sup&gt;) insert in the pccB coding region</td>
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<td>pTR68</td>
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<td>pTR78</td>
<td>pSU18 with pccB under the control of the lac promoter</td>
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- ACCase/PCCase assay. ACCase and PCCase activities in cell-free extracts were measured following the incorporation of H<sup>14</sup>CO<sub>3</sub> into acid non-volatile material (Huanaiti & Kolattukudy, 1982; Bramwell *et al.*, 1996). The reaction mixture contained 100 mM potassium phosphate pH 8.0, 300 µg BSA, 3 mM ATP, 5 mM MgCl<sub>2</sub>, 50 mM NaH<sup>14</sup>CO<sub>3</sub> [specific activity 200 µCi mmol<sup>−1</sup> (740 kBq mmol<sup>−1</sup>)], 1 mM substrate (acetyl-CoA or propionyl-CoA) and 100 µg cell-free protein extract in a total reaction volume of 100 µl. The reaction was initiated by the addition of NaH<sup>14</sup>CO<sub>3</sub>, allowed to proceed at 30 °C for 15 min and stopped with 200 µl 6 M HCl. The contents of the tubes were then evaporated to
dryness at 95 °C. The residue was resuspended in 100 µl water, 1 ml of Optiphase liquid scintillation (Wallac Oy) was added and ¹⁴C radioactivity determined in a Beckman scintillation liquid counter. Non-specific CO₂ fixation by crude extracts was assayed in the absence of substrate. One unit of enzyme activity catalysed the incorporation of 1 µmol ¹⁴C into acid-stable products per min.

**DNA manipulations.** Isolation of chromosomal and plasmid DNA, restriction enzyme digestion and agarose gel electrophoresis were carried out by conventional methods (Sambrook et al., 1989; Hopwood et al., 1985). Southern analyses were performed by using ³²P-labelled probes made by random oligonucleotide priming (Prime-a-gene kit; Promega).

**Gene cloning and plasmid construction.** The synthetic oligonucleotides HG5, 5'-CATGGATCCCTGCTATCTCCTCAT-(GC)GGTCATCTC, and HG2, 5'-CATGGATCCATTCACCGCC-(GC)CGGT(CTGCGTCG)TTCT (bracketed oligonucleotides indicate positions of degeneracy) were used in PCR to amplify an internal fragment of a gene encoding the 65 kDa bacteriolysin protein. The reaction mixture contained 10 mM Tris/HCl pH 8.3, 50 mM KCl, 1 mM MgCl₂, 6% glycol, 25 µM of each of the four dNTPs, 2.5 U Taq DNA polymerase, 20 pmol of each primer and 50 ng of S. coelicolor chromosomal DNA in a final volume of 100 µl. After denaturation at 95 °C for 2 min, the samples were subjected to 30 cycles of denaturation (95 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 2 min). PCR products were analysed by agarose (0.9 %) gel electrophoresis. The PCR product was digested with BamHI and cloned in BamHI-cleaved pBluescript SK(+) (Stratagene) in E. coli DH5α, yielding pTR10. The pTR10 insert was isolated as a BamHI fragment, labelled with ³²P by random oligonucleotide priming and used as a probe to isolate the accA1 and accA2 genes from S. coelicolor chromosomal DNA. A 7.5 kb SstI fragment hybridizing to the 1 kb probe was isolated from a size-enriched library and cloned in SstI-cleaved pBluescript SK(+), yielding pTR9. An EcoRI–KpnI fragment from pTR9 was isolated and cloned into EcoRI–KpnI-cleaved pBluescript SK(+), yielding pCL1. A second hybridizing band was also isolated from a size-enriched library and cloned as a 4 kb PstI fragment, yielding pTR45.

Oligonucleotides TCI, 5'-CAGAATTCAGCGACAGCC-AAGGGCAAGG- and TC2, 5'-CAGAATTCATGGCCGTCGTAATTCGCTCTAT- were used to amplify an internal fragment of the S. coelicolor pccB gene. The reaction mixture was the same as the one indicated above. Samples were subjected to 30 cycles of denaturation (95 °C, 30 s), annealing (65 °C, 30 s) and extension (72 °C, 1 min). A 1 kb PCR fragment was used as a ³²P-labelled probe to screen a size-enriched library. A 4.5 kb PstI fragment containing the complete pccB gene was cloned in PstI-cleaved pBluescript SK(+), yielding pTR58.

The synthetic oligonucleotide HG14, 5'-CATGGATCCAT-ATGGGGAAGACCGCCACTGGC, was used to introduce an NdeI site at the translational start codon of the S. coelicolor pccB gene, and HG15, 5'-CATGGATCCATGCGGGCTGGAA-GATCTCG, were used to amplify an internal fragment of the S. coelicolor pccB gene. The reaction mixture was the same as the one indicated above. Samples were subjected to 35 cycles of denaturation (95 °C, 30 s), annealing (65 °C, 30 s) and extension (72 °C, 1 min). The PCR product was digested with BamHI and cloned in BamHI-cleaved pBluescript SK(+) in E. coli DH5α, yielding pTR66. This plasmid was digested with BglII and HindIII, ligated with a BglII–HindIII fragment cleaved from pTR58 and introduced by transformation into E. coli DH5α, yielding pTR67. An NdeI–HindIII fragment from the plasmid pTR67 was cloned in NdeI–HindIII-cleaved pET22b(+) (Novagen) (pTR68), thus placing the pccB gene under the control of the powerful T7 promoter and ribosome-binding sequences. To obtain the same gene under the control of the lac promoter, an XbaI–HindIII fragment from pTR68 was cloned in XbaI–HindIII-cleaved pLJ2926 (Janssen & Bibb, 1993) and introduced by transformation into E. coli DH5α, yielding pTR71. Co-expression accA1 and pccB genes, we cloned a BamHI–HindIII fragment from pTR71 containing pccB into BamHI–HindIII-cleaved pSU18 (Bartolome et al., 1991) (compatible with the pBluescript SK plasmid), yielding pTR78.

**Nucleotide sequencing.** Double-strand plasmid DNA was prepared from deletion clones generated by nuclease ExoIII (Erase-a-base kit; Promega). Synthetic oligonucleotides were used to complete the sequence. The nucleotide sequence of the accA1 and accA2 region was determined by dideoxy sequencing (Sanger et al., 1977) using the Promega TaqTrack sequencing kit and double-stranded DNA templates.

**RESULTS**

**Cloning of accA1 and accA2 from S. coelicolor**

The α chain of all the acyl-CoA carboxylases isolated so far from actinomycetes is a multifunctional polypeptide that contains a covalently bound biotin prosthetic group located towards the carboxy terminus and a biotin carboxylase domain towards the N-terminal end of the polypeptide. Alignment of the amino acid sequences from the biotin carboxylase domain of the BCH and the biotin-carboxyl carrier protein (BCCP) domains of Mycobacterium leprae, Mycobacterium tuberculosis (Norman et al., 1994), E. coli (Kondo et al., 1991; Li & Cronan, 1992) and Pseudomonas aeruginosa (Best & Knauf, 1993) revealed several blocks of conserved amino acid residues (data not shown). Based on this knowledge, we attempted to clone an internal fragment of a gene encoding the biotinylated component of an acyl-CoA carboxylase complex of S. coelicolor by using PCR. Degenerate oligonucleotides, containing a BamHI site at their 5' end, were designed corresponding to the conserved sequences IHPGYGF and EAMKMM found at their 5' end. When single digests of the two genomic DNA had a site within the 1 kb probe was isolated from a size-enriched library. A 1 kb PCR product was used as a ³²P-labelled probe to screen a size-enriched library. A 4.5 kb PstI fragment containing the complete pccB gene was cloned in PstI-cleaved pBluescript SK(+), yielding pTR58. The synthetic oligonucleotide HG14, 5'-CATGGATCCAT-ATGGGGAAGACCGCCACTGGC, was used to introduce an NdeI site at the translational start codon of the S. coelicolor pccB gene, and HG15, 5'-CATGGATCCATGCGGGCTGGAA-GATCTCG, were used to amplify an internal fragment of the S. coelicolor pccB gene. The reaction mixture was the same as the one indicated above. Samples were subjected to 35 cycles of denaturation (95 °C, 30 s), annealing (65 °C, 30 s) and extension (72 °C, 1 min). The PCR product was digested with BamHI and cloned in BamHI-cleaved pBluescript SK(+) in E. coli DH5α, yielding pTR66. When single digests of S. coelicolor genomic DNA were probed with the 1.4 kb BamHI insert from pTR10 in Southern blots, two strongly hybridizing bands of similar intensities appeared in all digests (data not shown). None of the restriction enzymes used to cut the genomic DNA had a site within the 1 kb sequence,
Propionyl-CoA carboxylase in S. coelicolor A3(2)

Fig. 1. Analysis of an accA1 mutant obtained by transformation with pTR36 and selection for Th\(^{R}\). (a) Genomic region of wild-type S. coelicolor chromosome harbouring accA1 and accA2 genes. The restriction sites and their positions are indicated. Below is a map showing the expected organization after integration of pTR36 at accA1 and selection for Th\(^{R}\). Only the relevant sites are shown. Restriction sites: B, BamHI; Bs, BstEII; E, EcoRI; K, KpnI; P, PstI; S, SstI. B* and Bs* indicate BamHI and BstEII sites that were blunt-ended before cloning the fragment into the Smal site of pIJ2460. (b) Hybridization analysis of a Southern blot of BamHI-digested DNAs from M145 (lane 1) and Th\(^{R}\) transformat MA4 (lane 2). Lane 3 contains MA4 DNA digested with BamHI and EcoRI. The probe used was the internal 802 bp BamHI–BstEII fragment from accA2.

The target sequences were cloned from two different size-enriched libraries as described in Methods. The nucleotide sequences of the two DNA fragments revealed two highly homologous loci that were named accA1, located in cosmid 2C4, and accA2, located in cosmid K13. The amino acid sequences of the two ORFs, deduced from the nucleotide sequences, were nearly identical (the two polypeptides differ in only 5 amino acids), and both were very similar to BcpA1 and BcpA2 of Saccharopolyspora erythraea (Donadio et al., 1996), presumed to be the \(\alpha\) component of a PCCase.

Suggesting that the two hybridizing bands represented duplicate genes encoding this biotinylated protein. Moreover, when the same probe was used to screen the cosmid library of S. coelicolor M145 DNA (Redenbach et al., 1996), two unlinked cosmids gave positive signals (data not shown). One of the signals was eventually localized to cosmid K13 on AseI fragment K and the other to cosmid 2C4, recently mapped on AseI fragment B in the gap found between cosmids 2H4 and 10H5 (H. Kieser, personal communication).

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**Attempted disruption of accA1 and accA2**

To understand the roles of AccA1 and AccA2 in the formation of the acyl-CoA carboxylase complexes, we attempted to disrupt the respective genes. Based on the remarkably high nucleotide sequence identity of accA1 and accA2 (99 %), we designed an experiment that could generate insertion mutants in either of these two genes (Fig. 1a). An internal 802 bp BamHI–BstEII segment of the accA2 coding region was blunt-ended and cloned into the Smal site of pIJ2460 (Floriano & Bibb, 1996), resulting in pTR36. Several Th\(^{R}\) integrative transformants were obtained after transformation of M145 protoplasts with this plasmid. Total DNA of 10 independent transformants was isolated, digested with BamHI and analysed by Southern blot hybridization. In theory, the integration event should occur with comparable frequencies in both genes (the nucleotide sequence corresponding to the BamHI–BstEII fragment of accA2 and accA1 differs in only 4 nucleotides). Nevertheless, the hybridization profiles of all 10 clones were identical and showed that the integration event had occurred exclusively within the accA1 locus. Fig. 1(b) shows the result obtained with DNA from one of the accA1 mutants, MA4. Lane 1 shows the two hybridizing BamHI bands of M145 DNA; lane 2 shows that the 2·9 kb band containing the accA1 gene in the parental DNA is shifted to a larger-sized band in the MA4 mutant. The expected size of the BamHI fragment containing accA1 after integration of pTR36 was 8·1 kb; however, a band of approximately 20 kb was obtained (lane 2). When the same DNA was digested with BamHI and EcoRI (lane 3), the 20 kb band disappeared, yielding two new bands. The 5·2 kb hybridizing fragment corresponds to the size of pTR36 and, since this band is more intense than the 2·1 kb band, we suggest the integration of multimers of pTR36 in the chromosome at the accA1 locus.

Since we could not recover any strain containing pTR36 integrated within accA2, we tried to generate a mutant
by gene replacement, rather than single insertion. For this we used the conjugative vector pSET151 (Bierman et al., 1992), which does not replicate in Streptomyces. A PstI–KpnI chromosomal fragment containing accA2 previously disrupted by insertion of an Am resistance cassette was cloned in pSET151. The resulting plasmid, pTR57, was transferred to S. coelicolor by conjugation via the E. coli donor ET12567 containing the RP4 derivative pUZ8002. Several AmR ThR exconjugants were obtained and DNA from six of these colonies was digested with BamHI and analysed by Southern blot hybridization to confirm that integration had occurred by single crossover at the accA2 locus. One of the exconjugants which showed the expected hybridization profile was passed through four rounds on SFM containing Am. Several thousand colonies were screened for Th sensitivity (which would have reflected successful gene replacement) but no ThS isolates were obtained. This result, along with the inability of pTR36 to integrate in the accA2 locus, suggests that accA2 is likely to be essential for S. coelicolor viability.

Analysis of the biotinylated proteins produced by M145 and the mutant strain MA4 was carried out using a modified Western blot procedure (Nikolau et al., 1985). In the accA1 mutant a biotinylated protein of 65 kDa was still observed, which was presumably the product of accA2 (data not shown).

Cloning and disruption of pccB

An ORF with high similarity to PccB from Saccharopolyspora erythraea was found in a BamHI fragment of 5 kb containing the cya (cyclic-AMP synthase) gene (A. Danchin, personal communication), which is located in cosmid K13 of the S. coelicolor cosmid library (Redenbach et al., 1996). Using the sequence data kindly sent to us by Dr Danchin, we cloned the pccB gene as a 4–5 kb PstI fragment from a size-enriched library. Analysis of the predicted amino acid sequence of the major ORF contained within this fragment revealed that the best matching sequence in the GenBank database was the PccB homologue from Saccharopolyspora.
Table 2. ACCase and PCCase activities in cell-free extracts of *S. coelicolor* wild-type and mutant strains

<table>
<thead>
<tr>
<th><em>S. coelicolor</em> strain</th>
<th>Genotype</th>
<th>ACCase [mU (mg protein)⁻¹]</th>
<th>PCCase [mU (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>M145</td>
<td></td>
<td>1.12 ± 0.03</td>
<td>2.62 ± 0.06</td>
</tr>
<tr>
<td>MA4</td>
<td>accA1</td>
<td>1.13 ± 0.03</td>
<td>2.42 ± 0.06</td>
</tr>
<tr>
<td>MTC21</td>
<td>pccB</td>
<td>1.10 ± 0.03</td>
<td>1.20 ± 0.03</td>
</tr>
</tbody>
</table>

* Results are means of three determinations ± se.

eythraea* (77% amino acid sequence identity) (Donadio *et al*., 1996); it was somewhat less similar to AccD5 of *M. tuberculosis* (68% identity) and PccB of *M. leprae* (67% identity) (Cole *et al*., 1998). We therefore followed the same nomenclature used for *Saccharopolyspora erythraea* and named the gene *pccB*. Interestingly, the putative N-terminal sequence of *S. coelicolor* PccB does not match the N-terminal sequence of the 66 kDa protein reported as the carboxyl transferase subunit of the PCCase complex purified from this micro-organism (Bramwell *et al*., 1996). From these data we could not define whether we had cloned the carboxyl transferase subunit of a new PCCase complex or the β subunit of a yet unidentified acyl-CoA carboxylase complex of *S. coelicolor*.

To determine whether PccB was part of an acyl-CoA carboxylase complex, a *pccB* mutant was constructed by gene replacement (Fig. 2a). An Am resistance cassette was cloned in the unique BgIII site present in the coding sequence of *pccB* contained in pTR58, and the PstI fragment containing the mutated allele was cloned in the conjugative vector pSET151. The resulting plasmid, pTR61, was introduced into the *E. coli* donor strain ET12567/pUZ8002 and transferred by conjugation into M145, yielding Th⁺ Am⁺ exconjugants. Fig. 2(b) shows the Southern blot of one of the purified Th⁺ Am⁺ exconjugants and one Th⁺ Am⁺ segregant derived from it after three rounds of sporulation in a medium lacking Th. The hybridizing bands correspond to the expected sizes for integration of pTR61 in one of the *pccB* flanking regions (lane 3) and for the replacement of the wild-type *pccB* by the Am⁻ mutant allele after a second crossover (lane 2). The resulting mutant, MTC21, was studied by Western blotting using antibodies raised against PccB. Fig. 2(c) shows a strong band in M145 (*pccB⁺*) that is absent in the *pccB* mutant MTC21.

Acyl-CoA carboxylase activity in *S. coelicolor* M145 and in the *accA1* and *pccB* mutants

ACCCase and PCCase activities were assayed in cell-free extracts of M145 and the mutant strains MA4 (*accA1*) and MTC21 (*pccB*). Crude extracts of M145 contained both enzyme activities. However, the carboxylase activity for propionyl-CoA was considerably higher than that for acetyl-CoA (Table 2). Similar results were described for other actinomycetes such as *Mycobacterium smegmatis* (Henrikson & Allen, 1979) and *Saccharopolyspora erythraea* (Huanaiti & Kolattukudy, 1982). MA4 had levels of enzyme activities indistinguishable from those in M145, indicating that the product of *accA1*, if present in the cell, was either not involved in the formation of these enzyme complexes or was replaced by the product of the highly homologous *accA2* gene. On the other hand, the levels of PCCase found in the crude extract of the MTC21 mutant were much lower than those of M145, while the ACCase activity remained equal to the levels found in M145. These results strongly suggest that *pccB* encodes the β chain of a PCCase complex and we propose that other enzyme complexes with the ability to carboxylate acetyl-CoA or propionyl-CoA (or both) should also exist in *S. coelicolor*.

**Heterologous expression of the *accA1*, *accA2* and *pccB* genes in *E. coli***

Expression of *accA1* and *accA2* in *E. coli* was carried out in strains RG1 and RG2 obtained by transformation of DH5α with plasmids pCL1 and pTR45, respectively. These plasmids are pBluescript SK(+) derivatives, and transcription of the cloned genes is under the control of the inducible lac promoter of the vector. After IPTG induction, biotinylated proteins present in the crude extracts were analysed by a modified Western blotting procedure. Production of biotinylated AccA1 by RG1 was more efficient than production of biotinylated AccA2 by RG2 (Fig. 3a, lanes 3 and 4). This might reflect a difference in the transcriptional levels of *accA1* and *accA2* from plasmids pCL1 and pTR45, respectively. For instance, a basal level of expression is also obtained from pCL1 (Fig. 3a, lane 2) in the absence of IPTG induction, indicating the recognition of the *accA1* promoter in *E. coli*. In addition, titration of biotin ligase could have limited the production of high levels of the biotinylated proteins in *E. coli* (Cronan, 1990). In an attempt to increase the biotinylation of AccA2, we introduced the multicopy plasmid pBA11 (Barker & Campbell, 1981), which carries the *E. coli* birA gene, into the *E. coli* strain containing pTR45 to yield strain RG5. As shown in Fig. 3a, the levels of biotinylated AccA2 increased significantly when extra copies of BirA were present in the cell (compare lanes 4 and 5).
To express pccB in E. coli, we introduced a NdeI site at the ATG start codon of the ORF by PCR; after two intermediate constructions (see Methods), we cloned the gene in the expression vector pET22b(+), yielding pTR68. E. coli strain BL21(DE3) was transformed with this plasmid and when induced with IPTG, large amounts of insoluble protein, presumably resulting from the formation of inclusion bodies, were obtained. This turned out to be very convenient for the purification of the putative carboxyl transferase subunit (Fig. 3b, lane 3), which was used to raise antibodies, but not for the enzyme reconstitution studies that we had planned. To enhance the production of large amounts of soluble protein, we subcloned the XbaI–HindIII fragment from pTR68 into pJJ2926 (Janssen & Bibb, 1993), which had been digested with the same enzymes. The resulting plasmid, pTR71, had the pccB gene downstream of the transcriptional and translational sequences of the lac operon. After transforming DH5α with this plasmid to yield strain RG4, IPTG induction resulted in the production of high levels of a soluble 63 kDa protein (Fig. 3b, lanes 1 and 2).

**In vitro reconstitution of a PCCase complex**

Disruption of pccB in S. coelicolor and enzyme assays of the mutant MTC21 suggested that this gene encodes the β subunit of a PCCase. In order to study the substrate specificity of this subunit, and at the same time to identify the α component of the enzyme complex, we attempted in vitro reconstitution of the enzyme activity by mixing E. coli crude extracts containing each of the two available biotinylated proteins with the putative carboxyl transferase subunit PccB. It is important to recall that E. coli does not contain a PCCase enzyme and that ACCase activity cannot be assayed directly by carboxylation of acetyl-CoA in this organism (Polakis et al., 1972). Cell-free extracts of RG4 containing the putative β subunit were mixed with cell-free extracts of the E. coli strains containing each of the biotinylated proteins AccA1 or AccA2. After incubation for 1, 24 or 72 h at 4 °C, the mixtures were assayed for ACCase and PCCase activity (Table 3). A complex with PCCase activity was successfully reconstituted when the carboxyl transferase subunit, PccB, was incubated in the presence of comparable amounts of either AccA1 or AccA2. The reconstituted enzyme complex specifically catalyses the carboxylation of propionyl-CoA; therefore, we propose that the enzyme formed with these α and β components is a PCCase rather than an acyl-CoA carboxylase with dual substrate specificity as has been described for other actinomycetes (Erfle, 1973; Henrikson & Allen, 1979; Huanaiti & Kolattukudy, 1982).

When both accA2 and pccB were co-expressed in E. coli strain RG7, PCCase activity was successfully assayed in freshly made cell-free extracts (Table 3), indicating that this complex can be effectively assembled in the cytoplasm of E. coli. Co-expression of these genes in E. coli strain LA1-6, which harbours a temperature-sensitive mutation in one of the carboxyl transferase subunits (accD) (Harder et al., 1972), could not complement the mutation, showing that the enzyme complex formed in
Table 3. Heterologous expression of PCCase components in cell-free extracts of E. coli and in vitro reconstitution of enzyme activity

<table>
<thead>
<tr>
<th>E. coli strain*</th>
<th>Proteins induced by IPTG</th>
<th>Plasmids</th>
<th>ACCase [mU (mg protein)^{-1}]†</th>
<th>PCCase [mU (mg protein)^{-1}]†</th>
</tr>
</thead>
<tbody>
<tr>
<td>RG1</td>
<td>AccA1</td>
<td>pCL1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RG2</td>
<td>AccA2</td>
<td>pTR45</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RG5</td>
<td>AccA2</td>
<td>pTR45, pBA1‡</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RG4</td>
<td>PccB</td>
<td>pTR71</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RG7</td>
<td>AccA1, PccB</td>
<td>pCL1, pTR78</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RG1:RG4‡</td>
<td>AccA1: PccB</td>
<td>pCL1: pTR78</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RG2:RG4‡</td>
<td>AccA2: PccB</td>
<td>pTR45: pTR71</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RG5:RG4‡</td>
<td>AccA2: PccB</td>
<td>pTR45, pBA11:pTR71</td>
<td>ND</td>
<td>0.65 ± 0.04</td>
</tr>
</tbody>
</table>

ND, Not detectable. The amount of ^14C fixed into acid-stable products was not significantly higher than background levels (10–30 c.p.m., equivalent to 0.02–0.06 mU).

*All the RG strains are derived from E. coli DH5a.
† Results are means of three determinations ± se.
‡ pBA11 expresses BirA constitutively.
§ Mix of equal amounts of proteins from cell-free extracts prepared from each of the strains indicated.

Propionyl-CoA carboxylase in S. coelicolor A3(2)

**vivo**, like the one in vitro, does not possess ACCase activity.

PCCase activity was readily measured after 1 h of incubation of the cell-free extracts containing the individual subunits, and remained stable for as long as 72 h. Interestingly, the levels of PCCase activity in the crude extracts prepared from *S. coelicolor* increased (approx. 50% after 72 h) with the time of incubation at 4°C. This effect could be accounted for by the inactivation of an interfering activity present in the crude extracts.

**DISCUSSION**

The results presented here are a step forward in the characterization of the acyl-CoA carboxylases present in *S. coelicolor* and in the identification of some of the α and β components of these enzyme complexes. By using a PCR-based approach with degenerate oligonucleotides designed against conserved regions of several bacterial BC and BCCP domains, we successfully amplified a PCR product whose nucleotide sequence revealed an ORF highly homologous to the accBC gene product of *M. tuberculosis* (Norman et al. 1994), the α chain of rat PCCase (Browner et al. 1989), and BcpA1 and BcpA2 of *Saccharopolyspora erythraea* (Donadio et al. 1996). Attempts to clone the entire coding sequence of this ORF prompted us to identify two highly similar copies of this gene that were named accA1 and accA2 (for acyl-CoA carboxylase α subunit).

In order to establish the role of the accA1 and accA2 genes in the formation of acyl-CoA carboxylase complexes in *S. coelicolor*, we tried to isolate mutants in each of these genes and assay them for ACCase and PCCase activities. Cell-free extracts of the accA1 mutant showed the same enzyme activity levels as the parental strain (Table 2), suggesting that the product of this gene was not involved in the formation of any of these enzyme complexes. However, the high similarity between accA1 and accA2, and the fact that we could not generate a mutant in the latter gene because of its apparent essentiality, leave open the possibility that AccA2 could substitute for AccA1 in vivo.

Mutants in pccB showed reduced PCCase activity, suggesting that PccB is the carboxyl transferase subunit of a PCCase complex. This result agrees with that obtained with the pccB homologue of *Saccharopolyspora erythraea* (Donadio et al. 1996), although in this micro-organism only one PCCase complex seems to be present (less than 5% of the PCCase activity remained in the pccB mutant). In contrast, in *S. coelicolor*, 50% of the original PCCase activity is still present in the pccB mutant, in accordance with the results of Bramwell et al. (1996) who purified a complex with affinity for propionyl-CoA and with a carboxyl transferase component different from PccB.

Heterologous expression of accA1, accA2 and pccB in *E. coli* and in vitro reconstitution experiments established that either AccA2 or AccA1 could function as the α component of a PCCase in *S. coelicolor* and confirmed that PccB was the β component of this enzyme complex (Table 3). Based on the apparent inviability of accA2 mutants in *S. coelicolor*, and on the fact that we successfully assayed ACCase activity in crude extracts of M145 and in the accA1 mutant (Table 2), we hypothesize that accA2 could also encode the α subunit of an essential ACCase whose β subunit has not yet been identified.

In the genus *Streptomyces*, several pathways leading to mmCoA have been described, and in *S. coelicolor* many of these pathways may exist (Bramwell et al.,
1993; Redenbach et al., 1996). Our results and those of Bramwell et al. (1996) demonstrate that *S. coelicolor* contains at least two PCCase complexes, indicating that this enzyme activity may play a major role in the metabolism of this micro-organism. In *Streptomyces*, mmCoA is generally related to the production of secondary metabolites (Hopwood & Sherman, 1990). *S. coelicolor* is not known to make a natural secondary metabolite derived from mmCoA, although it must presumably be able to make the precursor since it has been shown to be able to synthesize significant quantities of the cyclic macrolactone of erythromycin after introduction of the ery polyketide synthase genes (Kao et al., 1994). Thus, the physiological role of mmCoA in *S. coelicolor* remains to be established; it might perhaps be used to feed the TCA cycle, after its conversion into succinyl-CoA catalysed by the mmCoA mutase (Birch et al., 1993), rather than in the biosynthesis of secondary metabolites.

As we have shown here, heterologous expression of the individual components of the *S. coelicolor* PCCase and *in vitro* reconstitution of a functional complex have proved to be successful. This approach will be very useful for the characterization of the α and β components of other acyl-CoA carboxylases, not only in *S. coelicolor*, but also in other actinomycetes such as *M. tuberculosis*, where several acyl-CoA carboxylases seem to be present (Cole et al., 1998).

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