Role of an Essential Acyl Coenzyme A Carboxylase in the Primary and Secondary Metabolism of *Streptomyces coelicolor* A3(2)

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Two genes, *accB* and *accE*, that form part of the same operon, were cloned from *Streptomyces coelicolor* A3(2). *AccB* is homologous to the carboxyl transferase domain of several propionyl coenzyme A (CoA) carboxylases and acyl-CoA carboxylases (ACCases) of actinomycete origin, while *AccE* shows no significant homology to any known protein. Expression of *accB* and *accE* in *Escherichia coli* and subsequent in vitro reconstitution of enzyme activity in the presence of the biotinylated protein *AccA1* or *AccA2* confirmed that *AccB* was the carboxyl transferase subunit of an ACCase. The additional presence of *AccE* considerably enhanced the activity of the enzyme complex, suggesting that this small polypeptide is a functional component of the ACCase. The impossibility of obtaining an *accB* null mutant and the thistreptin growth dependency of a *tipA* *accB* conditional mutant confirmed that *AccB* is essential for *S. coelicolor* viability. Normal growth phenotype in the absence of the inducer was restored in the conditional mutant by the addition of exogenous long-chain fatty acids in the medium, indicating that the inducer-dependent phenotype was specifically related to a conditional block in fatty acid biosynthesis. Thus, *AccB*, together with *AccA2*, which is also an essential protein (E. Rodriguez and H. Gramajo, Microbiology 143:3109–3119, 1999), are the most likely components of an ACCase whose main physiological role is the synthesis of malonyl-CoA, the first committed step of fatty acid synthesis. Although normal growth of the conditional mutant was restored by fatty acids, the cultures did not produce actinorhodin or undecylprodigiosin, suggesting a direct participation of this enzyme complex in the supply of malonyl-CoA for the synthesis of these secondary metabolites.

Malonyl coenzyme A (CoA) is an essential metabolite in most living organisms. It is a substrate for fatty acid synthases (4, 16), for polyketide synthases in plants, fungi, and bacteria (19), and for fatty acid chain elongation systems (37). It also plays a role as a modulator of the activity of some proteins (8). Since malonyl-CoA is used in the production of many of the pharmaceutically important polyketides made by streptomyces (19), there is considerable interest in understanding the pathway(s) that leads to its synthesis. Thus, knowledge of the enzyme(s) involved in the supply of this key metabolite will not only provide a better understanding of primary metabolism in streptomycetes but will potentially allow for the development of more rational approaches for improving the level of production of many useful secondary metabolites.

Biosynthesis of malonyl-CoA occurs in most species through ATP-dependent carboxylation of acetyl-CoA by an acetyl-CoA carboxylase (45). The reaction catalyzed by this enzyme is a two-step process that involves ATP-dependent formation of carboxybiotin, followed by transfer of the carboxyl moiety to acetyl-CoA. Acetyl-CoA carboxylase expression is essential for the normal growth of bacteria (27, 28, 32), yeasts (17), and isolated animal cells in culture (33), reflecting the importance of this biosynthetic pathway.

Several complexes with acyl-CoA carboxylase (ACCase) activity have been purified from a number of actinomycetes. These complexes also possess the ability to carboxylate other substrates, including propionyl- and butyryl-CoA (12, 18, 20). Consequently, these enzymes are referred to as ACCases, and all of them consist of two subunits, a larger one (the α chain) with the ability to carboxylate its covalently bound biotin group and a smaller one (the β chain) bearing the carboxyl transferase activity. Little is known about the physiological role of these enzymes.

The pathway for the biosynthesis of malonyl-CoA in *Streptomyces coelicolor* has not been established yet. However, acetyl-CoA carboxylase activity has been readily measured in crude extracts of *S. coelicolor* (7, 36), confirming the presence of this enzyme activity in this microorganism. Attempts to purify a complex with acetyl-CoA carboxylase activity from streptomycetes have been unsuccessful, probably reflecting its high instability in vitro (7). An alternative pathway for the biosynthesis of malonyl-CoA was described in *Streptomyces aureofaciens* (2, 25) and involved the anaerobic enzymes phosphoenolpyruvate carboxylase and oxaloacetate dehydrogenase. However, oxaloacetate dehydrogenase could not be detected in *S. coelicolor* A3(2) (6), where malonyl-CoA synthesis appears to occur exclusively through the acetyl-CoA carboxylase complex. Attempts to identify enzymes with carboxylase activity in *S. coelicolor* led to the characterization of two complexes exhibiting exclusively propionyl-CoA carboxylase (PCCase) activity. The PCCase purified by Bramwell et al. (7) consisted of a biotinylated protein, PccA, of 88 kDa and a nonbiotinylated...
organism. be the main pathway for malonyl-CoA synthesis in this micro-
zyme complex possesses unique characteristics and appears to
M145 (SCP1

S. coelicolor
terization of an essential ACCase from
components were selected on media supplemented with the appropriate antibiotics
cloning and was transformed according to the method of Hanahan (15). Trans-
YEME liquid medium.
The strain was grown on SFM, R2, and R5 agar media and in 50 ml of SMM or
Paget, John Innes Centre, Norwich, United Kingdom) was used for
BL21(DE3) is an
expresses the T7 RNA polymerase from the isopropyl-
m
VOL. 67, 2001 ANALYSIS OF AN ACYL-CoA CARBOXYLASE OF
S. coelicolor
Bacterial strains, culture, and transformation conditions. S. coelicolor strain
M145 was manipulated as described by Hopwood et al. (19).

TABLE 1. Strains and plasmids used in this study

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Description</th>
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<tr>
<td><strong>Strains</strong></td>
<td></td>
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<td>R7G</td>
<td>DH5s carrying pCL1 and pBA11 plasmids</td>
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<td><strong>Plasmids</strong></td>
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<td>recombinant proteins under tipAp promoter</td>
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component, the carboxyl transferase, of 66 kDa. More recently, we characterized, genetically and biochemically, the compo-
nents of a second PCCase in this bacterium. In vitro recon-
titution experiments showed that an active complex could be
obtained by mixing a carboxyl transferase component of 65
kDa, PccB, with either of the two almost identical biotinylated
components, AccA1 and AccA2 (36).

Here we present a detailed genetic and biochemical charac-
terization of an essential ACCase from S. coelicolor. The en-
zyme complex possesses unique characteristics and appears to
be the main pathway for malonyl-CoA synthesis in this micro-
organism.

MATERIALS AND METHODS

Bacterial strains, culture, and transformation conditions. S. coelicolor strain
M145 (SCP1, SCP2) was manipulated as described by Hopwood et al. (19).
The strain was grown on SFM, R2, and R5 agar media and in 50 ml of SMM or
YEME liquid medium. Escherichia coli strain DH5α was used for routine sub-
cloning and was transformed according to the method of Hanahan (15). Trans-
formants were selected on media supplemented with the appropriate antibiotics
at the following concentrations: ampicillin, 100 μg ml⁻¹; apramycin (APR), 100
μg ml⁻¹; chloramphenicol, 25 μg ml⁻¹; and kanamycin, 30 μg ml⁻¹. Strain
BL21(DE3) is an E. coli B strain lysogenized with λDE3, a prophage that
expresses the T7 RNA polymerase from the isopropyl-β-D-thiogalactopyranoside
(IPTG)-inducible lacUV5 promoter (43). ET12567/pUZ8002 (a gift from M.
Paget, John Innes Centre, Norwich, United Kingdom) was used for E. coli-
S. coelicolor conjugation experiments (3). For selection of Streptomyces transfor-
ments and exconjugants, media were overlaid with thiostrepton (TH) (300 μg per
plate), hygromycin (HYG) (1 mg per plate), or APR (1 mg per plate), respec-
tively. Strains and recombinant plasmids are listed in Table 1. Fatty acid sup-
plementation studies were performed in SMM containing APR (10 μg ml⁻¹) and
0.075% (vol/vol) Brij 58. The different fatty acids were added at a final concen-
tration of 100 μg ml⁻¹.

Growth conditions, protein production, and preparation of cell extracts. S. coelicolor M145 was grown at 30°C in shake flasks in YEME medium for 24 to
48 h. When necessary, 10 μg of APR ml⁻¹ or 5 μg of TH ml⁻¹ was added to the
medium. Mycelia were harvested by centrifugation at 5,000 × g for 10 min at 4°C,
wash in 100 mM potassium phosphate buffer, pH 8, containing 0.1 mM di-
thiothreitol (DTT), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10%
glycerol (buffer A) and resuspended in 1 ml of the same buffer. The cells were
admitted by sonic treatment (4- or 5-s bursts) using a VibraCell Ultrasonic
Processor (Sonics & Materials, Inc.). Cell debris was removed by centrifugation,
and the supernatant was used as cell extract. For the expression of heterologous
proteins, E. coli strains harboring the appropriate plasmids were grown at 37°C
in shake flasks in Luria-Bertani medium in the presence of 25 μg of chloro-
phenicol ml⁻¹ or 100 μg of ampicillin ml⁻¹ for plasmid maintenance. In order to
improve the biotinylation of AccA1 and AccA2 in E. coli, the strains containing
pCL1 or pTR204 were also transformed with pBA11 (1), which overexpresses the
E. coli biotin ligase; 10 μM d-biotin was also added to the medium. Overnight
cultures were diluted 1:10 in fresh medium and grown to an A600 of 0.4 to 0.5
before addition of IPTG to a final concentration of 0.1 mM. Induction was
allowed to proceed for 4 h. The cells were harvested, washed, and resuspended
in 1 ml of buffer A. Cell extracts were prepared as described above.

Protein methods. Cell extracts were analyzed by denaturing sodium dodecyl
sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (26) using a Bio-Rad
minigel apparatus. The final acrylamide monomer concentration was 12% (wt/vol) for the separating gel and 5% for the stacking gel. Coomassie brilliant blue was used to stain protein bands. Protein contents were determined by the method of Bradford (5) with bovine serum albumin as the standard. The relative contribution of the AccB and AccC proteins was assessed in a Coomassie-blue-stained gel determined by densitometric scanning of the polyacrylamide-SDS gels.

Acetyl-CoA carboxylase and PCCase assay. Acetyl-CoA carboxylase and PCCase activities in cell extracts were measured following the incorporation of HCO_3^- into acid nonvolatile material (7, 20). The reaction mixture contained 100 mM potassium phosphate (pH 8.0), 300 µg of bovine serum albumin, 3 mM ATP, 5 mM MgCl_2, 50 mM NaH_2CO_3 (specific activity, 200 µCi mmol^-1 [740 kBq mmol^-1]), 1 mM substrate (acetyl-CoA or propionyl-CoA), and 100 µg of cell-free protein extract in a total reaction volume of 100 µl. The reaction was initiated by the addition of NaH_2CO_3, allowed to proceed at 30°C for 15 min, and stopped with 200 µl of 6 M HCl. The contents of the tubes were then evaporated to dryness at 95°C. The residue was resuspended in 100 µl of 1 M Optiphase scintillation liquid (Wallac Oy) and was added, and the 3^2^C radioactivity was determined in a Beckman liquid scintillation counter. Nonradioactive CO_2, which was dried in a column of 2.5% NaOH, was quantified by measurement of 3^2^C in the gas phase.

DNA manipulations. Isolation of chromosomal and plasmid DNA, restriction enzyme digestion, and agarose gel electrophoresis were carried out by conventional methods (22, 38). Southern analyses were performed by using 32P-labeled probes made by random oligonucleotide priming (Prime-a-gene kit; Promega).

Gene cloning and plasmid construction. The synthetic oligonucleotides TC1 (5'-ACAATTCGAGCGACGCGCCGGAAGC) and TC2 (5'-CAAGATTCGACTCGTCTGCTCAGTGG) were used to amplify an internal fragment from the plasmid pTR82, yielding the plasmid pTR92. This plasmid was digested with XbaI and SmalI, and the 1-kb PCR fragment was used as a 32P-labeled probe to screen a size-enriched library. A 2.7-kb XbaI fragment from pTR82 was cloned in pBluescript SK(+), yielding pTR83. This plasmid was digested with XbaI and cloned in pBluescript SK(+), yielding pTR84. A 1-kb XbaI fragment from the plasmid pTR82 was cloned in XbaI-digested pBluescript SK(+), yielding pTR82. An XbaI fragment from the plasmid pTR82 was cloned in XbaI-digested pBluescript SK(+), yielding pTR83. In order to place the chromosomal copy of the accBE operon under the control of the lac promoter, we removed from pTR83 a HindIII fragment containing the int gene and att of 63C1, yielding pTR84.

Protein purification protocols. The His6-tagged fusion protein H6AccE was purified from cultures of RG12 [strain BL21(DE3) harboring pTR237] after the addition of 0.1 mM IPTG to induce the DE3-encoded T7 RNA polymerase. Cells were pelleted, resuspended in 50 mM phosphate buffer (pH 7.2)-300 mM NaCl-0.5 mM diithiothreitol-10% glycerol, and disrupted by sonication. Cell debris was removed by centrifugation, and the supernatant was purified by dialysis against a nitritolactic acid-agarose affinity column equilibrated with the same buffer. The H6AccE protein was recovered by elution with 100 mM imidazole and dialyzed against a solution containing 100 mM sodium phosphate (pH 7.2), 1 mM dithiothreitol, 1 mM EDTA, and 20% glycerol.

Nucleotide sequencing. The sequence of the SfiI fragment containing accBE was determined by subcloning ApoI fragments from pRM08 in pSKBluescript SK(+). Synthetic oligonucleotides were used where needed to complete the sequence. Dideoxy sequencing (39) was carried out using the Promega TagTrack sequencing kit and double-stranded DNA templates.

S1 nuclease mapping. For each S1 nuclease reaction, 30 µg of RNA was hybridized in trichloroacetic acid-sodium salt (NaTCA) buffer (solid NaTCA [Aldrich] was dissolved to 5 M in 50 mM PIPES, 5 mM EDTA, pH 7.0) to about 0.002 molal (approximately 10^7 cpm) of the following probes. For accA2, the oligonucleotide 5'-GAAGTACAGGCCGAAGACCAC (accA2up), which corresponds to a sequence upstream of the accA2 promoter region, to generate a 766-bp probe. For accA1, the oligonucleotide 5'-GGCATTCGACGGCGCCAAGGAC (accA1down), which corresponds to a sequence downstream of the accA1 promoter region, to generate a 563-bp probe. For accB, the oligonucleotide 5'-CTCGAATGAGGCAGACAA (accBup), which corresponds to a sequence upstream of the accB promoter region, to generate a 483-bp probe. For accC, the oligonucleotide 5'-GGGAGGCGCGTGAGGGCGGCG (accCdown), which corresponds to a sequence downstream of the accC promoter region, to generate a 563-bp probe. For accD, the oligonucleotide 5'-CTACGCCTGCGCTGCGCGC (accDdown), which corresponds to a sequence downstream of the accD promoter region, to generate a 483-bp probe. For accE, the oligonucleotide 5'-GGGAGGCGCGTGAGGGCGGCG (accEup), which corresponds to a sequence upstream of the accE promoter region, to generate a 563-bp probe. For accF, the oligonucleotide 5'-CTACGCCTGCGCTGCGCGC (accFdown), which corresponds to a sequence downstream of the accF promoter region, to generate a 483-bp probe. For accG, the oligonucleotide 5'-CTACGCCTGCGCTGCGCGC (accGdown), which corresponds to a sequence downstream of the accG promoter region, to generate a 483-bp probe. For accH, the oligonucleotide 5'-CTACGCCTGCGCTGCGCGC (accHdown), which corresponds to a sequence downstream of the accH promoter region, to generate a 483-bp probe. For accI, the oligonucleotide 5'-CTACGCCTGCGCTGCGCGC (accIdown), which corresponds to a sequence downstream of the accI promoter region, to generate a 483-bp probe. For accJ, the oligonucleotide 5'-CTACGCCTGCGCTGCGCGC (accJdown), which corresponds to a sequence downstream of the accJ promoter region, to generate a 483-bp probe. The S1 nuclease reaction was completely terminated by the addition of ice-cold 0.2 M EDTA, pH 9.0.
Cloning the accBE genes. Since pccB mutants of S. coelicolor produce wild-type levels of acetyl-CoA carboxylase (36), we foresaw that a second gene encoding a different carboxyl transferase β subunit capable of recognizing acetyl-CoA as a substrate should exist in this organism. Based on the high level of sequence homology shown by genes encoding putative carboxyl transferases in the same species (e.g., in Mycobacterium tuberculosis [10]), we attempted to clone this alternative β subunit gene using pccB as a hybridization probe. When a BamHI digest of S. coelicolor DNA was probed with pccB under conditions of low stringency, a second poorly hybridizing band was readily detected (data not shown). This hybridizing sequence was cloned from a size-enriched library as a 2.5-kb BamHI fragment. Sequencing revealed the presence of an incomplete open reading frame (ORF) with high homology to pccB; the complete gene was subsequently cloned on a 6-kb SstI fragment, yielding pRM08 (Fig. 1). Sequencing of this fragment revealed a putative protein with end-to-end similarity to a likely decarboxylase of Streptomyces cyanogenus (76% identity [46]), to PccB from S. coelicolor (57% identity [36]), and to the β subunit (PccB) of the Saccharopolyspora erythraea PCCase (56% identity [11]). The gene encoding this new putative carboxyl transferase was called accB.

The sequence also revealed the presence of a small ORF, accE, whose start codon was only 17 bp downstream of the termination codon of accB. A 17-nucleotide (nt) inverted repeat which could function as a factor-independent bidirectional transcriptional terminator separates accE from three convergent ORFs with homology to putative proteins of M. tuberculosis of unknown function. The putative AccE protein has a deduced molecular mass of 7.5 kDa and does not resemble any other known protein. The region upstream of accB encodes a putative protein which is highly homologous to several known hyaluronidases.

Heterologous expression of accB, accE, and in vitro reconstitution of an ACCase complex. Recently, we achieved reconstitution of a PCCase complex activity by mixing E. coli cell extracts containing PccB (the carboxyl transferase) with cell extracts containing the biotinylated subunits AccA1 and AccA2 (36). To assess whether AccB and AccE were components of a previously uncharacterized carboxylase complex, we attempted similar in vitro reconstitution experiments with crude extracts containing these proteins. Since E. coli does not contain PCCase and acetyl-CoA carboxylase activity cannot be assayed directly by carboxylation of acetyl-CoA (34), the acetyl-CoA carboxylase activity measured in these crude extracts represents the activity of heterologous complexes reconstituted in vitro.

Overexpression of accB and accE in E. coli was attempted with strain RG8, a BL21(DE3) strain containing pTR88 (Fig. 1). SDS-PAGE of crude extracts of RG8, prepared from IPTG-induced cultures, revealed overexpression of a 57-kDa protein, corresponding to the predicted size of AccB. In the same electrophoretic analysis no clearly identifiable AccE band was observed. In vitro reconstitution of ACCase activity was then obtained by mixing a crude extract prepared from an IPTG-induced culture of RG8 with a cell extract of E. coli strain RG11, which overproduces the biotinylated protein AccA2 and the E. coli biotin ligase BirA, harbored in plasmids pTR204 and pBA11, respectively. After incubation for 1 h at 4°C, the mixture was assayed for acetyl-CoA carboxylase and PCCase activities. As shown in Table 2, an enzyme complex not containing PCCase and acetyl-CoA carboxylase activity could be obtained when the reconstitution experiments were performed using cell extracts of strain RG7, a BL21(DE3) strain containing pCL1 that provides AccA1 instead of AccA2 as the biotinylated component of the ACCase. The lower levels of both activities were similar to those measured when PccB was used in reconstitution experiments with E. coli cell extracts.
acetyl-CoA carboxylase and PCCase activity are due to the lower level of expression of accA1 by pCL1 (36). These results confirmed that either AccA1 or AccA2 could be used efficiently, at least in vitro, as the α subunit of the enzyme complex (Table 2).

Is AccE a functional component of the ACCase complex? The genetic organization of accB and accE as members of the same transcription unit suggested that AccE could also be a functional component of the ACCase complex. To investigate this hypothesis we assayed acetyl-CoA carboxylase and PCCase activities in a mixture of cell extracts that contained AccB [strain RG8, a BL21(DE3) strain containing pTR90] and AccA2 [strain RG11, a BL21(DE3) strain containing pTR204] but not AccE. Although ACCase activity was readily detected in this mixture, indicating that AccE is not catalytically necessary for the successful reconstitution of an active complex in vitro, the levels of acetyl-CoA carboxylase and PCCase activities were considerably lower (approximately 30%) than those obtained with cell extracts that contained AccB and AccE (Fig. 2, compare mixes 1 and 2). Since the levels of AccB in the cell extracts of RG8 and RG9 were essentially the same, we inferred from these experiments that AccE was necessary to obtain a fully active ACCase complex. To confirm that the absence of AccE was responsible for the lower ACCase activity observed, we studied the effect that the addition of cell extracts containing high levels of soluble AccE [strain RG10, a BL21 (DE3) strain containing pTR107] had on the ACCase activity present in a mix of crude extracts containing AccB and AccA2. As shown in Fig. 2 (mixes 2 and 3) the specific activities of both acetyl-CoA carboxylase and PCCase were almost 3.5 times higher in the presence of AccE than in the control experiment that lacked this protein and resembled those values obtained by mixing RG8 (AccBE) and RG11 (AccA2) cell extracts. Similar results were obtained when purified H6AccE was added to the AccB-AccA2 mix (Fig. 2, mixes 4 to 9). The addition of different amounts of H6AccE (from values ranging from 0.1 to 10 μg of pure protein) increased the levels of acetyl-CoA carboxylase activity, reaching saturation when more than 2 μg of AccE was present in the reaction mix. The fact that the maximum level of enzyme activity was obtained at high concentrations of AccE proposed a direct participation of this protein in the activation of the complex formed by AccB and AccA2. Although the results presented in this section

### Table 2. Heterologous expression of ACCase components in cell extracts of E. coli and in vitro reconstitution of enzyme activity

<table>
<thead>
<tr>
<th>E. coli straina</th>
<th>Protein(s) induced by IPTG</th>
<th>Protein expression (mU mg of protein⁻¹) in cell extractsb</th>
<th>Acetyl-CoA carboxylase</th>
<th>PCCase</th>
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<td>RG7-RG8d</td>
<td>AccA1-AccB, AccE</td>
<td>1.85 ± 0.12</td>
<td>2.25 ± 0.13</td>
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a All the RG strains are derived from E. coli BL21(DE3), except RG7, which derives from DH5α.
b Results are the means of three determinations ± standard errors.
c Contains plasmid pBA11 that expresses BrrA constitutively.
d Mix of equal amounts of proteins from cell extracts of each of the strains indicated.
e The amount of 14C fixed into acid-stable products was not significantly higher than background levels (10 cpm, equivalent to 0.02 mU).

![Graph](https://example.com/graph.png)

**Fig. 2.** Effect of AccE on the catalytic activity of the ACCase complex. In mixes 1, 2, and 3, acetyl-CoA carboxylase and PCCase activities were measured after mixing equal amounts of proteins from cell extracts from each of the strains indicated. In mixes 4 to 9, ACCase activity was determined using a mix of RG9 and RG11 cell extracts containing different amounts of purified H6AccE. Results are the means of three determinations. When ACCase activity was measured in individual cell extracts, the amount of 14C fixed into acid-stable products was not significantly higher than background levels (10 cpm, equivalent to 0.02 mU).
suggest that AccE increases the rate of the ACCase reaction, kinetic analysis using purified components will be necessary to understand the precise role played in enzyme activity by this small polypeptide.

**accB** is an essential gene in *S. coelicolor*. To study the role of AccB in vivo, we attempted to construct an *accB* mutant by gene replacement (Fig. 3A). A HYG resistance cassette was cloned in the unique *Bam*HI site present in the coding sequence of *accB* contained in pTR80. After an intermediate cloning step in pIJ2925, a *Bgl*II fragment containing the mutated allele was inserted in the conjugative *E. coli* vector pSET151. The resulting plasmid, pTR124, was introduced into the *E. coli* donor strain ET12567/pUZ8002 and transferred by conjugation into M145. Thr *Hygr* exconjugants were selected in which the plasmid had integrated into the chromosome at the *accB* locus by a single crossover. One of the exconjugants, T124, was taken through four rounds of sporulation on SFM medium with HYG to allow for a second crossover and replacement of the wild-type *accB* by the mutant allele. Although several thousand colonies were screened for TH sensitivity (which would have reflected successful gene replacement), none were obtained, suggesting that *accB* could be an essential gene in *S. coelicolor*. If this were true, the presence of a second copy of *accB* in the chromosome of T124 ought to permit a second crossover event, leading to the replacement of the wild-type *accB* gene by the *Hyg*’ mutant allele. To confirm this hypothesis, we first integrated pTR149 (see Materials and Methods; Fig. 3B) containing *accBE* and the native promoter into the *φC31 att* site of T124 (to yield strain T149) allowed replacement of the wild-type *accB* by the mutant allele.

**Construction and characterization of an accBE conditional mutant.** In order to regulate the expression of the putative *accBE* operon and study its effect on the physiology of *S. coelicolor*, we constructed a conditional mutant strain in which the expression of these genes was under the control of the TH-inducible *tipA* promoter (30). For this, pTR94 was transformed into the *E. coli* strain ET12567/pUZ8002 and conjugated into the *S. coelicolor* strain M145. Integration of pTR94 by Camp-

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**FIG. 3.** Attempted disruption of *accB*. (A) Diagram showing integration of pTR124 through one of the *accBE* flanking regions and resolution of the cointegrate by a second crossover event. The *X* on top of the arrow indicates the inability to obtain the replacement of the wild-type *accB* by the *Hyg*’ mutant allele. (B) Integration of a second copy of *accBE* at the *φC31 att* site of T124 (to yield strain T149) allowed replacement of the wild-type *accB* by the mutant allele.
broad recombination through the accBE homologous sequences left the accBE operon under tipAp. The strain obtained was named M94 and the genetic modification introduced was confirmed by Southern blot experiments (data not shown).

Normal growth of strain M94 on SMM depended on the presence of 5 µg of TH/ml, which derepresses the expression of the accBE operon. In the absence of TH growth was strongly affected, and the low growth levels observed were probably due to a leakiness of the control system (Fig. 4) (E. Takano and M. Bibb, unpublished data); no antibiotic production was observed in these cultures. To determine the effect of TH on the acetyl-CoA carboxylase and PCCase enzyme levels, both activities were measured in 38-h cultures grown in SMM with or without the addition of 5 µg of TH/ml. We used this time point because both cultures were still in their exponential phase and we expected, at least for the acetyl-CoA carboxylase activity, its maximal levels. As observed in Table 3, the acetyl-CoA carboxylase activity present in crude extracts prepared from the uninduced cultures was almost 10 times lower than that found in the TH-induced cultures. This difference was not observed in the levels of PCCase, a result that was expected considering the adenine of the most likely translation start codon of hrdb corresponds to a transcript that would start 1 bp upstream of or at the adenine of the 35 promoter regions similar to those likely to be recognized by $\sigma^{acBE}$ (42) are located upstream of the transcription initiation site (Fig. 5B).

To determine if accB and accE were cotranscribed, a 563-bp probe was generated by PCR that spanned the intergenic region. For this we used a 5’ oligonucleotide corresponding to a sequence within the coding region of accB and a 3’ oligo-

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Acetyl-CoA carboxylase (mU mg of protein $^{-1}$)</th>
<th>PCCase (mU mg of protein $^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMM</td>
<td>0.12 ± 0.03</td>
<td>2.20 ± 0.06</td>
</tr>
<tr>
<td>SMM + TH$^a$</td>
<td>1.24 ± 0.06</td>
<td>3.90 ± 0.07</td>
</tr>
<tr>
<td>SMM + Oleate$^b$</td>
<td>0.15 ± 0.03</td>
<td>1.40 ± 0.05</td>
</tr>
</tbody>
</table>

$^a$ Results are means of three determinations ± standard errors.
$^b$ TH, 5 µg/ml; oleate, 0.01% (wt/vol).
cleotide corresponding to a sequence within \textit{accE}. The addition of a 13-nt tail to the 5' oligonucleotide allowed for facile discrimination of full-length protection (reflecting cotranscription) of the probe from probe reannealing. The results clearly showed that \textit{accB} and \textit{accE} were part of the same transcript (Fig. 5C). The pattern of transcription of \textit{accBE} during the different growth phases corresponded well to the profile observed with the \textit{accB} probe.

The transcripts of \textit{accA2} present during exponential phase were studied by high-resolution S1 mapping. The probe used was a 766-bp PCR-generated DNA fragment uniquely labeled at the 5' end of the oligonucleotide corresponding to a sequence within \textit{accA2}. The experimental data revealed the presence of two RNA protected fragments, consistent with transcripts initiated 25 bp (from \textit{accA2p1}) and 153 bp (from \textit{accA2p2}) upstream of the putative translation start site of \textit{accA2} (Fig. 6A and C). The putative -10 and -35 regions of these promoters also show some similarity to the consensus sequence of promoters that are likely to be recognized by $\sigma^{hrdB}$. The growth phase-dependent expression of \textit{accA2} from these two putative promoters closely resembled that observed for the \textit{accBE} operon; i.e., there was a constant and high level of expression during the exponential and transition phases of growth that declined markedly upon entry into stationary phase (Fig. 7A). However, a new RNA protected fragment of 185 bp was also detected during transition phase. Since the nucleotide sequences of \textit{accA1} and \textit{accA2} are identical from nt -2 to nt +200 with respect to the coding sequence (36), 185 bp of this probe should also be protected by the \textit{accA1} mRNA. Thus, although the existence of a third promoter for \textit{accA2} that is regulated in a different manner cannot be ruled out, this transcript could also correspond to \textit{accA1}.

S1 nuclease protection analysis of \textit{accA1} was performed using a 563-bp PCR product uniquely labeled at the 5' end of the downstream oligonucleotide corresponding to a sequence within \textit{accA1}. Two major RNA protected fragments were identified (as well as a faint band designated \textit{accA1p3} in Fig. 6B), with the most abundant representing a putative transcriptional start site located 88 bp upstream of the GTG initiation codon of \textit{AccA1}. Putative -10 and -35 regions resembling those likely to be recognized by $\sigma^{hrdB}$ are again located upstream of each of the two more prominent start sites (Fig. 6B and C). Two direct repeat sequences of 16 bp containing only two mismatches flank the putative -35 region of \textit{accA1p1} and the transcription start point of \textit{accA1p2} and might represent the binding sites of a putative transcriptional regulator (Fig. 6C). S1 nuclease protection experiments with RNA from different growth phases revealed \textit{accA1} transcripts exclusively during transition phase (Fig. 7B), showing a completely different regulation than \textit{accA2} and suggesting that the smallest RNA protected fragment detected for \textit{accA2} during transition phase most probably reflects transcription of \textit{accA1}.

**DISCUSSION**

In \textit{Streptomyces} malonyl-CoA is not only an essential metabolite used as the main elongation unit for fatty acid biosynthesis (4, 8) but also one of the most common building blocks utilized in the synthesis of several pharmaceutically important polyketide compounds (19). Therefore, the interest in establishing the pathway(s) leading to the biosynthesis of this metabolic intermediate in this microorganism has relevance not only from a fundamental view but also from a more applied point of view.

![FIG. 5. Growth phase-dependent expression and transcription start site of the \textit{accBE} operon. (A) S1 nuclease mapping of \textit{accB}, \textit{actII}-\textit{ORF4}, and \textit{hrdB}, using RNA isolated from a liquid-grown culture of \textit{S. coelicolor} M145. Exp, Trans, and Stat indicate the exponential, transition, and stationary phases of growth, respectively. (B) The nucleotide sequence of both strands of the \textit{accB} promoter region is shown. The arrow indicates the most likely transcription start point for the \textit{accBE} promoter, as determined by S1 nuclease mapping. Potential -10 and -35 regions for \textit{accBEp} are underlined. (C) S1 nuclease mapping of the \textit{accB-accE} intergenic region using a 563-nt probe. FLP, full-length protection of the probe reflecting transcription across the intergenic region.](image-url)
In most species, malonyl-CoA is synthesized through carboxylation of acetyl-CoA by an acetyl-CoA carboxylase (45), and this enzyme complex has been shown to be essential for many microorganisms, such as *E. coli*, *B. subtilis*, and *Saccharomyces cerevisiae* (17, 28, 32). Based on this knowledge and in an attempt to characterize the malonyl-CoA biosynthetic pathway in *S. coelicolor* we searched for a carboxyl transferase component that could function as the β subunit of an acetyl-CoA carboxylase complex. Thus, by using *pccB* (36) as a hybridization probe we isolated the *accBE* operon of *S. coelicolor*. Expression of *accB* and *accE* in *E. coli* and subsequent in vitro reconstitution of enzyme activity in the presence of the biotinylated proteins AccA1 and AccA2 confirmed that AccB was the carboxyl transferase subunit of an ACCase. The additional presence of AccE considerably enhanced the activity of the enzyme complex (Table 2), suggesting that this small polypeptide is a functional component of the ACCase. Whether this protein plays a role as an allosteric regulator of the enzyme or as a structural component of the complex remains to be elucidated. All the actinomycete ACCases studied so far contain three functional domains located in two polypeptides (18, 20). Thus, AccE, for which there are no known homologues, might be a distinctive feature of ACCases from *Streptomyces* spp.

Based on these biochemical studies we decided to prove in vivo whether AccB was the carboxyl transferase component of an essential ACCase. The impossibility of obtaining an *accB* null mutant and the TH growth dependency of a *tipAp-accB* conditional mutant (Fig. 3A and 4) confirmed that AccB is essential for *S. coelicolor* viability. Normal growth phenotype in the absence of the inducer was restored in the conditional mutant by the addition of exogenous long-chain fatty acids in the medium (Fig. 4), indicating that the inducer-dependent phenotype was specifically related to a conditional block in fatty acid biosynthesis and that the acetyl-CoA carboxylase activity of the ACCase complex, containing AccB as the carboxyl transferase subunit, is the main pathway of malonyl-CoA biosynthesis in *S. coelicolor*. Although normal growth was restored by unsaturated fatty acids in liquid SMM medium, we were unable to obtain an *accB* mutant of T124 in the presence of oleate after several rounds of sporulation in SFM medium (41) supplemented with oleate and APR. We suggest that de novo fatty acid synthesis may be essential for an efficient sporulation of this microorganism, as was shown in *B. subtilis* in
which fatty acid synthesis is essential to couple the activation of the mother cell transcription factors with the formation of differentiating cells (40). If this hypothesis was correct, accB mutants would not be able to sporulate, even in the presence of oleate, and would be lost in the isolation procedure utilized.

Considering the essential role played by AccB and taking into account the apparent inviability of accA2 mutants in S. coelicolor (36), we postulate that AccA2 and AccB are the α and β components of an ACCase, whose main physiological role is the synthesis of malonyl-CoA. Transcriptional studies of accBE and accA2 showed that the expression of these genes occurred principally during the exponential and transition phases of growth (Fig. 5A and 6A), in agreement with their essential role in this organism. Consistent with these results the levels of acetyl-CoA carboxylase and PCCase activity throughout growth were also found to be maximal during exponential phase (data not shown).

In S. coelicolor, in addition to the need for malonyl-CoA synthesis during vegetative growth, there is also a requirement for this metabolite during transition and stationary phase. At least two of the secondary metabolites produced by S. coelicolor, undecylprodigiosin and actinorhodin, are synthesized during these growth phases and require malonyl-CoA for their synthesis. If the essential ACCase characterized in this work is the only enzyme capable of synthesizing malonyl-CoA, then it will also be required during the production of these two antibiotics. In agreement with this hypothesis fatty acid-supplemented cultures of the M94 conditional mutant, for which the product of this gene was not essential for fatty acid synthesis during vegetative growth, were also found to be maximal during exponential phase (data not shown).

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sites flanking a modified multiple cloning site and that retain the ability to identify recombinant clones by visual screening of Escherichia coli colonies. Gene 124:133–134.


