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Relationships between Fatty Acid and Polyketide Synthases from *Streptomyces coelicolor* A3(2): Characterization of the Fatty Acid Synthase Acyl Carrier Protein

W. PETER REVILL,* MAUREEN J. BIBB, AND DAVID A. HOPWOOD

John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, United Kingdom

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We have characterized an acyl carrier protein (ACP) presumed to be involved in the synthesis of fatty acids in *Streptomyces coelicolor* A3(2). This is the third ACP to have been identified in *S. coelicolor*; the two previously characterized ACPs are involved in the synthesis of two aromatic polyketides: the blue-pigmented antibiotic actinorhodin and a grey pigment associated with the spore walls. The three ACPs are clearly related. The presumed fatty acid synthase (FAS) ACP was partially purified, and the N-terminal amino acid sequence was obtained. The corresponding gene (*acpP*) was cloned and sequenced and found to lie within 1 kb of a previously characterized gene (*fabD*) encoding another subunit of the *S. coelicolor* FAS, malonyl coenzyme A:ACP acyltransferase. Expression of *S. coelicolor acpP* in *Escherichia coli* yielded several different forms, whose masses corresponded to the active (*holo*) form of the protein carrying various acyl substituents. To test the mechanisms that normally prevent the FAS ACP from substituting for the actinorhodin ACP, *acpP* was cloned in place of *actI*-open reading frame 3 (encoding the actinorhodin ACP) to allow coexpression of *acpP* with the *act* polyketide synthase (PKS) genes. Pigmented polyketide production was observed, but only at a small fraction of its former level. This suggests that the FAS and PKS ACPs may be biochemically incompatible and that this could prevent functional complementation between the FAS and PKSs that potentially coexist within the same cells.

Polyketides are a class of secondary metabolites with a huge range of structures, some of which are used as medical or veterinary agents. For example, erythromycin and tetracyclines are used as antibiotics, daunorubicin is used as an anti-cancer agent, and avermectin is used as an anti-helminthic. Although the structures of polyketides vary enormously, they are all synthesized, in their initial stages, by a mechanism that is very similar to fatty acid biosynthesis: simple acyl precursors such as acetyl and malonyl units are condensed in a sequential fashion to give a long carbon chain, catalyzed by the polyketide synthase (PKS). The differences between polyketide and fatty acid biosynthesis (and among diverse polyketide biosyntheses) are in the number and type of acyl precursors used, the extent and position of keto-group reductions, and the cyclization pattern of the products (subsequent post-PKS modifications add to the structural variety observed) (for reviews, see references 11, 12, 16, and 24). Thus fatty acid and polyketide biosyntheses are mechanistically related, and often the same precursor molecules are used. We are interested in understanding the relationships between a fatty acid synthase (FAS) and two PKSs in *Streptomyces coelicolor* A3(2).

S. coelicolor, genetically the most studied member of the genus, synthesizes at least two aromatic polyketides at different stages during its development cycle on solid media. The blue-pigmented antibiotic actinorhodin is synthesized just as the vegetative mycelial cells begin to differentiate and enter the reproductive phase of development (9). The other, grey-pigmented polyketide is synthesized near the end of colony differentiation and is associated with the spore walls (5). The *act* and *whiE* PKS gene sets (*act* for actinorhodin and *whiE* for spore pigment, so called because the mutants have white spores) have been cloned and sequenced (7, 8). Key members of both gene sets encode the three subunits that make up the minimal-PKSs, so called because they are all that are required for assembly of the polyketide chain (22). The *act* and *whiE* minimal-PKSs are very similar to each other and to those involved in synthesis of aromatic polyketides from related *Streptomyces* spp. (12). The deduced products of the minimal-PKS genes also have a moderate similarity to subunits of the *Escherichia coli* FAS. Thus, the FASs and PKSs are related not only in their mechanism of carbon chain assembly but also in their primary sequences (for a review of *E. coli* FAS, see reference 20). It seems probable that they have evolved from a common origin after an early gene duplication. The minimal-PKS subunits are a β-ketoacyl carrier protein synthase, required for condensation of the acyl units to form the growing polyketide chain; a polyketide chain-length factor which has a high degree of homology to the ketoacylsynthase but lacks the active site cysteine residue; and an acyl carrier protein (ACP), which serves a dual purpose in receiving the malonyl extender units prior to condensation and in holding the growing carbon chain after each condensation (12).

Recently, an enzyme subunit in *S. coelicolor* has been identified which is potentially required for both fatty acid and polyketide biosynthesis within the same organism. This is the malonyl coenzyme A (CoA):ACP acyltransferase, thought to be responsible for charging the ACP subunit of the FAS and both PKSs prior to condensation (27). (Similar observations have been made in *Streptomyces glaucescens* [32].) The malonyltransferase appears to provide a tangible link between the FAS and the PKSs that are expressed within the same organism. The subunits of the minimal-PKSs, on the other hand, appear to be prevented from forming any functional link between the synthases: a mutation in any of the *act* minimal-PKS subunit genes results in loss of actinorhodin production and is not complemented by the naturally expressed equivalent subunits from either the *fab* (encoding the FAS) or *whiE* gene sets.

* Corresponding author. Mailing address: John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, United Kingdom. Phone: 44 (1603) 452571. Fax: 44 (1603) 456844. Electronic mail address: REVILL@BBSRC.AC.UK.

TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Description	Source or reference
Plasmids		
pCos-4A7	Cosmid isolated from a library of *S. coelicolor* M145 DNA in *E. coli* cosmid vector Supercos-1	26
pBluescript II	*E. coli* cloning vector	Stratagene
pUC118	*E. coli* cloning vector	35
pIJ8102	1.3-kb *Bam*HI-*Asp*718 fragment of pCos-4A7 that carries *S. coelicolor acpP* gene cloned in pBluescript II SK(+); used as PCR template to generate pIJ8130	This work
pIJ8103 and pIJ8104	2.3-kb *Bam*HI-*Pst*I fragment of pCos-4A7 that carries *S. coelicolor fabH* and *acpP* genes in pBluescript II SK(+) and KS(+), respectively; used for generation of templates for sequence analysis	This work
pIJ4256	Carries *whiE*-ORFV (for spore pigment PKS ACP); used as PCR template to generate pIJ8132	37
pET11c	*E. coli* vector for high-level expression of heterologous genes	31
pIJ8130	Engineered *S. coelicolor acpP* in *Nde*I-*Bam*HI sites of pET11c	This work
pIJ8131	Engineered *S. coelicolor actI*-ORF3 (for actinorhodin ACP) in *Nde*I-*Bam*HI sites of pET11c	This work and reference 6
pIJ8132	Engineered *S. coelicolor whiE*-ORFV in *Nde*I-*Bam*HI sites of pET11c	This work
pRM5	Bifunctional *Streptomyces-E. coli* vector carrying *act* genes for synthesis of aloesaponarin (shunt product of the actinorhodin biosynthetic pathway)	23
pIJ8120	pRM5, but with *S. coelicolor acpP* in place of *actI*-ORF3	This work
pIJ8121	*actI*-ORF3 cloned into a unique *Eco*RI site downstream of and cotranscribed with the *act*/*fab* genes in pIJ8120	This work
Strains		
S. coelicolor A3(2)		
M145	Prototrophic SCP1$^-$ SCP2$^-$	10
CH1	*proA1 argA1* SCP1$^-$ SCP2$^-$ *redE60*	17
CH999	*proA1 argA1* SCP1$^-$ SCP2$^-$ *redE60* Δ*act*	23
YU105	*proA1 argA1* SCP1$^-$ SCP2$^-$ *redE60* Δ*act* Δ*whiE*	37
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Described in reference 29
"*Epicurian*" *coli* SURE	Used for maintenance of cosmid pCos-4A7; e14$^-$(*mcrA*) Δ(*mcrCB-hsdSMR-mrr*) *171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC*:Tn*5* (Kan^r) *uvrC* [F′ *proAB lacI*^{q}ZΔM15 Tn*10*]	Stratagene
BL21 (DE3)/pLysS	Lysogen of bacteriophage DE3; contains a single copy of the gene for T7 RNA polymerase under control of the inducible *lacUV5* promoter; also contains the plasmid pLysS encoding T7 lysozyme; used as a host for high-level expression of heterologous genes; F$^-$ *ompT hsdS gal* r_B^- m_B^-	31

We would like to know why some of the enzymatic subunits of the FAS and PKSs do not naturally complement each other, while other subunits such as the malonyltransferase appear to interact with all three synthases.

This question has recently been addressed with respect to *act* and *whiE*: the *whiE* PKS genes were expressed ectopically in mutant strains of *S. coelicolor* from which the *act* ketoacylsynthase, chain-length factor, or ACP genes had been individually deleted. A functional hybrid PKS was formed in which the spore pigment PKS subunits had replaced their deleted homologs from the actinorhodin PKS, and pigment production was restored. This suggests that under normal circumstances, the two PKS complexes are kept apart by expression of the different PKS genes in different regions of the developing *Streptomyces* colony, rather than by biochemical incompatibility (18, 37). With respect to *fab*, we might expect this gene set to be expressed in all regions of the colony, including those expressing either *act* or *whiE*, because those expressing PKS genes would probably also require new fatty acids during bacterial cell differentiation. The mechanism that functionally separates the FAS and actinorhodin ACPs from each other forms the subject of this investigation.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are described in Table 1. Standard conditions for culture of *Streptomyces* spp. were as described previously (10). Conditions for growth of *S. coelicolor* in liquid minimal medium supplemented with Casamino Acids (SMM) were as described previously (33), except that 200-ml cultures were grown in 2-liter flasks and 10-ml samples were withdrawn at intervals during growth. *S. coelicolor* cells were also cultured on solid supplemented minimal medium (SMMS) with cellophane discs laid over the agar prior to inoculation, as described previously (4). *E. coli* strains were grown as described previously (29), except for BL21 (DE3)/pIJ8130, which was grown as described in reference 31.

General protein techniques. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (19) with the Mini-Protean II system (Bio-Rad). Native PAGE was conducted in a manner similar to that for SDS-PAGE, except that no SDS or reducing agent was used, and the sample was not boiled. Native PAGE used 17.5% total acrylamide, with bisacrylamide at 0.9% of the total. Proteins were visualized in the gels by staining with Coomassie brilliant blue. Protein concentrations were determined by the method of Bradford (3) (dye reagent concentrate from Bio-Rad), with bovine serum albumin as the standard. For N-terminal sequencing of proteins separated by native PAGE, proteins were transferred onto a 0.2-μm-pore-size polyvinylidene difluoride (PVDF) membrane (Trans Blot; Bio-Rad) and were sequenced by P. Barker at the Microchemical Facility, Institute of Animal Physiology and Genetic Research, Babraham, Cambridge, United Kingdom). Purified proteins were analyzed by electrospray mass spectrometry (ESMS) by J. Crosby, School of Chemistry, University of Bristol, Cantock's Close, Bristol, United Kingdom, as described previously (6).

Malonyltransferase assay for ACPs. *S. coelicolor* cells were grown in SMM

until, in those strains capable of producing actinorhodin, actinorhodin was produced. Endogenous ACPs were assayed essentially as described previously (6), except that the crude cell extracts were not fractionated by ammonium sulfate precipitation before the assay and were used at 1 mg/ml in each assay. The [2-^{14}C]malonyl ACP product was either monitored by trichloroacetic acid precipitation, as previously described (6), or visualized by autoradiography after electrophoresis of the assay mixture on 17.5% native PAGE and transfer to PVDF membrane, with a type BAS-IIIs imaging plate and a BAS1000 Bioimaging analyzer (Fuji).

Purification of a potential FAS ACP from *S. coelicolor* YU105. Cells were grown for 48 h in 1 liter of tryptone soya broth (10), harvested, washed in 50 mM potassium phosphate (pH 7.2)–0.5 M NaCl, and washed again in 50 mM potassium phosphate (pH 7.2). The cells were resuspended in cell disruption buffer (50 mM potassium phosphate [pH 7.2], 10% glycerol, 2 mM dithiothreitol) and disrupted in a Bead Beater (Biospec Products, Bartlesville, Okla.) with 0.15-mm-diameter glass beads in a 250-ml chamber (one-half full of beads) with 12 bursts of disruption, each 20 s, and 1 min of cooling between each burst. Cell debris was removed by centrifugation for 10 min at 12,000 × *g* at 4°C and then for 30 min at 27,000 × *g* at 4°C. ACP was precipitated overnight at 80 to 100% saturation with solid ammonium sulfate (0°C, adjusted to pH 4 with acetic acid). After centrifugation for 30 min at 27,000 × *g* at 0°C, the precipitate was redissolved in cell disruption buffer and dialyzed overnight at 4°C against the same buffer (2 liters). At this stage, ACP was assayed by incubation of each cell fraction with [2-^{14}C]malonyl CoA, as described above; this relied on the copurification of malonyltransferase (essential for catalysis of the reaction) with the ACP (27). ACP was further purified by fast-performance liquid chromatography (Pharmacia) on an anion-exchange column (Mono Q HR5/5; Pharmacia) with a linear gradient of 0 to 1 M KCl–50 mM potassium phosphate (pH 7.2). ACP-containing fractions were monitored at this stage by measuring the stimulation in acid-precipitable counts above the background level when column fractions were added to whole-cell extracts of *S. coelicolor* YU105 (1.0 mg/ml). Active fractions were pooled, and eight major protein bands (and several minor bands) were detected on native PAGE stained with Coomassie blue (see Fig. 3a, panel 2). Two of these Coomassie-stained proteins (labelled I and II) were identified as potential ACPs because they comigrated with radiolabelled proteins in panel 1. Both proteins were transferred to PVDF membrane for N-terminal amino acid sequence analysis.

Expression of *Streptomyces* ACP genes in *E. coli* and their purification. *act*I-open reading frame 3 (ORF3 [encoding the actinorhodin ACP]) was isolated as an *Nde*I-*Bam*HI fragment from pIJ5232 (6) and cloned into the expression vector pET11c to create pIJ8131. Expression of *act*I-ORF3 was induced in *E. coli* BL21(DE3)/pLysS/pIJ8131) as described previously (31), and the overproduced protein was purified as described previously (27). The *whiE* ACP and potential FAS ACP genes were modified for optimum expression in *E. coli* by introduction of silent mutations in the first few codons so that (i) the codon usage more closely reflected that used by highly expressed genes in *E. coli* (30) and (ii) an *Nde*I restriction enzyme site was introduced spanning the ATG start codon, allowing the use of the ϕ10 promoter and ribosome binding site in pET11c (31). The alterations were introduced by the PCR with the following 5′-oligonucleotides: 5′-CATATGACTGACCAGCAGCTGGACTACCAG (*whiE*-ORFV) and 5′-CATATGGCTGCTACTCAGGAAGAAATCGTTGCTGGTCTCGCGGAGATCGTGAA (FAS ACP). A *Bam*HI site was introduced at the 3′ end of each ACP gene with a synthetic oligonucleotide corresponding to that region of each gene (see reference 7 for the *whiE* gene sequence). The PCR products were cloned in the *Sma*I site of pBluescript II SK(+), and the predicted sequences were confirmed. The ACP genes were then cloned between the *Nde*I and *Bam*HI sites of pET11c and introduced into *E. coli* BL21(DE3)/pLysS by transformation. Cells were grown and treated as described previously (31). Production of ACP was monitored by native PAGE. ACPs were almost pure in a single step after preparative native PAGE (Prep Cell-491; Bio-Rad), as previously described for actinorhodin ACP purification (27). Anion-exchange chromatography (Mono Q HR5/5; Pharmacia) was used to remove any contaminating *E. coli* FAS ACP and to separate the various forms of *S. coelicolor* ACP. Purification was monitored by native PAGE. The malonyltransferase assay was used to monitor the presence of contaminating *E. coli* FAS ACP. (*E. coli* FAS ACP can be detected because it has a slightly greater mobility than the *S. coelicolor* ACPs on native PAGE.)

General DNA techniques. Recombinant DNA techniques with *S. coelicolor* were performed as described previously (10). Techniques with *E. coli* were performed as described in reference 29, except for the following: cells were transformed by electroporation with a Gene Pulser (Bio-Rad) according to the manufacturer's recommendations. Plasmids for subcloning were isolated as described previously (14); Vent DNA polymerase (New England Biolabs) was used in the PCR, according to the manufacturer's recommendations, with 10% glycerol in the reaction mix. Labelling of DNA probes, hybridization, and detection were done as recommended for the Dig-system (Boehringer Mannheim). Nucleotide sequencing was done by N. Hartley, John Innes Centre sequencing facility, John Innes Centre, Norwich Research Park, Colney, Norwich, United Kingdom. Overlapping deletion clones were generated from pIJ8103 and pIJ8104 with ExoIII nuclease. Sequence reactions were carried out with the ABI Prism cycle sequencing system (Perkin-Elmer) and analyzed on an ABI 373 A automated sequencer (Applied Biosystems). Some sequences were also determined with the Taqtrack system (Promega) with DNA templates generated by use of convenient restriction sites and with custom-made oligonucleotides. Nucleotide sequences were assembled into one contiguous fragment with the STADEN XBAP Development assembly program (V14.0 MRC Laboratory of Molecular Biology, Hills Road, Cambridge, United Kingdom) and analyzed with the UWGCG sequence analysis packages (version 8.0-UNIX; Genetics Computer Group, Madison, Wis.). The FRAME program (2) was used to identify ORFs and their direction of transcription.

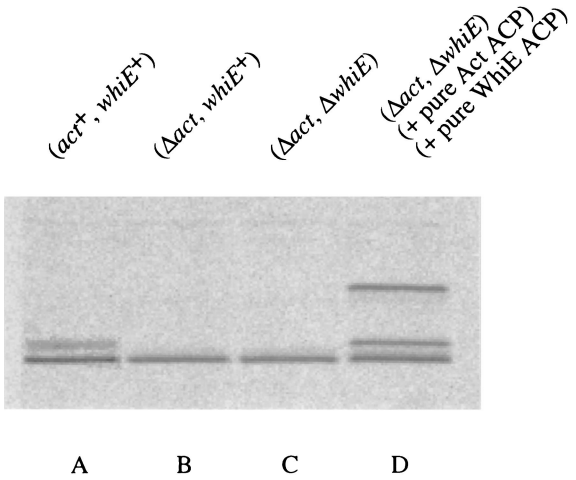

FIG. 1. Endogenous ACP species in *S. coelicolor*. Cultures were grown on solid medium (SMMS) and harvested for assay after sporulation. The ACP species were radiolabelled by incubation of the cell extract with [2-^{14}C]malonyl CoA, which served as a substrate for malonyl CoA:ACP malonyltransferase, also present in the cell extract, to form the [2-^{14}C]malonyl ACP seen in the figure. The figure shows an autoradiograph of the assay mixtures after electrophoresis on native PAGE and transfer to PVDF membrane. Lanes: A, *S. coelicolor* CH1, the parental strain (*act*$^+$ *whiE*$^+$); B, *S. coelicolor* CH999 (Δ*act whiE*$^+$); C, *S. coelicolor* YU105 (Δ*act* Δ*whiE*); D, *S. coelicolor* YU105 with pure actinorhodin and spore pigment ACPs added to the cell extract.

Nucleotide sequence accession number. The sequence of the *acpP* and *fabH* genes of *S. coelicolor* has been deposited in the EMBL database under accession number X86475.

RESULTS

A new, constitutively expressed ACP in extracts of *S. coelicolor*. An ACP that had not previously been characterized in *S. coelicolor* was detected by the transfer of [2-^{14}C]malonyl units from CoA onto ACP species present in cell extracts, catalyzed by the endogenous malonyl CoA:ACP acyltransferase. Extracts were prepared from cells grown as surface cultures on SMMS until sporulation and actinorhodin production were visible and then were assayed. Two radiolabelled malonyl ACP species, with slightly different electrophoretic mobilities, were visualized by autoradiography after separation by native PAGE (Fig. 1, lane A). One of these proteins was the actinorhodin ACP, because the corresponding band was absent from extracts of *S. coelicolor* from which the *act* genes had been deleted (*S. coelicolor* CH999) (Fig. 1, lane B), and addition of pure actinorhodin ACP to extracts of *S. coelicolor* CH999 resulted in reappearance of the radiolabelled form of this ACP. The second radiolabelled ACP species (with higher electrophoretic mobility) remained unaffected by deletion of the *act* cluster. When extracts of *S. coelicolor* YU105 (from which the *act* and *whiE* PKS gene sets had been deleted from the chromosome) were assayed, the second radiolabelled ACP species was unaffected (Fig. 1, lane C), indicating that it was not encoded by the *whiE* cluster. A potential reason for the inability to detect endogenous spore pigment PKS ACP in extracts of *S. coelicolor* CH1 (wild type with respect to *act* and *whiE*) is the difficulty in lysis of the spores in which the *whiE* PKS genes appear to be

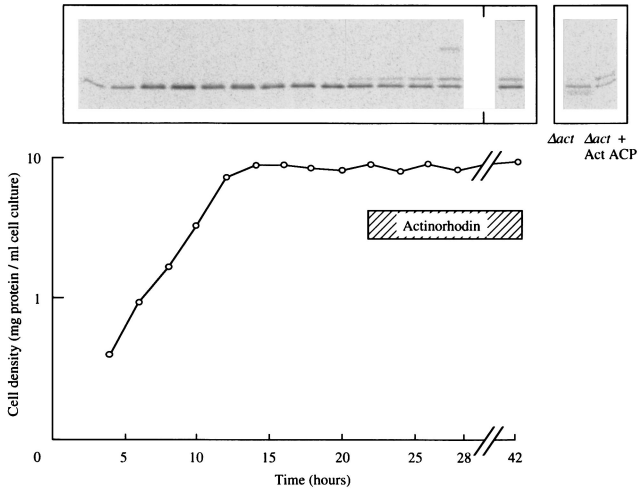

FIG. 2. Endogenous ACP species at different times during the growth of *S. coelicolor* CH1. Cultures were grown in liquid medium (SMM), and samples were taken for assay at the times indicated. Δ*act* is a control assay with *S. coelicolor* CH999 cell extracts, and Δ*act* + Act ACP is the same as Δ*act* but with pure actinorhodin ACP added to the cell extract prior to the assay. Actinorhodin (blue pigment) appeared at 24 h and was present in the culture at all subsequent times, as indicated by the box.

expressed (16a). Addition of pure spore pigment ACP to *S. coelicolor* YU105 (Δ*act* Δ*whiE*) cell extracts resulted in appearance of a radiolabelled acyl-ACP species with a different electrophoretic mobility from that of either of the other two ACPs (Fig. 1, lane D). These results indicated that a previously uncharacterized ACP had been detected in extracts of *S. coelicolor.*

To investigate further whether expression of the gene for the new ACP was developmentally regulated (characteristic of PKS genes), extracts prepared from *S. coelicolor* cells taken at different stages during growth in liquid culture were assayed (Fig. 2). Actinorhodin ACP was detected only in extracts from cells taken after 24 h, when the culture was producing the blue-pigmented actinorhodin. The new ACP, on the other hand, was expressed throughout all stages of growth in the liquid culture. This suggested that the protein was involved in a primary metabolic pathway, potentially fatty acid biosynthesis.

Partial purification and N-terminal sequence analysis of the constitutively expressed ACP from *S. coelicolor*. The constitutively expressed ACP was partially purified from *S. coelicolor* YU105 (Δ*act* Δ*whiE*). After ammonium sulfate fractionation and Mono-Q anion-exchange chromatography, two radiolabelled malonyl ACP species were detected on native PAGE (Fig. 3a, proteins I and II). The N-terminal amino acid sequence was obtained for both proteins. The sequences of the first 25 amino acids of protein I and the first 40 amino acids of protein II suggested that the two could be identical (Fig. 3b [some of the amino acids obtained for the sequence of protein I could not be assigned with confidence]). Subsequent hybridization experiments suggested that proteins I and II were the products of just one gene (see below), although it remains a possibility that two different ACPs have been identified. The sequences showed a reasonable similarity with the N termini of several FAS ACPs, including *E. coli* (25) and the recently sequenced *S. glaucescens* FAS ACP (32). This confirmed the identity of the sequenced protein as an ACP distinct from either of the two previously characterized PKS ACPs of *S. coelicolor.*

The slower-migrating protein I had previously been observed in the 30-h cell fraction assayed during the time-course experiment (Fig. 2). The appearance of protein I was not observed when the experiment was repeated with 30-h cell extracts that had been prepared in the presence of phenylmethylsulfonyl fluoride (1 mM [data not shown]), suggesting that protein I may be the product of proteolytic digestion. Despite this, protein I retained activity in the malonyltransferase assay.

Cloning and sequencing of the gene for the constitutively expressed ACP of *S. coelicolor*. A degenerate oligonucleotide probe was designed that corresponded to amino acids 13 to 25 of the newly sequenced ACP, on the basis of the identity of sequences between proteins I and II and the highly biased usage of synonymous codons in *Streptomyces* DNA across this region (36) (Fig. 3c). The probe hybridized to a single 3.7-kb *Bam*HI fragment of *S. coelicolor* genomic DNA under stringent conditions (the Southern blot was washed twice at 65°C with 0.1× SSC for 15 min each time), indicating the presence of just one gene on the *S. coelicolor* chromosome likely to encode proteins I and II. The oligonucleotide probe also showed strong hybridization to a previously isolated cosmid (pCos-4A7) that contains the gene (*fabD*) for a malonyltransferase, which is likely to be a subunit of the *S. coelicolor* FAS (27). Further gene probing localized the hybridizing region to a position approximately 1 kb downstream of *fabD* and immediately adjacent to *fabH*, which encodes the ketoacylsynthase III subunit of the presumed *S. coelicolor* FAS (reference 27 and described below).

Sequence analysis of part of the 3.7-kb *Bam*HI fragment revealed two ORFs which were assigned by the following cri-

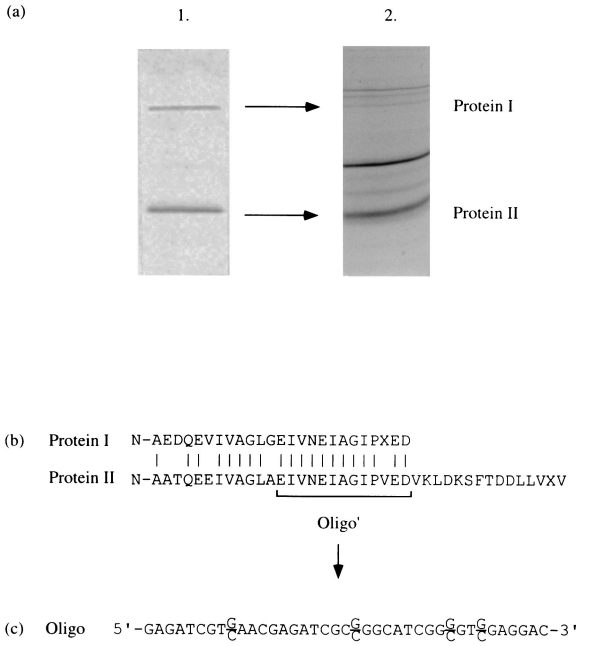

FIG. 3. Partial purification and N-terminal amino acid sequence analysis of ACPs from cell fractions of *S. coelicolor*. (a) Panel 1 shows the active ACP species present in the cell fraction after purification by anion-exchange chromatography and labelling with radioactive malonyl units, shown in panel 2. Panel 2 shows the native PAGE, stained with Coomassie blue, of the partially purified fraction assayed in panel 1. Proteins I and II were analyzed by N-terminal amino acid sequencing. (b) Results from the sequence analysis obtained for proteins I and II. (c) The "guessmer" oligonucleotide (16-fold degenerate) designed from the amino acid sequence shown above (b) and used as a probe to clone the *acpP* gene.

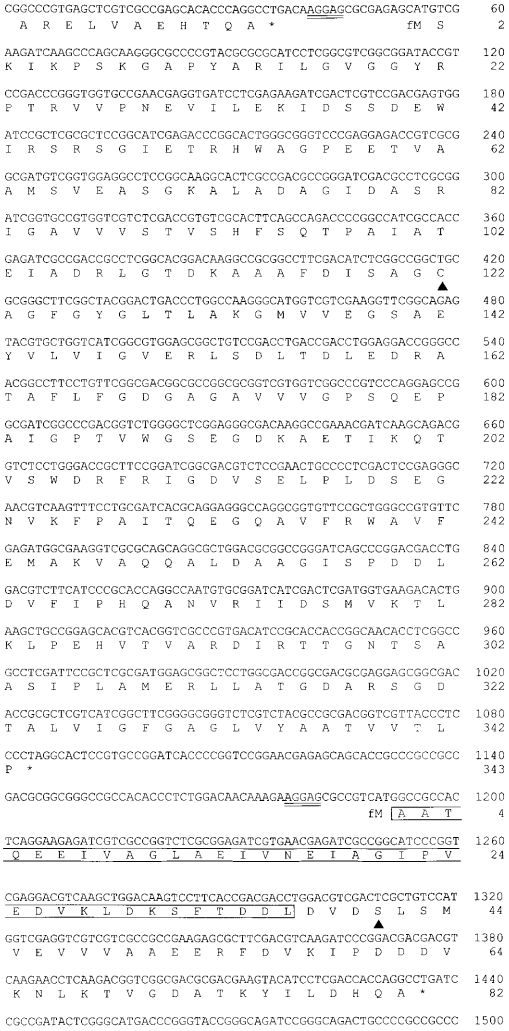

FIG. 4. Nucleotide and deduced amino acid sequences of the constitutively expressed ketoacylsynthase III and ACP of *S. coelicolor*. The experimentally determined N-terminal amino acid sequence of the ACP is boxed. ▲, active sites, Cys-122 of ketoacylsynthase III and Ser-41, for attachment of the 4′-phosphopantetheine prosthetic group of ACP. Putative ribosome binding sites are doubly underlined.

teria: (i) the high abundance of a G or C in the third codon position (>96%) of the potential reading frames, demonstrated by the use of FRAME (2), a computer program for predicting potential coding regions in *Streptomyces* DNA; (ii) strong end-to-end similarity of the deduced gene products with the subunits of *E. coli* FAS (21, 25, 34) and with the recently sequenced putative FAS of *S. glaucescens* (32); and (iii) the presence of a potential ribosome binding site a few nucleotides upstream of the start of each ORF (1).

The deduced product of one of the ORFs exactly matched the N-terminal amino acid sequence for the constitutively expressed ACP (Fig. 4). One hundred three nucleotides upstream of the start codon for the ACP gene is the stop codon for the other ORF, predicted to encode a homolog of the ketoacylsynthase III of *E. coli* FAS. (In *E. coli*, ketoacylsynthase III, encoded by *fabH*, catalyzes the first condensation reaction between the acetyl CoA starter unit and malonyl ACP extender to initiate fatty acid biosynthesis [13, 34].) The beginning of the *S. coelicolor fabH* homolog had previously been identified (27) and is cotranscribed with another subunit of the putative *S. coelicolor* FAS, *fabD*. The *fabD*/*fabH* operon is essential for viability of the cells (27). The proximity of the ACP gene to those encoding presumed subunits of the *S. coelicolor* FAS is such that the ACP gene could also be transcribed as part of the same operon. This is consistent with the observation that *fabD* and the gene for the ACP are constitutively expressed (reference 27 and this work) and implies a role for the ACP in fatty acid biosynthesis. We tentatively name this gene *acpP*, in keeping with the nomenclature used for genes encoding subunits of the *E. coli* FAS (20). *acpP* is predicted to encode a protein of 83 amino acids (M_r, 8,917) with a theoretical pI of 3.9, which is typical of ACPs. The small size and high negative charge explain the high electrophoretic mobility of ACPs on native PAGE.

***S. coelicolor acpP*, expressed in *E. coli*, forms acyl ACP thioesters.** *S. coelicolor acpP* was expressed to a very high level in *E. coli* (Fig. 5) with the pET11c expression system. The protein was all in the soluble fraction. When a cell extract of the recombinant *E. coli* host was analyzed by native PAGE, multiple ACP bands were seen. The number of ACP species varied according to the induction conditions; cells induced in late log phase (A_{600}, 1.0 or above) generally gave two or three bands on native PAGE, and those induced in early log phase (A_{600}, 0.4) generally gave the pattern seen in Fig. 5.

In order to characterize the different migratory species, each band was purified to homogeneity as described in Materials and Methods. The proteins were analyzed by electrospray mass spectrometry (ESMS), which allowed accurate determination of the mass of each ACP species. The experimentally determined mass values were in excellent agreement with those calculated for the *S. coelicolor* ACP without its N-terminal methionine. One of the ACPs corresponded to the *apo* form, which is inactive because it does not carry the prosthetic group 4′-phosphopantetheine. The mass values for the other proteins correspond to those of ACP species with different posttranslational modifications that had taken place in the *E. coli* host. These included (i) the active, or *holo* form of the ACP, which had been correctly modified by addition of 4′-phosphopantetheine to serine 41 of the *S. coelicolor* ACP, catalyzed by the *holo* ACP synthase of *E. coli*; and (ii) several acyl ACP species

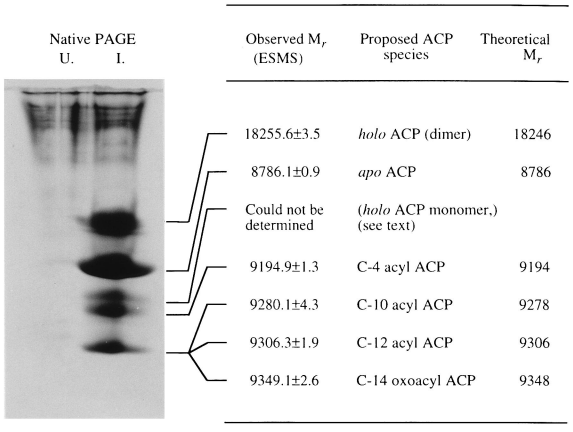

FIG. 5. High-level expression of the *S. coelicolor acpP* gene (for FAS ACP) in *E. coli*. On the left are cell extracts of induced *E. coli* cultures on native PAGE, stained with Coomassie blue. U, control *E. coli* strain without *S. coelicolor acpP* gene; I, recombinant *E. coli* host with *S. coelicolor acpP*. On the right are the experimentally determined M_rs for the ACP species identified in the gel after purification by preparative gel electrophoresis and anion-exchange chromatography together with their predicted structures.

(Fig. 5). This demonstrates that the *S. coelicolor holo* ACP can interact with the subunits of *E. coli* FAS to stimulate fatty acid biosynthesis in the heterologous host.

The M_r of the ACP species with the slowest migration on native PAGE (Fig. 5) is in reasonably good agreement with the calculated mass for a dimer form of the *holo* ACP, formed through a disulfide bond between the sulfur atoms at the end of the 4′-phosphopantetheine prosthetic groups. The dimer *holo* ACP was readily reduced back to the monomer form on addition of 5 mM dithiothreitol, as confirmed by native PAGE and ESMS. (We made use of the ability of *holo* ACP to dimerize in the absence of reducing agent for its purification; the dimer is eluted from the Mono-Q anion-exchange column at 2 M KCl [see Materials and Methods].) The M_r of the ACP species that lies directly below *apo* ACP on the native gel could not be directly determined by ESMS (not all forms of the ACP gave clean mass spectra), but since it comigrated with the reduced, monomeric *holo* ACP (generated from the dimeric ACP), it is thought to correspond to reduced *holo* ACP. All other forms of ACP migrated faster than *holo* ACP in the native gel; these are the acyl ACP species. The first is predicted to be *holo* ACP plus a reduced four-carbon acyl substituent. From ESMS, we could not determine whether this represents the expected product of the first condensation between acetyl CoA and malonyl ACP (and subsequently reduced) or whether a four-carbon unit, such as a butyryl or isobutyryl residue, had been transferred directly to the ACP. If the former had occurred, the *S. coelicolor* ACP would resemble the ACP of *E. coli* FAS, while the latter would be consistent with the observation that isobutyryl units can be used as starters to initiate long-chain fatty acid biosynthesis in *Streptomyces* spp. (15). This might also explain the observed accumulation of the C-4 ACP species, which, if an isobutyryl unit, would not be expected to serve as a substrate for further chain extension in *E. coli.*

No C-6 or C-8 chain-length intermediates were detected. ESMS analysis of the fastest migrating band demonstrated that there were three different ACP species comigrating with each other on native PAGE. The M_rs corresponded to those for C-10, C-12, and C-14 acyl ACPs. The M_r of the presumed C-14 acyl ACP corresponded to that predicted for an unreduced, or only partly reduced, oxoacyl substituent (by ESMS, it is not possible to differentiate between the two possibilities: C-14 β-ketoacyl ACP or C-14 β-hydroxyacyl ACP). Likewise, differentiation between the fully reduced and the enoyl ACP derivatives is not possible by ESMS; this is the subject of further investigation. Addition of different acyl chains to the ACP is not expected to greatly affect the size or charge of the protein; rather, this might reflect a change in the shape of the ACP as fatty acid biosynthesis proceeds (28).

The purified *holo* ACP was tested in the in vitro malonyltransferase assay with cell extracts from *S. coelicolor* and from *E. coli*. In both cases, the recombinant ACP was competent to act as a substrate for the malonyltransferase present in each respective crude cell extract (data not shown).

Ectopic expression of *acpP* with the *act* PKS. A mutant of *S. coelicolor*, altered in *actI*-ORF3 (the gene which encodes actinorhodin ACP), does not make actinorhodin (17). To test the mechanism that normally prevents *acpP* from complementing mutants of *actI*-ORF3, *acpP* was expressed ectopically with the *act* PKS gene set, in place of *actI*-ORF3, with the PKS expression system of McDaniel et al. (23). This system comprises two components: an *S. coelicolor* host strain, CH999 (Δ*act*), and a plasmid, pRM5, which carries the first six genes of the actinorhodin biosynthetic pathway. These genes (three for the minimal PKS and one each for a ketoreductase, aromatase, and cyclase) encode the components required for the synthesis of aloesaponarin, a shunt-product of actinorhodin biosynthesis that is easily identified by its yellow-brown pigmentation.

The sequence of the DNA flanking *acpP* was altered, so that the gene could be cloned in place of *actI*-ORF3 in pRM5 without changing any of the surrounding DNA (for details of the surrounding DNA sequence, see references 17 and 23). This would allow *acpP* to be expressed as part of a transcription unit that includes all of the genes required for aloesaponarin production, except *actI*-ORF3. The resulting plasmid, pIJ8120, was capable of directing synthesis of very small amounts of a yellow-brown-pigmented polyketide when introduced into *S. coelicolor* CH999 by transformation (about 2% in comparison with the amount produced by the parental strain CH999/pRM5 under the same growth conditions). We were concerned that the reduced amount of polyketide produced might have been a result of an unintended alteration in the *act* PKS gene set, introduced during construction of pIJ8120. To assess this possibility, pIJ8120 was reisolated from *S. coelicolor* CH999, and its integrity was confirmed by (i) isolation of *acpP* by PCR and resequencing of the gene, which showed that the sequence of *acpP* in pIJ8120 was correct; and (ii) construction of a new plasmid, pIJ8121, in which *actI*-ORF3 was placed downstream of the *act* biosynthetic genes in pIJ8120 to allow cotranscription with the *act*/*fab* hybrid gene set. On introduction of pIJ8121 that contained both *acpP* and *actI*-ORF3 into CH999, pigment production was restored to a level approximately half that observed in the parental strain *S. coelicolor* CH999/pRM5. This showed that the remaining *act* PKS genes in pIJ8121 (and presumably its parent plasmid pIJ8120) were expressed, and the gene products were functional.

DISCUSSION

A new ACP detected in extracts of *S. coelicolor* has reasonable end-to-end amino acid sequence similarity to the two previously characterized PKS ACPs of *S. coelicolor*, particularly around the site of attachment of the prosthetic group, 4′-phosphopantetheine. All three ACPs accept malonyl units transferred from malonyl CoA, thought to be catalyzed by a single malonyltransferase which appears to be a subunit of the *S. coelicolor* FAS (27). The malonyltransferase may provide a link between fatty acid and polyketide biosynthesis in the same organism. The ACP subunits, on the other hand, appear to define a point at which the different biochemical pathways diverge. We have been concerned with the mechanism which prevents the different ACPs of *S. coelicolor* from complementing each other in vivo.

The newly characterized ACP has several features which distinguish it from the previously characterized PKS ACPs (discussed below) and which are much more consistent with a potential role in fatty acid biosynthesis in *S. coelicolor*. We have tentatively named the corresponding gene *acpP*, in keeping with the name used for the ACP subunit gene of the *E. coli* FAS. *acpP* is expressed throughout all stages of growth in *S. coelicolor* (Fig. 2), unlike the PKS genes, which are generally expressed only in the secondary phase of growth. The new ACP is a relatively abundant protein in *S. coelicolor* and appears to be present in an active form at a level approximately two to five times the maximum observed for the actinorhodin ACP. These properties indicate a likely role for the new ACP in primary metabolism. The proximity of *acpP* to *fabD* and *fabH* (previously shown to be cotranscribed and essential for the viability of the cells) is consistent with the proposed role of the *acpP* gene product as a subunit of the *S. coelicolor* FAS. In agreement with this role, the *S. coelicolor acpP* gene product is

more similar to known FAS ACPs from other organisms (e.g., 42% amino acid sequence identity to the *E. coli* FAS ACP with no gaps) than to either of the two PKS ACPs from the same organism (31% identity with the actinorhodin ACP and two gaps; 28% identity with the spore pigment ACP and three gaps). The conservation of sequence among FAS ACPs from different organisms may reflect a functional constraint on the FAS to ensure the correct synthesis of long-chain fatty acids. The accumulation of fatty acyl ACP adducts of the *S. coelicolor* ACP, when expressed in *E. coli*, attests to the functional relatedness between the presumptive FAS ACP subunit from *S. coelicolor* and its homolog in *E. coli*. Acyl ACP adducts were never observed when the actinorhodin or spore pigment PKS ACPs were expressed in *E. coli*. In fact, the *holo* actinorhodin ACP accumulated only on prolonged incubation after induction of the recombinant *E. coli* host (6).

Under normal circumstances, the FAS ACP of *S. coelicolor* does not appear to function in place of its homolog from the PKS gene cluster; in mutants of *S. coelicolor* blocked in *actI*-ORF3 (encoding the actinorhodin ACP), polyketides do not accumulate (17). One possible explanation for this is that the FAS ACP might be prevented from replacing actinorhodin ACP because of expression of the two ACPs in different regions of the developing *S. coelicolor* colony, as was observed for the actinorhodin ACP and spore pigment ACPs (18, 37). Alternatively, the FAS ACP subunit may be biochemically incompatible with other subunits of the PKSs. The latter explanation is favored by the finding that *S. coelicolor acpP* could complement an *actI*-ORF3 deletion only very weakly to give the pigmented polyketides observed in the parental strain *S. coelicolor* CH999/pRM5 (23). (This is in keeping with earlier observations in which a presumed FAS ACP of *Saccharopolyspora erythraea* was cloned in place of *actI*-ORF3 on the *S. coelicolor* chromosome and shown to complement actinorhodin production, but only very weakly [17]). Such complementation may reflect expression of *acpP* in *cis* with the *act* PKS, rather than in the natural in *trans* situation, coupled with the elevated level of expression of the hybrid *act*/*fab* gene set in the pRM5-derived plasmid (the level of active actinorhodin ACP present in CH999/pRM5 was approximately 20-fold higher than that detected in the act^+ strain CH1 (26a).

A mechanism of biochemical incompatibility may allow the FAS to function in the same regions of an *S. coelicolor* colony as those expressing the PKSs, without biochemical interference. If this were the case, then de novo fatty acid synthesis might occur in all cell types of the *S. coelicolor* colony and so ensure the continued growth and development of the colony. Coexpression of the FAS and PKS genes in the same regions of a colony may also be a requirement for PKS function. Previous studies had indicated that the malonyltransferase, presumed to be a subunit of the FAS (encoded by *fabD*), may be essential for polyketide biosynthesis (27). Biochemical mechanisms would ensure that subunits such as the malonyltransferase could be available to interact with the PKS, while other subunits, such as the ACP, the gene for which is potentially cotranscribed with *fabD*, are prevented from doing so. We are currently investigating whether other subunits of the potential *S. coelicolor* FAS, such as ketoacylsynthase III encoded by *fabH*, play a role in polyketide biosynthesis in *S. coelicolor.*

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