

## Purification of a Malonyltransferase from *Streptomyces coelicolor* A3(2) and Analysis of Its Genetic Determinant

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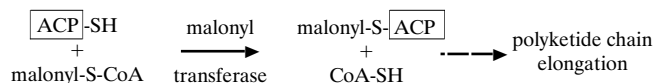
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*Streptomyces coelicolor* A3(2) synthesizes each half molecule of the dimeric polyketide antibiotic actinorhodin (Act) from one acetyl and seven malonyl building units, catalyzed by the Act polyketide synthase (PKS). The synthesis is analogous to fatty acid biosynthesis, and there is evident structural similarity between PKSs of *Streptomyces* spp. and fatty acid synthases (FASs). Each system should depend on a malonyl coenzyme A:acyl carrier protein malonyltransferase, which charges the FAS or PKS with the malonyl units for carbon chain extension. We have purified the Act acyl carrier protein-dependent malonyltransferase from stationary-phase, Act-producing cultures and have determined the N-terminal amino acid sequence and cloned the structural gene. The deduced amino acid sequence resembles those of known malonyltransferases of FASs and PKSs. The gene lies some 2.8 Mb from the rest of the *act* cluster, adjacent to an open reading frame whose gene product resembles ketoacylsynthase III of *Escherichia coli* FAS. The malonyltransferase was expressed equally as well during vegetative growth (when other components of the *act* PKS were not expressed) as in the stationary phase, suggesting that the malonyltransferase may be shared between the FAS and PKS of *S. coelicolor*. Disruption of the operon containing the malonyltransferase gene proved to be impossible, supporting the idea that the malonyltransferase plays an essential role in fatty acid biosynthesis.

The aromatic polyketide actinorhodin (Act) (Fig. 1) is a secondary metabolite produced by *Streptomyces coelicolor* A3(2), genetically the most studied member of the genus. A C<sub>16</sub> carbon chain forms the backbone of each half-molecule of Act and is synthesized from one acetyl coenzyme A (acetyl CoA) starter and seven malonyl CoA extender units. These units are condensed together (with loss of CO<sub>2</sub>) in a sequential fashion by the Act polyketide synthase (PKS) (14), a process which shows clear similarities to the mechanism of fatty acid biosynthesis (21, 33). The linear intermediate then undergoes cyclizations and further, "tailoring" modifications to yield the final molecule. A set of genes (*act*) involved in the synthesis of actinorhodin has been identified and located on one continuous (22-kb) section of the *S. coelicolor* chromosome (28). The *act* cluster has been completely sequenced (4, 10–12, 16); it contains a set of some 22 genes that include the structural genes for the Act PKS, genes which encode the enzymes involved in the subsequent modification of the PKS product, and genes for activation of expression of the *act* cluster and for actinorhodin export.

The *act* PKS itself consists of a series of discrete open reading frames (ORFs) encoding several proteins that share considerable amino acid sequence similarity with the components of *Escherichia coli* fatty acid synthase (FAS) (11). These are (i) a ketosynthase (KS), which catalyzes the condensation of the acyl building units; (ii) an acyl carrier protein (ACP), which serves a dual purpose as the recipient for the malonyl extender units prior to condensation and as an anchor to hold the growing polyketide chain; and (iii) a ketoreductase involved in reduction at C-9 of the polyketide chain intermediate. A further component of the *act* PKS is a protein with a high degree of homology to the KS but lacking its active site. This protein is involved, to a large degree, in determining the polyketide chain length (29, 30). However, one enzyme expected by anal-

ogy with the FAS of *E. coli* has not been identified by genetic means or by analysis of the sequence of the *act* gene cluster: this is a malonyl CoA:ACP malonyltransferase, responsible for transfer of the malonyl extender unit onto the ACP subunit of the Act PKS by the following reaction:



Here we describe the purification of a malonyltransferase from *S. coelicolor* which can transfer malonyl units to Act ACP in vitro. The corresponding gene has been cloned and sequenced, and attempts at gene disruption showed that the transcription unit in which the gene resides is essential. We suggest that the malonyltransferase described here might be shared by the Act PKS pathway and the FAS of *S. coelicolor*.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and phages.** The strains, phages, and plasmids used are described in Table 1. Standard conditions for culture of *Streptomyces* spp., isolation of  $\phi$ C31 phages, and lysogenization were as previously described (18). Conditions for growth of *Streptomyces* spp. in minimal medium supplemented with Casamino Acids were as previously described (43), except that 200-ml cultures were grown in 2-liter flasks and 5-ml samples were withdrawn at intervals during growth. In attempts to isolate lysogens disrupted for the FAS, lysogenization was conducted on agar (3) supplemented with 25  $\mu$ g of 13-methyltetradecanoic acid per ml, 25  $\mu$ g of 12-methyltetradecanoic acid per ml, and 400  $\mu$ g of Brij 58 per ml (fatty acids and Brij were from Sigma). *E. coli* strains were grown as previously described (38), except for *E. coli* K38(pIJ5232, pGP1-2), which was grown as described elsewhere (7).

**General protein techniques.** Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (25), using the Mini-Protein 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. Proteins were visualized in the gels by staining either with Coomassie brilliant blue or with silver stain. Protein concentrations were determined by the method of Bradford (protein assay dye reagents from Bio-Rad), with bovine serum albumin as the standard. For N-terminal sequencing of proteins separated by SDS-PAGE, proteins were transferred onto a 0.2- $\mu$ m-pore-size polyvinylidene difluoride 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.

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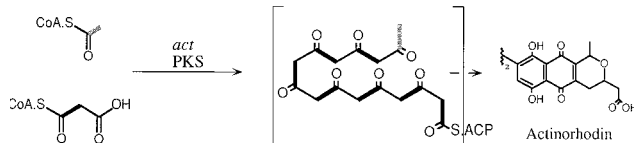


FIG. 1. The Act PKS synthesizes the full-length polyketide chain (hypothetical intermediate shown in brackets) from one acetyl starter and seven malonyl extender residues, in a manner resembling fatty acid biosynthesis. Subsequent modification of the polyketide chain yields Act. ACP is the component of the Act PKS which serves to anchor the growing chain.

**Purification of Act ACP.** *E. coli* K38(pIJ5232, pGP1-2) was grown and induced as described previously (7). Cells from 500 ml of culture were resuspended to a thick fluid consistency in cell disruption buffer (50 mM Tris-Cl [pH 7.5], 10% glycerol, 2 mM dithiothreitol), broken by sonication, and kept cool on ice water. Cell extracts were maintained on ice or at 4°C in all subsequent steps. Debris was cleared by centrifugation for 10 min at 12,000 × g and then for 30 min at 27,000 × g, and the supernatant was loaded directly onto a native polyacrylamide gel column (Prep Cell-491; Bio-Rad) (18 ml of resolving gel at 15% total acrylamide, with bisacrylamide at 1.9% of the total, and 3.5 ml of stacking gel at 5% total acrylamide, with bisacrylamide at 0.6% of the total). Act ACP was eluted in 50 mM Tris-Cl (pH 7.5) at 12 W for 150 to 200 min. Purification was monitored by SDS-PAGE (at the acrylamide concentrations shown above and with glycerol in the running gel at 10%) to detect a protein with an apparent  $M_r$  of 2,500. Pure Act ACP was dialyzed against 20 mM  $\text{NH}_4\text{HCO}_3$ , lyophilized, and stored at 4°C.

**Assay for malonyltransferase.** *S. coelicolor* M145 cells were grown in minimal medium supplemented with Casamino Acids until Act was produced. The cell extract was prepared and assayed for malonyl CoA:ACP malonyltransferase, essentially as described previously (7), except that endogenous ACP species were not removed from the cell extracts. The contribution to malonyltransferase activity from endogenous ACP species was found to be very low, close to the background level, so activity was measured as the difference between cell extracts with and without exogenously added Act ACP. A unit is defined as nanomoles of  $^{14}\text{C}$ -malonyl ACP formed per milligram of cell extract per minute, when malonyltransferase was determined to be the limiting component in the assay.

**Purification of malonyltransferase from *S. coelicolor*.** Cells from 1 liter of an Act-producing culture were washed in 50 mM potassium phosphate (pH 7.2)–0.5 M NaCl, washed again in 50 mM potassium phosphate (pH 7.2), resuspended in buffer A (50 mM potassium phosphate [pH 7.2], 10% glycerol, 2 mM dithiothreitol), and disrupted by sonication. Cell debris was removed by centrifugation for 10 min at 12,000 × g and then for 30 min at 27,000 × g, both at 4°C, and malonyltransferase was precipitated at between 70 and 100% saturation (at 0°C) with solid ammonium sulfate. After centrifugation for 20 min at 27,000 × g at 2°C, the precipitate was redissolved in buffer A and dialyzed overnight at 4°C against buffer A (2 liters). Malonyltransferase was further purified by chromatography on an anion exchange column (Mono Q HR5/5; Pharmacia), using a linear gradient of 0 to 1 M KCl–50 mM Tris-Cl, pH 7.4, followed by elution from a hydrophobic interaction column (Phenyl-Superose HR5/5; Pharmacia), using a linear gradient of 1.8 to 0 M  $(\text{NH}_4)_2\text{SO}_4$ –50 mM Tris-Cl, pH 7.4. The salt gradients were pumped through the columns with a fast protein liquid chromatography system (Pharmacia). Purification was monitored by activity assays and by SDS-PAGE (12% total acrylamide, with bisacrylamide at 1.4% of the total).

**General DNA techniques.** Recombinant DNA techniques in *Streptomyces* spp. and isolation of *Streptomyces* total DNA were performed as described previously (18). Techniques using *E. coli* were performed as previously described (38), except that cells were transformed by electroporation with a Gene Pulser (Bio-Rad) according to the manufacturer's recommendations; plasmids for subcloning were isolated as previously described (20), and plasmids for sequence analysis were isolated with the Magic-Miniprep system from Promega. Labelling of DNA probes, hybridization, and detection were done as recommended for the Dig-system (Boehringer Mannheim). Nucleotide sequence was determined with the Taqtrack system (Promega), according to the manufacturer's recommendations for double-stranded DNA. Convenient restriction sites and custom-made oligonucleotides were used to obtain the DNA sequence on both strands of the malonyltransferase gene (*fabD*). Sequences were analyzed by using the STADEN (41) and UWGCG (9) computer packages. The FRAME program (2) was used to identify ORFs and their direction of transcription.

**Pulsed-field gel electrophoresis.** Pulsed-field gel electrophoresis of *S. coelicolor* M145 DNA and hybridization of *S. coelicolor fabD* DNA to the separated fragments were done as described previously (23).

**Nucleotide sequence accession number.** The sequence of the *fabD* gene of *S. coelicolor* A3(2) has been deposited in the EMBL database under the accession number X86475.

TABLE 1. Strains, phages, and plasmids used

Plasmid, phage, or strain	Description <sup>a</sup>	Reference or source
<b>Plasmids</b>		
pCos-4A7	Cosmid isolated from a library of <i>S. coelicolor</i> M145 DNA in <i>E. coli</i> cosmid vector Supercos-1	Gift of M. Redenbach
pGP1-2	Helper plasmid to pIJ5232	42
pIJ68	Activator gene ( <i>actII</i> -ORF4) for Act production in <i>S. coelicolor</i> on <i>Streptomyces</i> high-copy-number plasmid pIJ699	34
pIJ2925	Derivative of pUC19 with <i>Bgl</i> II sites flanking the polylinker region	19
pIJ5232	Contains <i>act</i> ACP gene under control of the T7 gene 10 promoter	7
pUC118	<i>E. coli</i> cloning vector	45
<b>φC31 phages</b>		
KC466	$c^+ \Delta attP::vph::!gyl!$	39
KC467	$c^+ \Delta attP::tsr::vph::!gyl!::!fab!$	This study
KC515	$c^+ \Delta attP::tsr::vph$	35
KC928	$c^+ \Delta attP::tsr::vph::!gyl!::!hrdC!$	3
<b><i>S. coelicolor</i> A3(2)</b>		
M145	Prototrophic, SCP1 <sup>-</sup> SCP2 <sup>-</sup>	18
CH1	<i>proA1 argA</i> SCP1 <sup>-</sup> SCP2 <sup>-</sup> <i>redE60</i>	22
CH999	<i>proA1 argA1</i> SCP1 <sup>-</sup> SCP2 <sup>-</sup> <i>redE60 Δact</i>	29
J1501	<i>hisA1 strA1 uraA1 pgl</i> SCP1 <sup>-</sup> SCP2 <sup>-</sup>	18
<b><i>S. lividans</i> 66</b>		
1326	Prototrophic, SLP2 <sup>+</sup> SLP3 <sup>+</sup>	6
<b><i>E. coli</i></b>		
DH5α	<i>supE44 ΔlacU169</i> (φ80 <i>lacZΔM15</i> ) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	38
K38	HfrC(λ)	42
Epicurian coli	$e14^-$ ( <i>mcrA</i> ) $\Delta$ ( <i>mcrCB-hsdSMR-mrr</i> )171 <i>endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC</i>	Stratagene
SURE	<i>umuC::Tn5</i> (Kan <sup>r</sup> ) <i>uvrC</i> [F' <i>proAB lacI<sup>q</sup>ΔM15 Tn10</i> (Tet <sup>r</sup> )]	

<sup>a</sup> The use of flanking exclamation marks indicates fragments internal to transcription units (e.g., !gyl!).

## RESULTS

**A novel method for purification from *E. coli* of Act ACP, a substrate for malonyltransferase.** The source of Act ACP for malonyltransferase assays was a recombinant *E. coli* in which the carrier protein had been expressed to a high level under control of a T7 promoter (7). Initially, the Act ACP was purified in several steps by routine chromatographic methods, but here we describe a novel procedure for purification by gel electrophoresis. Act ACP is a small, highly charged protein ( $M_r = 9,117$  [minus its formylmethionyl], with a theoretical pI of 3.8) which can be resolved from the other *E. coli* proteins by native PAGE. This property was exploited to purify Act ACP in a single step, using a preparative-scale Prepcell-491 gel apparatus (Bio-Rad; see Materials and Methods). Typically, 10 mg of pure Act ACP was obtained from 78 mg of *E. coli* crude protein extract and was found not to be contaminated with the FAS ACP of *E. coli*, as judged by malonyltransferase assays (see below), electrophoresis of the assay mixture by native PAGE, and visualization of the radiolabelled ACP species by autoradiography (*E. coli* ACP can be acylated by *S. coelicolor* malonyltransferase and has a different mobility from that of Act ACP by native PAGE [34a]). The method described ensured a ready supply of the Act ACP, and though only 2 to 3% of this was in the active holo-form (requiring correct posttranslational modification by the *E. coli* holo-ACP synthase, which catalyzes the addition of 4'-phosphopantetheine), this was enough for characterization of a malonyltransferase from *S. coelicolor* and for its subsequent purification.

**Initial characterization of the malonyltransferase in extracts of *S. coelicolor*.** Malonyltransferase activity was determined as the ability of whole cell extracts of *S. coelicolor* to catalyze the formation of [2- $^{14}$ C]malonyl ACP from radiolabelled malonyl CoA and exogenously added Act ACP (adapted from reference 7; see Materials and Methods). This assay was used to determine if the malonyltransferase is encoded within the cluster of *act* genes and if its activity is regulated by the same mechanisms that regulate *act*.

The specific activity of malonyltransferase in extracts of the *act*<sup>+</sup> strain M145 was  $17.3 \pm 0.5$  U. Deletion of the *act* cluster (*S. coelicolor* CH999 [29]) had no significant effect on malonyltransferase activity ( $16 \pm 0.5$  U). Similarly, the introduction of pIJ68 (34), a derivative of the multicopy plasmid pIJ101, carrying *actII-ORF4*, the pathway-specific activator of the *act* cluster (10), did not increase the malonyltransferase activity ( $16.5 \pm 0.1$  U), though it is known to cause overexpression of the *act* genes resulting in a 10-fold increase in Act production compared to that observed with M145 (15). These data indicated that, under the assay conditions described, the *act* cluster does not express transferase activity and that expression of the malonyltransferase is not regulated by the same mechanisms that control the rest of the *act* genes.

To investigate further the regulation of the malonyltransferase, extracts were prepared from cells of *S. coelicolor* taken at different stages during growth in liquid culture. All the cell extracts were competent in the transfer of malonyl units onto Act ACP, even at the very earliest time points, when the *act* genes are not expressed (Fig. 2) (15, 43).

**Purification of the Act ACP-dependent malonyltransferase from *S. coelicolor*.** *S. coelicolor* M145 was grown until the cells became blue-purple, indicating that they were producing Act. During purification, cell fractions were assayed for malonyltransferase activity by measuring the stimulation in  $^{14}$ C-malonyl ACP formation upon the addition of exogenous Act ACP. The malonyltransferase was purified to near homogeneity (Table 2; Fig. 3). Only one peak of activity, corresponding to a

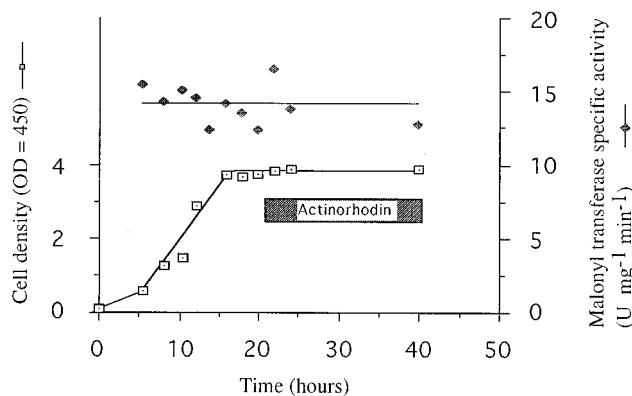


FIG. 2. Specific activity of malonyltransferase at different time points during the growth of *S. coelicolor* M145. Cells were grown in minimal medium supplemented with Casamino Acids, and samples were taken from the culture for assay at the times indicated. Act appeared at 20 h and persisted in the culture, as indicated by the box. OD, optical density.

protein with an  $M_r$  of 35,000 (estimated by SDS-PAGE) and similar to the monofunctional malonyltransferase of *E. coli* FAS ( $M_r$ , 32,286), could be resolved. Malonyltransferase was transferred from the SDS-polyacrylamide gel to a polyvinylidene difluoride membrane (Transblot; Bio-Rad). The sequence of the first 15 amino acids (Fig. 3C) showed a good alignment with amino acids at the N termini of known acyltransferases from both FASs and PKSs.

The N-terminal sequence of the malonyltransferase contains amino acids for which there is a low redundancy of codon usage in *Streptomyces* spp. (46), making possible the design of a synthetic oligonucleotide with relatively little variation in its sequence (Fig. 3C). The "guessmer" oligonucleotide was then used as a hybridization probe to isolate the gene.

**Identification and sequence analysis of the gene for Act ACP-dependent malonyltransferase from *S. coelicolor*.** The oligonucleotide hybridized to a 3.6-kb *Bam*HI fragment of *S. coelicolor* genomic DNA under stringent conditions (washed twice at 65°C with 0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] for 15 min). By using these hybridization conditions, we identified one positively hybridizing clone (Cos-4A7) in a cosmid library of *S. coelicolor* DNA. The hybridizing 3.6-kb *Bam*HI fragment in the clone was partly sequenced (Fig. 4). An ORF was assigned, using the following criteria: (i) the deduced protein sequence encoded by the potential reading frame, starting at nucleotide 112, matched the N-terminal amino acid sequence determined previously (except that Gly-14, towards the end of the experimentally determined protein sequence which was more difficult to assign unambiguously, was found to be a proline); (ii) the extent of the reading frame was consistent with the FRAME analysis (2), which makes use of a strong bias towards G or C in the third position of a codon in *Streptomyces* DNA; (iii) the deduced protein sequence has end-to-end similarity to the malonyltransferase of *E. coli* FAS and active site identity with various acyltransferases from both FASs and PKSs (Fig. 5); (iv) identification of a potential ribosome binding site 10 nucleotides upstream of the GTG start codon, with moderate complementarity to the 3' end of 16S rRNA from *Streptomyces lividans* (1); and (v) identification of a new ORF starting 16 nucleotides from the predicted stop of the malonyltransferase gene. Analysis of the sequence immediately upstream of the *S. coelicolor* malonyltransferase gene did not reveal any significant similarities to known gene products (data not shown).

TABLE 2. Purification of malonyl CoA:Act ACP malonyltransferase

Cell fraction	Total protein (mg)	Sp act (U mg <sup>-1</sup> min <sup>-1</sup> )	Total activity (U)	Yield (%)	Purification (fold)
Crude extract	303	13.9	4,212	100	1
Ammonium sulfate precipitation (70–100%)	11.25	22	247.5	5.9	1.6
Anion exchange chromatography (Mono Q HR5/5)	0.35	84.2	29.5	0.7	6.1
Hydrophobic interaction chromatography (Phenyl-Superose HR5/5)	0.01	1,484	15.6	0.4	106.8

Initial sequence data indicate that the deduced product of the downstream ORF resembles one of the ketoacylsynthases (KS III) of *E. coli* (Fig. 6). KS III catalyzes the condensation of the starter acetyl unit with the first malonyl extender unit, to initiate fatty acid biosynthesis (44).

The gene encoding malonyltransferase of *E. coli* FAS is called *fabD* (26). In keeping with this terminology, we tentatively name the genes encoding malonyltransferase and KS III of *S. coelicolor* *fabD* and *fabH*, respectively.

*fabD* is predicted to encode a protein of 317 amino acids with an *M<sub>r</sub>* of 32,077. This is smaller than the 35,000 estimated by SDS-PAGE, but a similar discrepancy was observed for the malonyltransferase of *E. coli* (27). The sequence resembles that of the *E. coli* malonyltransferase involved in fatty acid biosynthesis (38% identity and 53% similarity over 315 amino acids, with 5 gaps introduced to optimize the alignment). It also clearly resembles a recently sequenced malonyltransferase

from *Streptomyces glaucescens* (41a); it has been suggested that this protein is a component of the FAS of *S. glaucescens*, but this awaits confirmation. Ser-97 of the *S. coelicolor* malonyltransferase is predicted to be the amino acid involved in catalysis of acyl transfer, on the basis of similarity with the *E. coli* malonyltransferase for which this has been determined experimentally (36, 37).

**Location of the gene for malonyltransferase on the *S. coelicolor* chromosome.** The malonyltransferase gene hybridized to the *DraI*-G fragment on the physical map of the *S. coelicolor* chromosome (23); it is thus separated by approximately one third of the length of the chromosome (~2.8 Mb) from the *act* cluster.

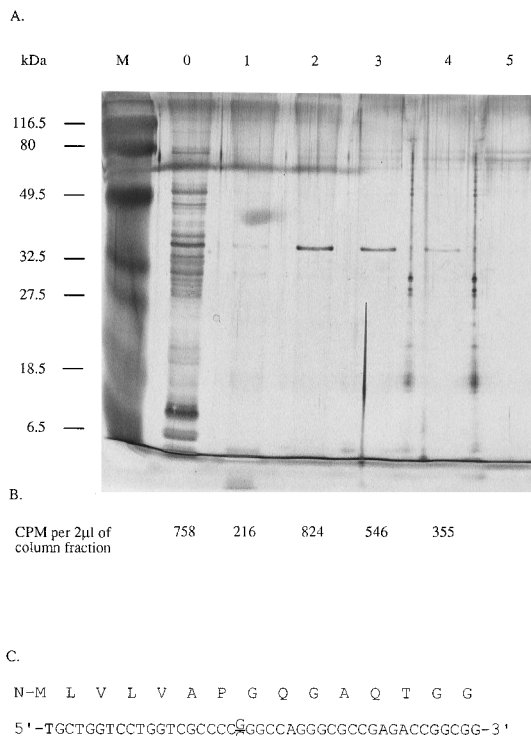


FIG. 3. Purification and N-terminal amino acid sequence of the malonyltransferase. (A) Silver-stained SDS-polyacrylamide gel of fractions eluted from a Phenyl-Superose hydrophobic interaction chromatography column. Lane 0, protein sample loaded onto the Phenyl-Superose column. Lanes 1 to 5, fractions 1 to 5, respectively, eluted from the column. Lane M, molecular mass markers. (B) Activity (in counts per minute) of 2 µl of each fraction recovered from the Phenyl-Superose column. (C) N-terminal amino acid sequence obtained from the purified malonyltransferase and sequence of the guessmer oligonucleotide used as a probe to clone the gene.

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CGACGGAGATCCGCAGCTCTAGGATTTGTGTGCGAGGTCACAAAACCCGCTCTGTCTCT 60
TCGTCCCTGTCCCCACGGCCGCTCGTGGCCGTCTCAAGAGAGAGTGTGAGAGTGCCTCGT 120
                                     FM L V 3
ACTCGTCGCTCCCGGCCAGGGCCGCCAGACGCGCCGCTCTCTGACTGACTGGCTCGCCCT 180
L V A P G Q G A Q T P G F L T D W L A L 23
CCCCGGTGGCGCTGACCGCGTCCGCGCGTGGTCGGACGCCATCGGACTCGATCTCGCCCA 240
P G A A D R V A A W S D A I G L D L A H 43
CTTCGGCACCAAGCCGACGCGGACGAGATCCGAGACAGTCCGTGGCCAGCCGCTGCT 300
F G T K A D A D E I R D T S V A Q P L L 63
GGTCGCGCCGGAATCTGTCCGCGCGGCACTCGGTACGCGAGACATCTGTCTGCTGACGC 360
V A A G I L S A A A L G T Q T S V A D A 83
GACGGGCCCCGGTTACCCCCCGCGCGTCCGCGGACACAGCGTCGGCGAGATCACCCG 420
T G P G F T P G A V A G H S V G E I T A 103
CGCGTCTTCGCGGGCGTCTCGAGACACCGCCGCGTGTCCCTCGTACGCGCTCGCGG 480
A V F A G V L D D T A A L S L V R R R H 123
CCTGGCCATGGCCGAGCCGCGCGGTACCCGAGACCGCATGTCCGCGCTGCTCGGGGG 540
L A M A E A A A V T E T G M S A L L G G 143
CGACCCGAGGTGAGCGTCCGCGACCTGGAGCGGCTCGGCTGACCCCGCGCAACGTGAA 600
D P E V S V A H L E R L G L T P A N V R 163
CGCGCCGTCAGATCGTGGCGCGGGCCACCATGGAGCAGTGGCCGCGCTGAACAGGGA 660
G A G Q I V A A G T M E Q L A A L N E D 183
CAAGCCGAGGTTGTGCGCAAGTCTCCCGTGAAGTGGCCGCGCGTCCACACCCG 720
K P E G V R K V V P L K R A V A S G F H T R 203
CCACATGGCCCCCGCGTGGACAAGCTCGCCGAGGCCCAAGCGCTGACCGCCGCCGA 780
H M A P A V D K L A E A A K A L T P A D 223
CCGGAAGGTGACGTACGTCTCCAACAAGGACGGCCGCGCGTCCGCGCACCGAGGT 840
P K V T Y V S N K D G R A V A S G T E V 243
GCTGGAACGGCTGGTCCGCGAGTCCGCAACCCGCTGCGCTGGGACCTGTGCATGGAGAC 900
L D R L V G Q V A N P V R W D L C M E T 263
GTTCAAGGAGTGGCGCTCACCGCATCATCGAGTGTGTCGCGGCGGACGCTGACCCG 960
F K E L G V T A I I E V C P G G T L T G 283
GCTGGCCAAGCGCGCTGCCCGAGTGAAGACGCTGGCCCTGAAGACCCCGCAGCAGCT 1020
L A K R A L P G V K T L A L K T P D D L 303
                                     *****
CGACGGCCCGTGGTCTGTCGCGGACACACCCAGGCTGACCAAGGAGCGCGAGAGCA 1080
D A A R E L V A E H T Q A * FM
TGTCGAGATCAAGCCAGCAAGGGCGCCCGTACGCGGCATCTCGCGTCCGCGGAT 1140
S K I K P S K G A P Y A R I L G V G G Y
ACCGTCCGACCCGGTGGTCCGCAACGAGGTGATCCTCGAGAAGAT 1186
    
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FIG. 4. Nucleotide and deduced amino acid sequences of the *S. coelicolor* malonyltransferase. ▲, active-site amino acid Ser-97. The putative ribosome binding sites for the malonyltransferase and for the KS III genes are marked above the sequence with asterisks, and an inverted repeat is underlined with arrows.

ChickFAS	IAQIDVLKAAAGLQPDGILGHVSVGELACGYADNSLSHEEA
RatFAS	IALIDLTLTSMGLKPDGIIGHSLGEVACGYADGCLSQREA
SeryPKS1	VSLASMMRRAHGVEPAVIGHSGEIAAACVAGALSLLDDA
SeryPKS1a	TSLAALWRSFGVTFDAVVGHSIGELAAHVCGAAGAADA
PpaPKS	IGLSALLQSNGITPQAVIGHVSVGEIAASVWAGALSPAEG
YeastFAS	AFEDLKSRLIPADATFAGHSLGEYAAALASLADVMIES
BamFAS	QIAELAEAGVKSQDTYFAGHSVGEYNALAAAYQVLSLEA
EcoFAS	ALYRVWQQGGKAPAMMAGHSLGEYSALVCGVDFADA
ScoFAS	QTSVADATGPGFTPGAVAGHSVGEITAAVAFVGLDDTAA
Consensus	.....GHS.GE.....*

FIG. 5. Amino acid sequence alignment at the active site region of acyltransferases and transferase domains. The active-site serine is indicated by an asterisk. Sequences have been obtained from the National Biomedical Research Foundation databases, and the corresponding database and accession numbers are as follows: ChickFAS, acetyl-malonyl transferase domain (PIR1:XYCHFA); RatFAS, acetyl-malonyl transferase domain (PIR1:XYRTFA); SeryPKS1, acetyl-malonyl transferase domain from module 1 of 6-deoxyerythronolide B synthase of *Saccharopolyspora erythraea*; SeryPKS1a, presumed propionyl transferase domain from module 1 (PIR2:S13595); PpaPKS, acetyl-malonyl transferase domain from 6-methyl salicylic acid synthase of *Penicillium patulum* (PIR3:S13178); YeastFAS, malonyl-palmitoyl transferase domain from *Saccharomyces cerevisiae* (PIR2:A31107); BamFAS, malonyl-palmitoyl transferase domain from *B. ammontigenes* (PIR3:S20473); EcoFAS, *fabD* gene product of *E. coli* (PIR3:S20443); ScoFAS, this study.

**Targeted disruption of the DNA segment containing the malonyltransferase gene suggests that it is part of an essential transcription unit.** The short distance between *fabH* and *fabD* (16 nt; Fig. 4) and the likelihood that they are involved in the same metabolic pathway suggested that the two genes are probably cotranscribed. Therefore, disruption of the upstream *fabD*, by most methods, could have a polar effect on the downstream gene (or any other downstream gene encoded within the same potential transcription unit), and the phenotype might be that of *fabH*, rather than that of a *fabD* mutant. Hence, for our first disruption experiment, a strategy which would test if the two genes were cotranscribed, as well as test the viability of a strain disrupted in the potential transcription unit, was chosen. The strategy involved the use of a derivative of the *Streptomyces* phage  $\phi$ C31, KC467, which contains a 0.8-kb *Sma*I fragment of DNA overlapping the stop codon of *fabD* and the start of *fabH* (nucleotides 370 to 1152; Fig. 4). Only fragments internal to a transcription unit are mutagenic (5); thus, if the corresponding region of the *S. coelicolor* chromosome were disrupted by homologous recombination and no phenotypic change was seen, this would suggest that *fabD* and *fabH* were not part of the same transcription unit, and further experiments would be needed to explore the effect of disrupting them individually. If, however, there were a phenotypic change, this would automatically imply that the two genes are cotranscribed (Fig. 7). KC467 contains a second fragment of DNA internal to *gylB* (7a, 39), which serves as an internal

CwrFASa	KLVGSGSAMFALQVSNDDLKIVDNDIEWISVRTGIRNRRV
CwrFASb	KLIGSGSAVPLQISNDDLAKIVDNDIEWISVRTGIRNRRV
SolFAS	KLVGCGSAVFKLQISNDDLKIVDNDIEWISVRTGIRQRHV
AthFAS	KLIGCGSAVPLQISNDDLAKIVDNDIEWISVRTGIRTRRV
PumFAS	HILSTGSSVPEFVSVENQDFEIIETSDHWISTRTGIRKLSL
EcoFAS	KLIGTGSYLPQVTRNADLEKMDVTSDEWIVTRTGIRERHI
RcaHIM	VVRGVGHYLPARVVENAEFEGKLDTTDEWIRSTRGIERRHF
ScoFAS	RILGVGGYRPTRVVENEVILEKIDSSDEWIRSRSGIETRRHW

FIG. 6. Amino acid sequence alignment near the N terminus of KS III homologs. Sequences have been either obtained from the National Biomedical Research Foundation or translated from the European Molecular Biology Laboratory databases. The corresponding databases and accession numbers are given in parentheses as follows: CwrFASa and CwrFASb, *Cuphea wrightii* (translated from EMN:CWAS3A and -B); SolFAS, *Spinnacea oleracea* (translated from EMN:SOKASIII); AthFAS, *Arabidopsis thaliana* (translated from EMN:ATKASIII); PumFAS, *Porphyra umbilicalis* (PIR2:S30933); EcoFAS, *E. coli* *fabH* gene product (PIR3:A42431); RcaHIM, *Rhodobacter capsulatus*, upstream of a gene encoding host integration factor (*himA*) (PIR2:C41608); ScoFAS, this study.

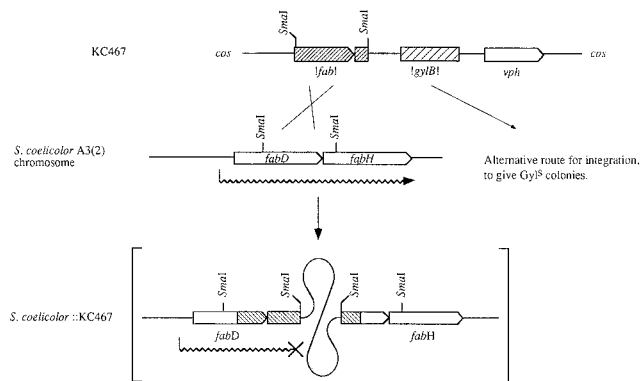


FIG. 7. Strategy for attempted disruption of the *fabD-fabH* operon of *S. coelicolor*. If insertion of  $\phi$ C31 were to occur at the *fab* operon by homologous recombination, a copy of each gene would be left intact but transcription of *fabH* would be prevented (shown in brackets). If the operon were essential, integration would occur through *gylB* only. Open bars represent open reading frames on the chromosome of *S. coelicolor*. !-! indicates fragments internal to transcription units (e.g., *fabI*). ~ represents transcription. *vph* is the viomycin phosphotransferase gene.

control for lysogen formation. Disruption of the *S. coelicolor* chromosome by homologous recombination through *gylB* results in inactivation of the glycerol-3-phosphate dehydrogenase and produces a mutant severely impaired in its ability to grow on agar containing glycerol as a sole carbon source ( $Gyl^s$ ; glycerol-3-phosphate, which is toxic to the cells, is accumulated). Because the phage lacks *attP*, it can form lysogens only by homologous recombination into either the *fab* or the *gyl* DNA sequence on the chromosome. As an additional control, a second  $\phi$ C31 derivative, KC928, was used to test the efficiency of lysogenization. KC928 contains both the 1.25-kb fragment of *gyl* DNA (the same as for KC467) and a 0.9-kb fragment of DNA (similar in size to the *fab* DNA) internal to *hrdC*. *hrdC* encodes an RNA polymerase  $\sigma$  factor dispensable for growth and differentiation in *S. coelicolor* (3); lysogens involving *hrdC* will be  $Gyl^r$ .

Lysogens of *S. coelicolor* J1501 were obtained by selection for viomycin resistance (conferred by the prophage) and then replicated to a medium containing glycerol as the sole carbon source. With KC928, containing the *hrdC* fragment, 287 of 1,125 lysogens grew in the normal, healthy manner and were presumably disrupted in the nonessential *hrdC* sequence, whereas the remainder were  $Gyl^s$ . More than 5,000 lysogens of KC467 (containing the *fab* fragment) were tested, and all were  $Gyl^s$  (two of the KC467 lysogens were further analyzed by Southern hybridization, and this confirmed that integration of the phage had been mediated by the *gyl* sequence). This indicated both that *fabD* and *fabH* are cotranscribed and that disruption of the operon is a lethal event. (This experiment does not rule out the possibility, unprecedented in *Streptomyces* genetics, that the *fab* fragment used was unusually low in recombination proficiency.)

Subsequent attempts to isolate  $Gyl^r$  lysogens using KC467 on medium supplemented with 13-methyltetradecanoic acid and 12-methyltetradecanoic acid, the major fatty acids of *S. coelicolor*, also proved unsuccessful. This was not entirely unexpected, because it has been impossible to isolate fatty acid auxotrophs of *E. coli* defective in both unsaturated and saturated fatty acid biosynthesis, unless as temperature-sensitive mutants (17, 26).  $\beta$ -Hydroxymyristoyl ACP is an intermediate of both biosynthesis of long-chain fatty acids and synthesis of lipid A, a component of lipopolysaccharide in *E. coli*. When

$\beta$ -hydroxymyristic acid is supplied in the medium, it cannot be incorporated into lipid A (17). This is presumed to be because there is no transferase to synthesize the  $\beta$ -hydroxymyristoyl ACP intermediate and may explain the lack of representative mutants for all of the *E. coli fab* genes. Lipopolysaccharides are not an essential component of the *Streptomyces* cell wall, but, in principle, a similar intimate association may exist between fatty acid biosynthesis and production of some other cell component that cannot be supplemented in the medium. The results of the gene disruption experiments suggest that one or more of the genes cotranscribed with *fabD* are essential and may be components of the (still uncharacterized) *S. coelicolor* FAS.

## DISCUSSION

The malonyltransferase described here was detected in extracts of *S. coelicolor* by its ability to charge Act ACP with radiolabelled malonyl units. When this assay was used, a single detectable malonyltransferase was isolated from extracts of *S. coelicolor* which were producing Act. A notable feature of the transferase is that it is not encoded by the *act* cluster, since extracts of *S. coelicolor* CH999 (from which the entire set of *act* genes has been deleted) had full transferase activity. The only likely candidate for an acyltransferase encoded within the *act* cluster was a potential acyltransferase domain, identified within the Act KS. The domain has the sequence GHS, which is conserved throughout all actinomycete PKS KS homologs (11) and which is also a motif found at the active site of acyltransferases. Recent experimental evidence, obtained by mutagenesis of the actinorhodine and the tetracenomycin KS, has cast doubt on whether the serine at the center of the putative acyltransferase domain is essential for PKS function (24, 32).

The corresponding *S. coelicolor* gene for malonyltransferase (*fabD*) has been cloned and sequenced. Consistent with the detection of just one transferase, no close homolog of *fabD* could be detected by Southern hybridization to the *S. coelicolor* chromosome. The gene lies approximately one-third (2.8 Mb) of the length of the chromosome away from the cluster of *act* genes. Immediately downstream of *fabD* is a gene whose product resembles the KS III of *E. coli* FAS (*fabH*; Fig. 6) and which is cotranscribed with *fabD*. Disruption of the *S. coelicolor* chromosome in the region of the *fabD-fabH* junction, which would inactivate the KS (or the product of any gene that is downstream of and cotranscribed with *fabH*), appears to be lethal. This supports the cotranscription of *fabD* and *fabH* and suggests that this operon is involved in primary metabolism, most likely, fatty acid biosynthesis. Recent work (34a) has shown that the gene for another constitutively expressed protein, an ACP from *S. coelicolor*, lies approximately 1 kb downstream of *fabD*. It will be of considerable interest to determine if the remaining genes for the *S. coelicolor* FAS are to be found close to *fabD*.

Earlier reports had suggested that *S. coelicolor* might contain a large multifunctional FAS (13) similar to that of vertebrates (type I [40]) or to the "yeast-type" synthase of *Brevibacterium ammoniagenes* (31). However, it was not possible to purify the presumed FAS to homogeneity. The nucleotide sequence analysis presented here indicates that *fabD* encodes a discrete protein (rather than a fragment generated by adventitious proteolysis of a larger type I FAS), more like the type II system of the kind found in *E. coli* and in *Bacillus subtilis*. (*B. subtilis*, like *S. coelicolor*, produces long-chain fatty acids with a methyl branch at the terminus [8]). It is possible that more than one type of FAS exists in *S. coelicolor*, though the lethal effect of attempted gene disruption suggests that the type II system is

likely to be the only one active in *S. coelicolor* at a level sufficient for cell viability.

Direct genetic evidence proving a link between the FAS and the Act PKS of *S. coelicolor*, by the generation of fatty acid auxotrophs, has not been possible. It is the accumulation of persuasive indirect evidence which helps support the hypothesis that a single malonyltransferase is shared by the two pathways.

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