Multiple Pathways for Triacylglycerol Biosynthesis in *Streptomyces coelicolor*[▽]

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The terminal reaction in triacylglyceride (TAG) biosynthesis is the esterification of diacylglycerol (DAG) with a fatty acid molecule. To study this reaction in *Streptomyces coelicolor*, we analyzed three candidate genes (*sco0958*, *sco1280*, and *sco0123*) whose products significantly resemble the recently identified wax ester synthase/acyl-coenzyme A (CoA):DAG acyltransferase (DGAT) from *Acinetobacter baylyi*. The deletion of either *sco0123* or *sco1280* resulted in no detectable decrease in TAG accumulation. In contrast, the deletion of *sco0958* produced a dramatic reduction in neutral lipid production, whereas the overexpression of this gene yielded a significant increase in de novo TAG biosynthesis. In vitro activity assays showed that *Sco0958* mediates the esterification of DAG using long-chain acyl-CoAs (C₁₄ to C₁₈) as acyl donors. The *K_m* and *V_{max}* values of this enzyme for myristoyl-CoA were 45 μM and 822 nmol mg⁻¹ min⁻¹, respectively. Significantly, the triple mutant strain was not completely devoid of storage lipids, indicating the existence of alternative TAG-biosynthetic routes. We present strong evidence demonstrating that the residual production of TAG in this mutant strain is mediated, at least in part, by an acyl-CoA-dependent pathway, since the triple mutant still exhibited DGAT activity. More importantly, there was substantial phospholipid:DGAT (PDAT) activity in the wild type and in the triple mutant. This is the first time that a PDAT activity has been reported for bacteria, highlighting the extreme metabolic diversity of this industrially important soil microorganism.

Triacylglycerols (TAGs) are the most common lipid-based energy reserves in animals, plants, and eukaryotic microorganisms (3). In bacteria, the most abundant class of neutral lipids are polyhydroxyalkanoic acids (3), but a few examples of substantial TAG accumulation have been reported, mainly in the actinomycetes *Mycobacterium* (4), *Nocardia* (1), *Rhodococcus* (2), and *Streptomyces* (27). Furthermore, the biosynthesis of wax esters (WEs) (oxoesters of long-chain primary fatty alcohols and long-chain fatty acids) has been frequently reported for members of the genus *Acinetobacter* (23). Recently, storage lipid accumulation in the hydrocarbonoclastic marine bacterium *Alcanivorax borkumensis* was reported (19), representing the first example of significant TAG accumulation in a gram-negative prokaryote.

Three different classes of enzymes are known to mediate TAG formation from diacylglycerol (DAG) (22). Acyl-coenzyme A (CoA):DAG acyltransferase (DGAT) catalyzes the acylation of DAG using acyl-CoAs as substrates. In eukaryotes, two DGAT families (DGAT1 and DGAT2) with no sequence resemblance to each other have been identified and characterized. Members of the DGAT1 gene family were found in animals and plants (7, 16, 31), whereas members of the DGAT2 gene family are found in animals (8), plants (5), and *Saccha-*

romyces cerevisiae (32). Acyl-CoA-independent TAG synthesis in yeast and plants is mediated by a phospholipid (PL):DGAT (PDAT) that uses PLs as acyl donors and DAG as an acceptor. This enzymatic activity has been found in plants and yeasts, and some of the genes encoding these enzymes have been identified (9, 25, 35). A third alternative mechanism present in animals and plants is TAG synthesis by a DAG-DAG-transacylase, which uses DAG as an acyl donor and as an acceptor, yielding TAG and monoacylglycerol (21, 36). The gene coding for this putative transacylase has not been identified. It is noteworthy that none of the eukaryotic TAG-synthesizing enzymes significantly resemble any bacterial protein.

Recently, a key enzyme involved in storage lipid biosynthesis in *Acinetobacter baylyi* strain ADP1, the WE synthase/acyl-CoA:DGAT (WS/DGAT) AftA, was characterized (18). This enzyme has a relaxed substrate specificity since it can synthesize WE and TAG by utilizing different-chain-length acyl-CoAs as acyl donors and different-chain-length fatty alcohols or DAG as an acyl acceptor, respectively (18, 37). Remarkably, this novel enzyme does not resemble known acyltransferases involved in TAG or WE biosynthesis in eukaryotes, although it is widely distributed among TAG-accumulating actinomycetes (40).

Only a few members of this novel prokaryotic acyltransferase family have been characterized. In *Mycobacterium tuberculosis*, 4 of 15 proteins homologous to AftA exhibited high DGAT activity but only a very low WE synthase activity when expressed in *Escherichia coli* (10). Furthermore, the disruption of one of these genes, *tgs1*, in *M. tuberculosis* impaired TAG accumulation under several stress conditions (33). In *A. borkumensis* SK2, two AftA homologues were found (AftA1 and

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
<i>S. coelicolor</i>		
M145	Parental strain; SCP1 [−] SCP2 [−]	20
AA0123	sco0123 disruption mutant; Am ^r ; derivative of M145	This study
AA0958	sco0958 disruption mutant; Am ^r ; derivative of M145	This study
AA1280	sco1280 in-frame deletion mutant; derivative of M145	This study
AA21	sco0958 and sco0123 double disruption mutant; Am ^r Hyg ^r ; derivative of AA0958	This study
AA22	sco0958 and sco1280 double disruption mutant; Am ^r ; derivative of AA1280	This study
AA3	sco0958, sco0123, and sco1280 triple disruption mutant; Am ^r Hyg ^r ; derivative of AA22	This study
TR0123	M145 derivative carrying the integrative plasmid pTR0123; Km ^r	This study
TR0958	M145 derivative carrying the integrative plasmid pTR0958; Km ^r	This study
TR1280	M145 derivative carrying the integrative plasmid pTR1280; Km ^r	This study
AA0958C	sco0958 disruption mutant; Am ^r ; carrying the integrative plasmid pTR0958 (Km ^r)	This study
TR285	M145 derivative carrying the integrative plasmid pTR285; Km ^r	This study
<i>E. coli</i>		
DH5 α	<i>E. coli</i> K-12 F [−] lacU169 (ϕ 80lacZ Δ M15) endA1 recA1 hsdR17 deoR supE44 thi-1-l2 gyrA96 relA1	14
BL21 λ (DE3)	<i>E. coli</i> B F [−] ompT r _B [−] m _B [−] (DE3)	38
Rosetta(DE3)	F [−] ompT hsdS _B (r _B [−] m _B [−]) gal dcm (DE3) pRARE2 (Cm ^r)	Novagen
ET 12567	supE44 hsdS20 ara-14 proA2 lacY galK2 rpsL20 xyl-5 ml-1 dam dcm hsdM (Cm ^r)	28
RZ60	malB ⁺ dgk-6 transductant of ES430 (RZ6 donor)	30
RZ6033	RZ60 derivative carrying pBAD33	This study
RZ60123	RZ60 derivative carrying pBAD0123	This study
RZ60958	RZ60 derivative carrying pBAD0958	This study
RZ601280	RZ60 derivative carrying pBAD1280	This study
Plasmids		
pET28a	Phagemid vector for expression of recombinant proteins under the control of strong T7 transcription and translation signals	Novagen
PCR-Blunt	Used for cloning PCR products	Invitrogen
pRT802	Integrative vector based on ϕ BT1 integrase	12
pTR0958	pRT802 derivative plasmid carrying the sco0958-His tag gene under the control of <i>perme</i> *	This study
pTR0123	pRT802 derivative plasmid carrying the sco0958-His tag gene under the control of <i>perme</i> *	This study
pTR1280	pRT802 derivative plasmid carrying the sco0958-His tag gene under the control of <i>perme</i> *	This study
pRT281	pRT802 derivative carrying a 108-bp in-frame deletion of the sco1280 gene	This study
pTR285	pRT802 derivative carrying the <i>ermE</i> * promoter with no gene under its control	This study
pTR257	pET28(a) with an insert carrying the sco0958-His tag fusion gene under the control of strong T7 transcription and translation signals	This study
pTR270	pET28(a) with an insert carrying the sco0123-His tag fusion gene under the control of strong T7 transcription and translation signals	This study
pTR271	pET28(a) with an insert carrying the sco1280-His tag fusion gene under the control of strong T7 transcription and translation signals	This study
pBAD33	Vector for recombinant protein expression under the control of the P _{BAD} promoter; Cm ^r	13
pBAD0123	pBAD33 with an insert carrying the sco0123-His tag fusion gene under the control of the P _{BAD} promoter; Cm ^r	This study
pBAD0958	pBAD33 with an insert carrying the sco0958-His tag fusion gene under the control of the P _{BAD} promoter; Cm ^r	This study
pBAD1280	pBAD33 with an insert carrying the sco1280-His tag fusion gene under the control of the P _{BAD} promoter; Cm ^r	This study

AtfA2), exhibiting robust acyltransferase activity during in vitro tests, but only AtfA1 seems to be involved in vivo in TAG and WE biosynthesis in this organism (19).

Streptomyces coelicolor synthesizes neutral lipid storage compounds during its postexponential phase of growth in submerged liquid culture (27). The lipid bodies are composed mainly of TAG; no WE accumulation has been detected in this microorganism (26, 27). A BLAST search with the amino acid sequence of AftA (WS/DGAT) against the *S. coelicolor* database revealed three sequences with significant similarity to this protein, Sco0958, Sco0123, and Sco1280, with 25.7%, 20.0%, and 22.1% amino acid identities to AftA, respectively. Only the Sco0958 acyltransferase candidate contained a conserved pu-

tative active-site motif (HHXXXDG), which has been proposed to be essential for catalytic activity (19).

The goal of this study was to investigate TAG biosynthesis in *S. coelicolor* and to elucidate the roles of the three putative WS/DGAT proteins Sco0958, Sco0123, and Sco1280 in the accumulation of neutral lipids.

MATERIALS AND METHODS

Strains, media, and growth conditions. The strains and plasmids used in this study are described in Table 1. *E. coli* strains were grown either on solid or in liquid Luria-Bertani medium at 37°C and supplemented when needed with the following antibiotics: 100 μ g ampicillin (Ap) ml^{−1}, 50 μ g kanamycin (Km) ml^{−1}, 20 μ g chloramphenicol (Cm) ml^{−1}, or 100 μ g apramycin (Am) ml^{−1}. *Streptomy-*

TABLE 2. Oligonucleotides used in this study as primers for PCR

Primer	Sequence ^a
0958dn.....	5'-CGGATTGGATCCAGCGCGCGCGTGGATCCAAAC-3'
0958up.....	5'-CATGCGTCGTCTCTTACGAGGCAAGCATATGACT-3'
EZR1.....	5'-ATGCGCTCCATCAAGAAG-3'
Am2.....	5'-CGGCATCGCATTTCTTCGATCC-3'
Am1.....	5'-CCATTGCCCTGCCACCTCACTC-3'
0123up.....	5'-TTTCATATGTCCGCCCCGCCACCGCG-3'
0123dn.....	5'-TTGGATCCACTAGTCAACCCGCTGCACGCTC-3'
1280L1.....	5'-TTTGGCGCCGCTACGCCGTCAGGCGGTG-3'
1280L2.....	5'-TTTGGATCCGAGATAGACGTCCTGTGAC-3'
1280R1.....	5'-TTTGGATCCGGGCAACCGCATGGTAC-3'
1280R2.....	5'-TTTCTAGAGCACTGAACATCCACGAGC-3'
1280up.....	5'-TTTCATATGCGACCCGACTTCGGTAC-3'
1280dn.....	5'-TTGGATCCACTAGTCAGGGCCGCTCCAGCTCC-3'

^a Restriction sites used for cloning purposes are underlined.

ces strains were grown at 30°C on MS agar, R5, YEME, or SMM medium supplemented with glucose (1%, wt/vol) (SMM-Glu) (20). SMM medium is a nitrogen-limiting medium that promotes storage lipid accumulation. The antibiotics Am, hygromycin (Hyg), and Km were added to solid medium at final concentrations of 50, 50, and 200 µg ml⁻¹ and added to liquid medium at final concentrations of 10, 5, and 25 µg ml⁻¹.

Generation of *sco0123*-, *sco0958*-, and *sco1280*-disrupted mutants of *S. coelicolor*. To disrupt *sco0958* and *sco0123*, we used two cosmids from the transposon mutant ordered cosmid library of *S. coelicolor* (15). Cosmids m11-1.G05 and j21-2.BO4, carrying individual Tn5062 insertions in *sco0958* and *sco0123*, respectively, were introduced into *S. coelicolor* M145 by conjugation using *E. coli* ET12567/pUZ8002 as a donor. For each mutant, three independent Am^r Km^s exconjugants were isolated and checked by PCR, verifying that allelic replacement had occurred. The *sco0958* disruption was analyzed with the following primer pairs: 0958dn-ERZ1 (15); Am2-Am1, which amplifies the Tn5062 Am resistance cassette; and 0958up-0958dn (Table 2). To analyze the *sco0123* disruption, we employed the following primer pairs: 0123up-EZR1, Am1-Am2, and 0123up-0123dn (Table 2). For the inactivation of *sco1280*, a 108-bp in-frame deletion was generated by PCR. Two fragments flanking the 108-bp deletion were PCR amplified from *S. coelicolor* M145 genomic DNA using the following primers: 1280L1 and 1280L2 for the left flanking region and 1280R1 and 1280R2 for the right flanking region (Table 2). The two fragments were ligated into a NotI-XbaI fragment of pRT802 (12) to make pTR281. This plasmid was used as a suicide vector for delivery and integration by stepwise double crossover to generate an *S. coelicolor* *sco1280* mutant. Two independent strains carrying the mutated allele of *sco1280* were selected and confirmed by PCR analysis.

To isolate the *sco0958*-*sco0123* double mutant, the Am marker of cosmid j21-2.BO4 was replaced by the Tn5066 Hyg^r cassette from plasmid pQM5066 (P. Dyson, personal communication). This j21-2.BO4 Hyg^r cosmid was introduced into strain AA0958 to obtain the strain AA21. The inactivation of *sco0958* and *sco1280* in *S. coelicolor* was achieved by the conjugal transfer of cosmid m11-1.G05 into strain AA1280 to obtain strain AA22. Triple mutant strain AA3 was generated by the conjugal transfer of cosmid j21-2.BO4 (Hyg^r) into strain AA22.

Lipids and fatty acid analysis. Total lipids of the *S. coelicolor* and *E. coli* strains were extracted twice from lyophilized cell material (1.5 to 3 mg) with chloroform-methanol (2:1, vol/vol). The combined extracts were evaporated and analyzed by thin-layer chromatography (TLC) on Silica Gel 60 F254 plates (0 ± 2 mm; Merck), as described previously (39), using the solvent hexane-diethylether-acetic acid (80:20:1, vol/vol/vol) for TAG analysis. Lipid fractions were visualized by Cu-phosphoric staining. Olive oil was used as the TAG reference substance.

For de novo TAG biosynthesis, *S. coelicolor* mycelium was grown in SMM-Glu to stationary phase and labeled for 3 h with 3 µCi [¹⁴C]acetate (58.9 mCi/mmol; Perkin-Elmer). Lipids were extracted and analyzed by TLC as described above. The radioactivity incorporated into each lipid fraction was quantified using a Storm 860 PhosphorImager (Molecular Dynamics), and the corresponding bands were scraped into vials for scintillation counting and/or quantified using ImageQuant software (version 5.2). Metabolite identity was based on the mobility of known standards.

In order to assess in vivo DGAT activity for the putative acyltransferases under study, *E. coli* RZ60 (30) and RZ60 derivatives (RZ6033, RZ60958, RZ60123, and RZ601280) (Table 1) were grown in LB medium at 37°C to the mid-log phase of growth (optical density at 600 nm of ~0.6 nm), supplemented with 0.2% (vol/vol) L-arabinose and oleic acid (0.15%, vol/vol), and labeled with 3 µCi [¹⁴C]acetate (58.9 mCi/mmol; Perkin-Elmer) for 10 h at 23°C. The cells were collected, and total lipids were extracted and analyzed by TLC as described above.

[¹⁴C]lipid substrates were prepared from radiolabeled mycelium. Lipids were fractionated as described above, visualized by iodine vapor staining, and then isolated from the Silica Gel 60 F254 plates (Merck). In this manner, we obtained endogenous radiolabeled TAG, DAG, free fatty acids (FFAs), and PLs.

Fatty acid analysis of TAG inclusions was done by preparing fatty acid methyl esters by transesterification of isolated lipids bodies with 0.5 M sodium methoxide in methanol and then analyzing them using a Perkin-Elmer Turbo Mass gas chromatograph-mass spectrometer on a capillary column (30-m by 0.25-mm internal diameter) of 100% of dimethylpolysiloxane (PE-1; Perkin-Elmer). Helium at 1 ml min⁻¹ was used as the carrier gas, and the column was programmed at 4°C min⁻¹ from 140°C to 240°C. Branched-chain fatty acids, straight-chain fatty acids, and unsaturated fatty acids used as reference compounds were obtained from Sigma Chemical Co.

Cloning of *sco0958*, *sco1280*, and *sco0123* from *S. coelicolor*. *sco0958*, *sco1280*, and *sco0123* were amplified by PCR from total genomic DNA of *S. coelicolor* M145 using 0958up and 0958dn, 1280up and 1280dn, and 0123up and 0123dn as primers, respectively (Table 2). The resulting PCR products were verified by DNA sequencing and cloned as NdeI-BamHI fragments into the expression vector pET28a, which contain six His codons upstream of the NdeI site, to make pET28a::0958 (pTR257), pET28a::1280 (pTR271), and pET28a::0123 (pTR270), respectively. For complementation and overexpression in *S. coelicolor*, each XbaI-BamHI fragment from the three pET28a derivatives (pTR257, pTR270, and pTR271) plus a NotI-XbaI PCR product containing the *ermE** promoter were cloned into the integrative vector pRT802 to make pTR0958, pTR0123, and pTR1280, placing *sco0958*, *sco0123*, and *sco1280*, respectively, under the *ermE** promoter. As a control, we constructed pTR285 by cloning the *ermE** promoter with the NotI-BamHI fragment of pRT802.

Expression and purification of DGATs. Different *E. coli* host strains [including BL21 λ(DE3) Codon Plus (Stratagene), C41(DE3) (24), C43(DE3) (24), and *E. coli* Rosetta λ(DE3) (Novagen)] and different culture conditions were assayed for the optimization of protein expression. For the three putative DAGTs contained in each of plasmids pTR257, pTR270, and pTR271, the highest levels of soluble protein were obtained with Rosetta λ(DE3). For protein expression, transformants containing each of the plasmids were grown in Luria-Bertani medium at 37°C, induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and incubated for 6 to 8 h at 20 or 25°C. Cells were harvested by centrifugation at 4,000 × g for 20 min at 4°C, washed twice with 100 mM potassium phosphate buffer (pH 7.0) containing 200 mM NaCl-10% glycerol (buffer A), and resuspended in buffer A. Cell disruption was carried out in a French pressure cell at 1,000 MPa in the presence of 1% (vol/vol) protease inhibitor cocktail (Sigma-Aldrich). The lysate was cleared by ultracentrifugation at 35,000 × g for 1 h at 4°C, and the supernatant was applied to a Ni²⁺-nitrilotriacetic acid-agarose affinity column (Qiagen) equilibrated with the same buffer supplemented with 20 mM imidazole. The column was washed, and the His₆-tagged proteins were eluted using buffer A containing 60 to 250 mM imidazole. Fractions were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The fractions containing purified proteins were dialyzed against a solution containing 100 mM potassium phosphate (pH 7.0), 100 mM NaCl, 1 mM EDTA, and 20% (vol/vol) glycerol at 4°C overnight. Proteins were stored at -80°C.

Determination of DGAT and WE synthase activities with purified proteins. Acyltransferase activity assays for the putative WS/DGAT purified recombinant proteins were developed based on the detection of released CoA using 5,5'-dithiobis(2-nitrobenzoic acid), a compound which specifically reacts with thiol groups. The formed adduct was measured spectrophotometrically. DGAT activity was determined in a total volume of 200 µl containing 1.25 mg/ml of bovine serum albumin, 100 mM potassium phosphate buffer (pH 7.0), 5% (vol/vol) ethanol, and 10 mM MgCl₂ (buffer B) plus 20 to 100 µM long-chain acyl-CoA and 2 mM 1,2-dipalmitoyl-*sn*-glycerol. Water-insoluble substrates were dissolved in chloroform, dried under a stream of N₂, rinsed with ether, and evaporated again under a stream of N₂. The mix solution was emulsified by ultrasonication. The reaction was initiated by adding the purified proteins (0.5 µg/µl) to the mixture and incubating the mixture at 30°C. Aliquots (60 µl) were taken at time intervals, followed by the immediate addition of 60 µl 1% (wt/vol) trichloroacetic acid in order to terminate the reaction. After pelleting of the protein by centrifugation (20,000 × g for 10 min), 100 µl of the supernatant was taken and added to 100 µl 5,5'-dithiobis(2-nitrobenzoic acid) (2 mM dissolved in Tris-HCl, pH 8.2) in a microtiter plate. The absorbance was measured at 412 nm ($\epsilon = 13.7$ mM⁻¹ cm⁻¹) using a microplate reader (SpectraMax Plus; Molecular Devices). WE synthase activity was initially assayed using the same reaction mix described above for DGAT but with 1-hexadecanol (2 mM) instead of 1,2-dipalmitoyl-*sn*-glycerol as the acyl acceptor substrate. Since no activity was detected with this reaction mix, we modified the assay conditions by using different buffer systems

(Tris-HCl, HEPES, and phosphates), detergents (Triton and Tween), pHs (pH 6.5 to 8), and incubation temperatures (30°C to 37°C).

Spectrophotometric analysis of actinorhodin. One milliliter whole broth was added to KOH to give a final concentration of 1 M; the solution was mixed vigorously and centrifuged at $4,000 \times g$ for 5 min. The A_{640} of the supernatant was determined and the actinorhodin concentration was calculated using a molar absorption coefficient at 640 nm of 25,320 (6).

Determination of TAG biosynthesis activities in cell extracts. Cell extracts were prepared from stationary-phase cultures of strains M145 and AA3 grown in SMM-Glu. The mycelium was harvested, washed in 100 mM phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride, and resuspended in 1 ml of the same buffer. Finally, the mycelium was disrupted by sonic treatment with an ultrasonic processor (Vibrocell VCX600 sonicator) and centrifuged at $20,000 \times g$ for 30 min. DGAT activity was determined using buffer B with [14 C]DAG and 50 μ M of stearyl-CoA as substrates. The acyl-CoA synthase-dependent DGAT activity was determined using buffer B plus [14 C]FFA, 5 mM ATP, 2 mM reduced CoA (CoASH), and 2 mM 1,2-dipalmitoyl-*sn*-glycerol. PDAT activity was determined using buffer B with [14 C]PLs and 2 mM 1,2-dipalmitoyl-*sn*-glycerol or, alternatively, 0.075 to 0.15 μ g/ μ l phosphatidylethanolamine (PE) (Sigma) and [14 C]DAG as the acyl donor and acyl acceptor substrates, respectively. Water-insoluble substrates were dissolved in chloroform, dried under a stream of N_2 , rinsed with ether, and dried again under N_2 . The mix solution was emulsified by ultrasonication, and the reaction was initiated with 50 to 300 μ g of cell extract. The assay mixtures were incubated at 30°C for 5 h and stopped by extraction with chloroform-methanol (2:1, vol/vol). The reaction products were separated by TLC using the solvent hexane-diethylether-acetic acid (80:20:1, vol/vol/vol). The radioactivity incorporated into each lipid fraction was analyzed using a Storm 860 PhosphorImager (Molecular Dynamics).

RESULTS

Heterologous expression and biochemical characterization of Sco0958, Sco0123, and Sco1280. To test the abilities of the three putative acyltransferases identified in the *S. coelicolor* genome to catalyze TAG or wax biosynthesis, purified recombinant enzymes (His-Sco0123, His-Sco0958, and His-Sco1280) were assayed with various linearly saturated acyl-CoAs as acyl donors and with dipalmitoylglycerol or 1-hexadecanol as the acyl acceptor.

Of the three putative acyltransferases, only His-Sco0958 exhibited DGAT activity (Fig. 1A). The enzyme activity increased linearly with protein levels up to 5 μ g and 90 min of incubation time (data not shown). As shown in Fig. 1A, Sco0958 had a slight preference for $C_{14:0}$ -CoA over $C_{16:0}$ -CoA and $C_{18:0}$ -CoA. At saturating concentrations of dipalmitoylglycerol and at different concentrations of myristoyl-CoA, a hyperbolic saturation curve was obtained, indicating that the DGAT reaction followed Michaelis-Menten kinetics (Fig. 1B). Assuming substrate saturation for 1,2-dipalmitoyl-*sn*-glycerol, the analysis of the plot revealed a K_m value for myristoyl-CoA of 45 μ M and a V_{max} of 822 nmol mg^{-1} min^{-1} . Considering that Sco0958 contains the conserved putative DGAT active-site motif (HHXXXDG), which was previously proposed to be essential for catalytic activity (19), it is not surprising that this protein could catalyze the acylation of dipalmitoylglycerol, but the lack of this activity in Sco0123 and Sco1280 cannot be accounted for simply by the absence of a conserved active site (see Discussion). In order to study the DGAT activities of the three putative acyltransferases of *S. coelicolor* in vivo, we expressed the three open reading frames under study in a *dgk* mutant of *E. coli* (30), which accumulates high concentrations of DAG. We found out that only Sco0958 was able to raise the intracellular levels of TAGs (10-fold higher than that of the parental strain) (Fig. 1C), confirming the DGAT activity of this protein. However, no modification in TAG biosynthesis was observed in the strains containing the other two putative acyl-

FIG. 1. Enzymatic properties of Sco0958. (A) The substrate specificity of purified recombinant His-Sco0958 was assayed using different long-chain acyl-CoAs (myristoyl-CoA [C_{14}], palmitoyl-CoA [C_{16}], and stearyl-CoA [C_{18}]) and 1,2-dipalmitoyl-*sn*-glycerol as substrates as described in Materials and Methods. DGAT activity represents the average value of three independent experiments. (B) Enzyme kinetics of the His-Sco0958 DGAT. Enzyme activity was determined using a spectrophotometric assay. (C) Total lipids extracted from 5 mg of lyophilized [14 C]acetic acid-labeled cultures of the indicated *E. coli* strains were analyzed on silica gel TLC plates and developed in hexane-diethylether-acetic acid (80:20:1, vol/vol/vol). The radiolabeled lipid species were visualized using a PhosphorImager screen, and the bands were identified by their comigration with standards.

transferases (Sco0123 and Sco1280), suggesting either that these two proteins are not functional in this background or that they catalyze a different reaction.

WS activity was initially monitored using the same reaction

conditions as those used for DGAT but with 1-hexadecanol as the acyl acceptor. Since no activity was detected with any of the three purified enzymes, we made several modifications to the reaction mix and assay conditions to try to detect WS activity. The main changes were the use of different buffer systems, the supplementation of the reaction mix with a variety of detergents, and the use of different pH values and incubation temperatures (see Materials and Methods). Again, none of the purified enzymes could catalyze the esterification of 1-hexadecanol under any of the conditions tested, in agreement with the fact that WEs have not been found in *Streptomyces*.

Functional analysis of Sco0123, Sco0958, and Sco1280 in TAG accumulation. The *in vivo* role of *sco0123*, *sco0958*, and *sco1280* in storage lipid synthesis was studied by generating three single mutants, each with a knockout in one of the three genes, as well as a set of double and triple mutant strains (Table 1). Single mutants for *sco0123* and *sco0958* were obtained by disrupting each coding region with a Tn5 derivative transposon (see Materials and Methods). To evaluate the abilities of these mutant strains to synthesize TAG *de novo*, pulse-labeling experiments were carried out using early-stationary-phase mycelium and [14 C]acetic acid. TAG formation was analyzed by total lipid organic solvent extraction and fractionation by normal-phase TLC.

The inactivation of *sco0958* resulted in a strong decrease in [14 C]acetate incorporation into TAG during cultivation on SMM-Glu (Fig. 2A, lane 2). In contrast, the disruption of either *sco0123* or *sco1280* did not show a significant effect on [14 C] incorporation into TAG (Fig. 2A, lanes 3 and 4). Additionally, the double mutants AA21 [*sco0123*::Tn5066(Hyg^r) *sco0958*::Tn5062(Am^r)] and AA22 [*sco0958*::Tn5062(Am^r) Δ *sco1280*] and the triple mutant AA3 [*sco0123*::Tn5066(Hyg^r) *sco0958*::Tn5062(Am^r) Δ *sco1280*] (Fig. 2A, lanes 5, 6, and 7) also showed lower levels of acetate incorporation into TAG, but the levels were similar to those observed for the *sco0958* single mutant, where *de novo* TAG biosynthesis was approximately 30% of the wild-type level.

To confirm that the reduced TAG production phenotype was exclusively related to the absence of *sco0958*, we complemented the AA0958 mutant with the integrative shuttle vector pTR0958 to yield AA0958C and assayed it for *de novo* TAG biosynthesis in parallel with the M145 and the AA0958 mutants. As shown in Fig. 2B, lane 2, the incorporation of labeled acetate into TAG could be restored by the presence of an intact copy of the *sco0958* gene under the control of the constitutive *ermE** promoter, confirming that Sco0958 is directly involved in the biosynthesis of neutral lipids. Additionally, analysis of the absolute TAG content in the wild type and in the mutant by chemical staining of TLC plates demonstrated that cultures of AA0958 grown to stationary phase had a reduced TAG mass in all the media tested, SMM-Glu, YEME, and R5 (Fig. 2C). Under the same conditions, strains AA0123 and AA1280 showed no significant change in the total TAG mass (data not shown). These experiments are in agreement with the *in vitro* studies and suggest that, at least under the conditions tested, the *sco0958* gene product is a functional DGAT involved in TAG biosynthesis and that Sco0123 and Sco1280 either are not functional DGATs or are expressed at low levels under the growth conditions used.

FIG. 2. Characterization of *S. coelicolor* *sco0123*, *sco0958*, and *sco1280* gene disruption strains. (A) Total lipids extracted from 3 mg of lyophilized [14 C]acetic acid-labeled cultures of the indicated *S. coelicolor* strains were analyzed on silica gel TLC plates developed in hexane-diethylether-acetic acid (80:20:1, vol/vol/vol). The radiolabeled lipid species were visualized using a PhosphorImager screen, and the bands were identified by their comigration with standards. FA, fatty acid; MAG, monoacylglycerol. (B) Total lipids extracted from 3 mg of lyophilized [14 C]acetic acid-labeled cultures of the indicated *S. coelicolor* strains were analyzed as described above (A). (C) Total lipid extracts from 1.5 mg of lyophilized mycelium from stationary-phase cultures of M145 and AA0958 grown in different media were fractionated on silica gel TLC plates using hexane-diethylether-acetic acid (80:20:1, vol/vol/vol) and detected by chemical staining with Cu-phosphoric stain.

TABLE 3. Distribution of ¹⁴C in the different lipid forms^a

Genotype	% TAG ± SD	% DAG ± SD	% PL ± SD
TR285	60 ± 2	3.5 ± 0.7	31 ± 3
TR0123	64 ± 1.5	2.9 ± 0.5	28 ± 1.6
TR0958	83.0 ± 0.5	1.8 ± 0.6	14 ± 1
TR1280	72 ± 0.5	2.4 ± 0.6	23 ± 1.5

^a The strains were grown as described in the text and labeled with [¹⁴C]acetate for 3 h. Total lipids extracted from 1.5 mg of lyophilized labeled cells were analyzed on silica gel TLC plates developed in hexane-diethylether-acetic acid (80:20:1, vol/vol/vol). The percentage of each indicated lipid class was determined by quantifying the PhosphorImager data and is presented as a percentage of total incorporation with standard deviations. The data shown are representative of three independent experiments.

Lipid analysis of strains overexpressing *sco0958*, *sco0123*, or *sco1280*. To further analyze the in vivo roles of *sco0123*, *sco1280*, and *sco0958* in TAG biosynthesis, we constructed three M145-derivative strains, each containing an extra copy of one of these genes under the transcriptional control of *permE*^{*}. The strains were named TR1280 (*permE*^{*}-*sco1280*), TR0123 (*permE*^{*}-*sco0123*), and TR0958 (*permE*^{*}-*sco0958*) (Table 1). The successful expression of the individual genes in each strain was confirmed by Western blot analysis using anti-His₆ antibodies (data not shown). The recombinant strains were grown to early stationary phase in SMM-Glu medium, and their abilities to synthesize TAG de novo were measured by pulse-labeling with [¹⁴C]acetic acid. As revealed by TLC assays, no significant alteration in the total lipid profile was observed for these strains, but, as shown in Table 3, the increased expression of *sco0958* led to a significant increase (38%) in label incorporation into TAG compared with that of strain TR285 (M145/pRT285). The rise in de novo TAG synthesis and the decreased DAG and PL labeling are consistent with a rate-limiting role for this enzyme in TAG biosynthesis. Interestingly, strain TR1280 also showed slightly higher levels (20%) of radiolabeled TAG than did strain TR285, suggesting

that *Sco1280* is a functional DGAT and is involved in TAG production, at least when overexpressed.

Altered fatty acid composition of TAG in *S. coelicolor* mutant strains. In order to investigate the consequences of mutations in *sco0958*, *sco0123*, and *sco1280* on the fatty acid composition of TAG, we performed a detailed analysis of the fatty acids present in the lipid bodies purified from the wild type and from the mutant strains (Fig. 3). While the single-disruption *sco0123* strain had a fatty acid composition almost identical to that of the wild type, the *sco1280* mutant accumulated significantly larger amounts of i-C_{14:0} and slightly smaller amounts of n-C_{16:0} than did the wild type. Reduced levels of i-C_{14:0} and a-C_{15:0} and higher levels of i-C_{16:0} and a-C_{17:0} were found in the strain lacking the *sco0958* gene. Moreover, double mutant strain AA21 showed the same altered fatty acid profile as the *sco0958*-deficient strain (data not shown). The alteration of the fatty acid profiles in *S. coelicolor* strains lacking *Sco0958* could reflect the substrate preference of this enzyme.

Relationship between TAG formation and antibiotic production. Since TAG mobilization was proposed to be a possible carbon source for antibiotic biosynthesis in *S. coelicolor* (27), and considering that strain AA0958 showed a significant reduction in TAG content, we analyzed the effect of the *sco0958* mutation on the production of actinorhodin (Act), whose biosynthesis occurs through the condensation of one acetyl-CoA and seven malonyl-CoA molecules. M145 and AA0958 were grown in the minimal SMM-Glu medium and in rich YEME medium, and Act production was monitored throughout growth. As shown in Fig. 4A, when grown in SMM-Glu, the *sco0958* mutant showed higher levels of Act synthesis after 60 h of growth than did M145. However, after 140 h, only a slight difference (14%) in the production levels of this acetate-derived antibiotic was observed between these two strains. In YEME medium (Fig. 4B), instead, the timing of Act production was not affected, although we did find an increase in Act accumulation of nearly 20% in AA0958 compared to the

FIG. 3. Fatty acid composition of TAG isolated from wild-type M145 and the different mutant strains. Cells were cultivated to the stationary phase of growth in SMM-Glu medium. Total lipid extracts from lyophilized mycelium were fractionated by preparative TLC and developed in hexane-diethylether-acetic acid (80:20:1, vol/vol/vol), and TAG was purified prior to subsection to gas chromatography analysis.

FIG. 4. Growth phenotype and Act production of wild-type M145 and AA0958. The strains were grown in SMM-Glu (A) and in YEME medium (B). Open and filled circles represent the growth curves for M145 and AA0958, respectively. Open and filled squares represent the levels of Act production in M145 and AA0958, respectively. Each time point represents data from three independent experiments and the standard deviation $\pm 0.5\%$. OD, optical density.

wild type after 140 h of growth. The reduced but still substantial amounts of TAG present in the AA0958 mycelium cultivated in either medium could explain the lack of a more obvious phenotype regarding antibiotic production.

Alternative pathways for TAG biosynthesis in *S. coelicolor*.

The persistence of considerable intracellular levels of TAG in triple mutant strain AA3 clearly indicated the presence of additional pathways for the biosynthesis of neutral lipids in *S. coelicolor*. Therefore, we set out to assay for DGAT, DAG: DAG acyltransferase, and PDAT activities in cell extracts of wild-type M145 and the AA3 mutant.

DGAT activity was assayed in cell extracts prepared from stationary-phase cultures of M145 and AA3 grown in SMM-Glu; different long-chain acyl-CoAs (C_{14} to C_{18}) and [^{14}C]DAG were used as acyl donors and the acyl acceptor, respectively. After the reaction, total lipids were extracted, fractionated by TLC, and visualized by phosphorimaging. As shown in Fig. 5A, cell extracts of M145 and AA3 revealed significant radiolabeled incorporation into TAG (lanes 2 and 5). Remarkably, cell extracts from the triple mutant still contained considerable DGAT activity levels. On the other hand, none of these extracts showed radiolabeled incorporation into neutral lipids during incubation with [^{14}C]DAG in the pres-

ence of dipalmitoylglycerol (Fig. 5A, lanes 3 and 6), suggesting that a DAG:DAG acyltransferase activity could not be detected under these assay conditions and therefore was not responsible for TAG biosynthesis. Incubation of the crude extracts with [^{14}C]FFA and dipalmitoylglycerol revealed that these substrates could be incorporated into TAG only in the presence of exogenous ATP and CoASH, suggesting the need for previous acyl-CoA biosynthesis (Fig. 5B, lanes 2 and 5). This activity depended on the DAG concentration and on the amount of crude extract used (Fig. 5C). In addition, levels of TAG biosynthesis were higher in the M145 cell extract than in those measured in the AA3 mutant, confirming that FFA had to be activated to its acyl-CoA derivative before entering the TAG biosynthetic pathway. All these results are consistent with the notion that the residual TAG in strain AA3 is made, at least in part, by an acyl-CoA-dependent reaction.

When cell extracts of M145 and AA3 were supplied with [^{14}C]PLs and unlabeled dipalmitoylglycerol, substantial biosynthesis of radiolabeled TAG was detected in both reactions (radioactive FFA and DAG were also detected, probably as the result of PL degradation) (Fig. 5D). The amount of labeled TAG depended on both the concentration of crude extract employed and the amount of DAG present in the reaction mix (Fig. 4E). Further incubation of M145 cell extracts with [^{14}C]DAG and PE also demonstrated a significant formation of radiolabeled TAG (Fig. 5F). Thus, as shown in Fig. 5D to F, we concluded that in this microorganism, PLs could act as acyl donors for TAG biosynthesis and that this reaction could be catalyzed by a PDAT enzyme. Levels of TAG biosynthesis through the PDAT pathway were comparable in both cell extracts, indicating that the simultaneous mutation of the three genes under study did not affect this novel PDAT activity in *S. coelicolor*.

DISCUSSION

The distinctive reaction associated with TAG biosynthesis comprises the acylation of DAG using different acyl donors; all the other reactions from *sn*-glycerol 3-phosphate to DAG share steps involved in phospholipid biosynthesis (34, 40). The most prominent enzymes catalyzing this step are DGATs, which use acyl-CoAs as acyl donors and DAG as an acceptor (34). In prokaryotes, dual-function acyltransferases that mediate both WE and TAG formation have been identified. The first member of this novel enzyme family, AftA from *A. baylyi*, has been characterized at both the genetic and biochemical levels (18, 37) and has been extensively used for homology searching in different bacterial genomes (40). Using this approach, we found three *S. coelicolor* genes, sco0123, sco0958, and sco1280, which could potentially encode acyltransferase enzymes involved in TAG biosynthesis.

In an attempt to determine if the *S. coelicolor* AftA homologues could catalyze TAG biosynthesis, the three genes encoding the putative WS/DGATs were expressed in *E. coli*, and their purified products were assayed for DGAT and WS activities. Only one of the three purified enzymes, Sco0958, exhibited robust acyltransferase activity in vitro (Fig. 1), while the other two, Sco0123 and Sco1280, showed no detectable DGAT activity with any of the acyl-CoAs tested, either as pure proteins or as membrane-associated proteins obtained from *E. coli*

FIG. 5. Conversion of ¹⁴C-labeled substrates into the neutral lipid fraction by crude extracts of M145 and AA3. Shown is an autoradiogram of total lipid fractions separated on TLC plates developed in hexane-diethylether-acetic acid (80:20:1, vol/vol/vol) after incubation (overnight at 30°C) with crude extracts obtained from M145 and AA3 in the presence of different acyl acceptor and diverse labeled acyl donors (as indicated). Each enzymatic assay was performed as described in Materials and Methods. (A) DGAT activity for M145 and AA3. (B and C) Acyl-CoA synthase-dependent DGAT enzymatic activity in M145 and AA3. (D and E) PDAT enzyme activity in M145 and AA3. CE, crude extracts; FA, fatty acid.

strains overexpressing sco0123 or sco1280 (data not shown). As mentioned above, the highly conserved acyltransferase domain HHXXXDG found in AftA and in nonribosomal peptide synthases (40) is also present in Sco0958 but differs at the first histidine in Sco0123 and Sco1280 (40). It is likely that the altered active-site motif of the last two proteins could influence their ability to catalyze the esterification of acyl-CoAs to dipalmitin, although there are examples of DGATs in *M. tuberculosis* and *Mycobacterium smegmatis* where this motif is either incomplete or absent, and the proteins are still active (10). A

broader and more extensive characterization of the DGAT/WS enzymes will be needed to clearly understand the role of this motif in enzyme activity. Furthermore, none of the three purified proteins could mediate the esterification of 1-hexadecanol using straight-chain acyl-CoAs as donors. Likewise, most of the *M. tuberculosis* AftA-like proteins exhibited either very low or undetectable levels of WS activity (10). To assess the physiological role of the sco0123, sco0958, and sco1280 loci in TAG biosynthesis, we constructed and analyzed single- and multiple-disruption mutants. Disruption of sco0958

provoked a drastic reduction in both de novo TAG biosynthesis and the total TAG content under all conditions tested. Consistently, the amount of TAG was significantly reduced in the doubly disrupted strains (sco0123::Hyg, sco0958::Am, and sco0958::Am Δ sco1280) lacking sco0958, indicating that neither of the other putative DGATs Sco0123 and Sco1280 could substitute for Sco0958 in its role in TAG synthesis. Moreover, the absence of Sco0958 also produced a clear effect on the composition of the fatty acids esterified to TAG (Fig. 3), implying a preference of this enzyme for terminally branched-chain C₁₄-C₁₅ acyl-CoAs.

Regarding the functional role of Sco1280, the in vitro and in vivo results were slightly more difficult to interpret. The lack of enzyme activity in vitro is in agreement with the absence of changes in TAG content in the AA1280 mutant; however, the overexpression of sco1280 under the *ermE* promoter in M145 provoked a 20% increase in de novo TAG biosynthesis. Moreover, although the sco1280 deletion did not alter the total TAG mass, the absence of the Sco1280 protein resulted in a twofold reduction in C_{16:0} and a 2.2-fold increase in i-C_{14:0}. All these results suggest that Sco1280 is an active DGAT but with a minor contribution to *S. coelicolor* TAG synthesis. The lack of in vitro activity for Sco1280 is intriguing; however, we could raise some plausible explanations for this observation, such as the utilization of a very narrow set of acyl-CoAs by this enzyme as substrates or the need for interactions with a specialized oil droplet surface protein.

The lack of DGAT/WS activity in vitro and the absence of an obvious phenotype of the sco0123 mutant strain suggested that this gene might play a completely different physiological role in *S. coelicolor*. Incidentally, an examination of the genomic neighborhoods of the sco0123 locus revealed that it is located upstream of a putative biosynthetic eicosapentanoic acid cluster (sco0124 to sco0129), suggesting that this enzyme either may have an entirely different set of substrates or might be involved in the production of an unknown fatty acid ester.

The presence of substantial TAG accumulation in the triple-knockout mutant was compelling evidence for the existence of one or more alternative TAG-biosynthetic pathways in *S. coelicolor* that are not dependent on an AftA-like enzyme. Similar observations were recently made for *Acinetobacter baylyi* and the gram-negative bacterium *Alcalinovoxax borkumensis* (18, 19); however, the nature of the remaining TAG-biosynthetic pathway was not characterized in either study. Our studies using cell extracts of wild-type and triple mutant strains identified at least two remaining activities for the last step of TAG biosynthesis in this organism. In vitro acyltransferase activity assays, using stearyl-CoA and [¹⁴C]DAG as substrates, detected significant radiolabeled incorporation into TAG in the AA3 crude extracts, indicating the existence of substantial residual DGAT activity in the triple-knockout strain. The *S. coelicolor* genome contains numerous putative acyltransferase genes of unknown function, but none of these putative proteins exhibit reasonable homology to any of the DGAT/WS enzymes already characterized (19, 33, 37). Therefore, the remaining DGAT activity present in AA3 identifies an alternative DGAT isoenzyme(s) responsible, at least in part, for TAG biosynthesis.

Our in vitro assays also unmasked a previously uncharacterized TAG-biosynthetic pathway in bacteria, which uses phos-

pholipids instead of acyl-CoA as acyl donors (9, 25, 35). The PDAT activity was detected using either [¹⁴C]phospholipids and dipalmitin or PE and [¹⁴C]DAG as substrates for the transacylation reaction; importantly, PE is the main class of phospholipids in *Streptomyces* (17). The PDAT activities in yeast and plants were previously described, and the *Saccharomyces cerevisiae* Lro1 enzyme (9, 25) was biochemically characterized. Lro1 resembles the well-studied enzyme lecithin: cholesterol acyltransferase, which catalyzes sterol ester synthesis in blood plasma (11). The absence of sequence similarity to any of the PDATs characterized in the *S. coelicolor* genome suggests that a previously unknown class of PDAT enzymes exists in this microorganism. The extent to which this novel activity contributes to TAG formation under different growth conditions remains to be investigated. However, since PDAT activity utilizes phospholipids as one of its substrates, it is conceivable that in addition to its role in TAG synthesis, PDAT might function to modulate membrane lipid composition (9).

Neutral lipids such as TAG or WE are accumulated as depots of energy and carbon in actively growing cells and as a sink for FFAs, diminishing their potential damaging effects on cell membranes. In *Streptomyces*, it was proposed previously that TAG accumulation might serve as the source of acetate units for the biosynthesis of polyketide compounds once the carbon source from the medium is exhausted (27). Our physiological studies, however, suggest the opposite. For instance, the AA0958 mutant, which contains only 30% of wild-type TAG levels, produced almost 20% more Act than did M145 (Fig. 4). This suggests that TAG competes with Act for precursors and is not itself readily available as a source of acetate units for Act biosynthesis, at least under the conditions that we have examined. Incidentally, studies carried out previously by Plaskitt and Chater demonstrated a higher content of TAG in a *bldA* mutant, which is known to be unable to produce Act and undecylprodigiosin (29).

Our studies reveal once again the extraordinary complexity of *S. coelicolor* metabolism and shed light on the different enzymes and pathways that determine the final levels of TAG biosynthesis in this bacterium. The redundancy of the last step of TAG biosynthesis might well be related to the complex life cycle of these microorganisms on surface growth, which may impose different requirements for storage metabolism, including its interactions with membrane biosynthesis. However, a mutant completely depleted of TAG will be required before we can really understand the physiological role of neutral fats in these microorganisms.

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