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Analysis of neutral lipid biosynthesis in *Streptomyces avermitilis* MA-4680 and characterization of an acyltransferase involved herein

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Abstract The physiology of lipid production in *Streptomyces avermitilis* MA-4680 with regard to the fatty acid composition of the accumulated lipids and their cellular distribution was analyzed. Cells were able to accumulate about ten to 30 lipid granules with diameters between 100 and 500 nm filling about 70–80% of the cell cytoplasm. Gas chromatography/mass spectrometry analyses of total cellular lipids and from isolated triacylglycerols (TAG) confirmed a similar fatty acid composition with a large portion of *iso*- and *anteiso*-methyl-branched fatty acids. De novo biosynthesis of wax esters (WE) appeared only during cocultivation on glucose and hexadecanol as carbon source. Homology alignments with the wax ester synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT; AtfA) from *Acinetobacter baylyi* strain ADP1 yielded one open reading frame in the genome databases of *S. avermitilis* MA-4680 referred to as SAV7256 with 25.3% homology. The highly conserved HHAxxDG active site motif found in AtfA, which is present in SAV7256, as well as the similar hydrophobicity profiles of AtfA and SAV7256 indicate a similar structure and function of both proteins. High acyl-CoA:diacylglycerol acyltransferase activity (DGAT; 143 pmol (mg min)$^{-1}$) but low wax ester synthase activity (WS; 1.3 pmol (mg min)$^{-1}$) were detected in crude extracts of *S. avermitilis*, which were consistent with the high TAG and negligible WE content of the cells. This indicates that TAG accumulation in *S. avermitilis* MA-4680 is mediated by the classical acyl-CoA-dependent DGAT pathway. Heterologous expression experiments in recombinant *Escherichia coli* BL21(DE3) demonstrated both WS and DGAT enzyme activity of SAV7256. Furthermore, substrate specificities of the acyltransferase SAV7256 will be discussed.

Keywords *Streptomyces* · Avermectin · Triacylglycerol · Wax ester · Acyl-CoA:diacylglycerol acyltransferase

Introduction

Triacylglycerols (TAG) and wax esters (WE) are besides the predominant polyhydroxyalkanoic acids widespread hydrophobic intracellular storage compounds for carbon and energy in bacteria (Steinbüchel 1996). TAG and WE inclusions (oxoesters of long-chain fatty acids with glycerol or primary fatty alcohols, respectively) are formed by the wax ester synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT; the *atfA* gene product) at the cytoplasmic surface of the plasma membrane (Wältermann and Steinbüchel 2005). WS/DGAT was recently characterized as a novel class of acyl-CoA-dependent acyltransferase that exhibits no sequence homology to any known acyltransferase involved in storage lipid synthesis in eukaryotes. The promiscuous WS/DGAT (AtfA) is the key enzyme for biosynthesis of TAG and WE in *Acinetobacter baylyi* strain ADP1 (ATCC 33305) and probably also in most other lipid accumulating bacteria (Kalscheuer and Steinbüchel 2003; Wältermann et al. 2007). AtfA is an unspecific acyltransferase which simultaneously synthesizes WE and TAG by utilizing long-chain fatty alcohols and diacylglycerols as acceptor substrates and fatty acid CoA thioesters (acyl-CoA) as acyl donor substrates (Kalscheuer and Steinbüchel 2003). The biochemical analyses showed that AtfA of *A.*

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baylyi strain ADP1 exhibits a very broad substrate range, accepting primary, secondary, cyclic, and phenolic acyl alcohols, diols and dithiols as well as mono- and diacylglycerols (Kalscheuer et al. 2003; Stöveken et al. 2005; Uthoff et al. 2005). Depending on the metabolism of the microorganism, the media composition, or genetic engineering, diverse types of fatty acid esters can be synthesized in vivo by expressing WS/DGAT in different recombinant hosts leading to new biological, technological, or therapeutic applications (Stöveken and Steinbüchel 2008).

Numerous homologous proteins especially in actinomycetes were identified by an alignment of the amino acid sequence of WS/DGAT with the publicly accessible sequence databases. A general overview on occurrence and characteristics of putative WS/DGAT proteins in bacteria is given by Wältermann et al. (2007). It is remarkable that a large group of up to 14 homologous WS/DGAT proteins is present in most actinomycetes. In *Mycobacterium tuberculosis*, 15 homologous proteins were discovered suggesting an important function of these proteins (Daniel et al. 2004). The capability of TAG accumulation is also widely distributed among members of the genus *Streptomyces*. Streptomycetes are filamentous Gram-positive bacteria with unusual complex cell morphology. The complete genome sequences of *Streptomyces coelicolor* A3(2) (DSM40783) and *Streptomyces avermitilis* MA-4680 (DSM 46492) were published recently by Bentley et al. (2002) and Ikeda et al. (2003). In contrast to the large number of homologs found in mycobacteria, only three WS/DGAT homologous proteins in *S. coelicolor* A3(2) and a single WS/DGAT homologous protein in *S. avermitilis* MA-4680 were identified (Kalscheuer et al. 2003). The accumulation of TAG as insoluble cytoplasmic granules in the cell up to 5% of the cellular dry weight (CDW) has already been described for *S. coelicolor* A3(2) and *Streptomyces lividans* 66. It has been proposed that the accumulated TAGs might act as a carbon source for the biosynthesis of antibiotics (polyketide compounds) derived from acetyl-CoA/malonyl-CoA precursors (Olukoshi and Packter 1994; Packter and Olukoshi 1995). However, this hypothesis was disproven by the recently published report on the TAG biosynthesis pathway of *S. coelicolor* M145 (Arabolaza et al. 2008). The opposite behavior was observed in a disruption mutant, which stored about 70% less TAG but produced almost 20% more actinorhodin compared to the wild type, and although all three WS/DGAT homologous genes of *S. coelicolor* M145 were deleted, a decreased but still residual storage lipid accumulation remained in the triple mutant, indicating the presence of alternative neutral lipid biosynthesis pathways. Similar observations of residual lipid production were also made for WS/DGAT deletion mutants of *A. baylyi* strain ADP1 (Kalscheuer and Steinbüchel 2003), *Alcanivorax borkumensis* SK2 (Kalscheuer et al. 2007), and *Rhodococcus opacus* strain PD630 (Alvarez et al. 2008).

In this study, we investigated the role of WS/DGAT homologous proteins in the neutral lipid metabolism of actinomycetes with regard to the question whether TAG biosynthesis in *Streptomyces* sp. occurs exclusively by this new type of acyltransferases, by so far undiscovered DGAT isoenzymes, or even by alternative acyl-CoA-independent biosynthesis pathways.

Materials and methods

Bacterial strains and plasmids Bacterial strains and plasmids used in this study are listed in Table 1.

Media and cultivation conditions S. avermitilis MA-4680 was grown in modified mineral salts medium (MSM; Schlegel et al. 1961) containing 0.05% (*w*/*v*) yeast extract, 1.0% (*w*/*v*) glucose as carbon source, and 0.1% (*w*/*v*) NH_4Cl. Cells were cultivated for 72 h at 30°C. For induction of storage lipid biosynthesis, a reduced concentration of 0.01% (*w*/*v*) NH_4Cl was used ("storage conditions"). For preparation of protoplasts, cells were cultivated in yeast extract–malt extract medium (Hopwood et al. 2000); after protoplast transformation, R5 medium was used for regeneration (Okanishi et al. 1974) and CASO (Merck) medium for selection. After conjugational transfer, cells were regenerated on mannitol soy flour medium (Hobbs et al. 1989) and selected on modified MSM. Recombinant strains of *Escherichia coli* were cultivated at 37°C on Luria–Bertani medium (Sambrook et al. 1989) containing 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce expression of the protein encoded by pET23a(+). For preparation of electrocompetent cells of *E. coli* DY378, cells were cultivated at 30°C and induced for 15 min at 42°C (Yu et al. 2000). All liquid cultures were incubated aerobically in baffled Erlenmeyer flasks on an orbital shaker and were inoculated (1%, *v*/*v*) from a well-grown preculture. Solid media contained 1.8% (*w*/*v*) agar–agar. If required, antibiotics were added at the following concentrations: 75 μg ampicillin (Ap) ml^{-1}, 50 μg apramycin (Apra) ml^{-1}, 25 μg chloramphenicol (Cm) ml^{-1}, 50 μg kanamycin (Km) ml^{-1}, 12 μg thiostreptone (Tsr) ml^{-1}, and 20 or 10 μg nalidixic acid (Ndx) ml^{-1} for *E. coli* or *Streptomyces*, respectively.

Electron microscopy studies For transmission electron microscopy (TEM), cells of *S. avermitilis* MA-4680 were cultivated in modified MSM under storage conditions, harvested, and washed twice with 125 mM sodium phosphate buffer (pH 7.4). Cells were fixed with 4.6% (*w*/*v*) paraformaldehyde and postfixed with 1% (*w*/*v*) osmium tetroxide in 0.1

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Streptomyces avermitilis MA-4680	Wild type	DSM 46492
Streptomyces coelicolor A3(2)	Wild type	DSM40783
Acinetobacter baylyi ADP1	Wild type	ATCC 33305
Escherichia coli BL21(DE3)	F^- *ompT hsdSB* (rB^-, mB^-), *gal*, *dcm* (DE3)	Novagen
Escherichia coli ET12567	*dam-13*::Tn*9*, *dcm-6*, *hsdM*, *hsdR*, *recF-143*, *zjj-201*::Tn*10*, *galK-2*, *galT-22*, *ara-14*, *lacY-1*, *xy-l5*, *leuB-6*, *thi-1*, *tonA-31, rpsL-136*, *hisG-4*, *tsx-78*, *mtli*, *glnV-44*, F^-, Cm^r	MacNeil et al. 1992
Escherichia coli DY378	W3110 cI857 (*cro-bioA*)	Yu et al. 2000
Plasmids		
pGEM-T Easy	Ap^r, *lacPOZ'*	Promega
pET23a(+)	Ap^r, T7 promoter-based expression vector	Novagen
pGM446	*ori* pSG5, *rep* pSG5, *ori* pUC, *oriT*, *cos*, Tsr^r, $Apra^r$	Rose and Steinbüchel 2002
ΔpGM446	Deleted *Bam*HI/*Eco*RV fragment, *oriT*, *ori* pUC, *cos*, $Apra^r$, Streptomycetes suicide vector	This study
pUZ8002	*tra* genes of plasmid RP4, *oriT*, Km^r	Sia et al. 1996
pET23a::*atfA*	*atfA* from *Acinetobacter baylyi* strain ADP1, Ap^r	Stöveken et al. 2005
pET23a::SAV7256	SAV7256 gene as a 1.4-kbp *Hin*dIII/*Xho*I fragment, Ap^r	This study
pGEM-T Easy::SAV7256ΩKm	deleted 0.4-kbp *Dra*II fragment in SAV7256, *Dra*II Km-cassette cloned into singular *Dra*II site of SAV7256 and finally ligated as 1.9-kbp fragment into pGEM-T Easy via *Eco*RI, Ap^r, Km^r	This study
pKU402::CL_222_G04	*ori*, *cos*, Ap^r, containing via *Bam*HI site a 44.1-kbp DNA fragment of the chromosome of *S. avermitilis* MA-4680 including SAV7256	http://avermitilis.ls.kitasato-u.ac.jp/
pKU402::CL_222_G04::SAV7256ΩKm	Intact SAV7256 in CL_222_G04 replaced by linear PCR fragment SAV7256ΩKm via double crossover event (λ recombination system), 44.5-kbp fragment, Ap^r, Km^r	This study
ΔpGM446::CL_222_G04::SAV7256ΩKm	CL_222_G04::SAV7256ΩKm cloned via *Eco*RI, $Apra^r$, Km^r, Streptomycetes suicide vector	This study

M phosphate buffered saline (pH 7.3). For thin sectioning, samples were embedded in Lowicryl K4M (Polyscience); sections were made with an Ultracut S apparatus (LEICA Mikroskopie und Systeme) using a diamond knife and were then positioned on a 200-mesh copper grid. Imaging was performed with an H-500 TEM (Hitachi) in the bright-field mode at 75 kV acceleration voltage at room temperature. Micrographs were taken with an Agfa-Gevaert 23 D 56 film.

Lipid analysis Thin layer chromatography (TLC) of lipid extracts from whole cells was done as described previously (Kalscheuer and Steinbüchel 2003). Cetylpalmitate or oleyl oleate and triolein were used as reference substances for WEs and TAGs, respectively. Fatty acid analysis of whole cells and purified TAGs was done by gas chromatography (GC) and gas chromatography/mass spectrometry analysis (MS) according to Kalscheuer et al. (2004). Fatty acid methyl esters were analyzed on an Agilent 6850 GC (Agilent Technologies) equipped with a BP21 capillary column (50 m×0.22 mm, film thickness 250 nm; SGE) and a flame ionization detector (Agilent Technologies).

Preparation of crude cell extracts Cultivated cells of *Streptomyces* sp. were centrifugated (20 min, 6,238×*g*, 4°C) and washed with 125 mM sodium phosphate buffer (pH7.4). Cells were resuspended in the same buffer, incubated with 1 mg lysozyme ml^{-1} for 45 min on ice, and passaged through a French pressure cell (1,000 MPa) to obtain crude extracts. Cells of *E. coli* were grown to an $OD_{600\ nm}$ of 0.5 before IPTG was added to a final concentration of 1 mM. Induced cultures were incubated for further 3 h, harvested by centrifugation (3,345×*g*, 20 min, 4°C), washed twice, and resuspended in sodium phosphate buffer. Cells were disrupted by ultrasonification and protein concentrations were determined by the Bradford (1976) method.

Determination of enzyme activities WS/DGAT activity was determined in a total volume of 250 µl containing 12.5 µg bovine serum albumin ml^{-1}, 4.72 µM [1-^{14}C]palmitoyl-CoA (specific activity 1.961 Bq $pmol^{-1}$; Hartmann Radiochemicals), 125 mM sodium phosphate buffer (pH 7.4), and different acceptor molecules at a concentration of 3.75 mM. 1-Hexadecanol and 1,2-dipalmitoylglycerol were used as

standard substrates for assaying WS and DGAT activity, respectively. Alternatively, a range of various-chain-length linear alcohols (1-butanol, 1-decanol, 1-hexadecanol, 1-tetracosanol), various acyl-glycerols (1,2- and 1,3-dipalmitoylglycerol, 1-, 2-, and 3-monopalmitoylglycerol) as well as cyclic and aromatic alcohols (cyclohexanol, 2-cyclohexylethanol, phenylethanol) were tested as acyl acceptors. The enzyme reaction was performed as described previously by Stöveken et al. (2005). The reaction products were separated by TLC using different solvent systems for the separation of linear, cyclic, and aromatic WEs, monoacylglycerols, DAGs, or TAGs. After separation of lipids and staining of the TLC plates with iodine vapor, spots corresponding to the reaction products were scraped from the plates, and radioactivity was measured by scintillation counting.

Cloning of AtfA homologous gene from S. avermitilis MA-4680 The gene encoding the AtfA homologous protein SAV7256 of *S. avermitilis* MA-4680 was amplified from total genomic DNA by polymerase chain reaction (PCR). The resulting product was cloned as *Hin*dIII-*Xho*I fragment into the expression vector pET23a(+) colinear to the T7 promoter to obtain pET23a::SAV7256. This construct was then transformed into *E. coli* BL21(DE3).

SDS-PAGE and immunoblot analysis Protein content was analyzed according to Bradford (1976). Protein samples were resuspended in gel loading buffer and separated in 12.5% (*w*/*v*) sodium dodecyl sulfate (SDS) polyacrylamide gels as described by Laemmli (1970). Proteins in the polyacrylamide gels were visualized using Coomassie brilliant blue R250 according to Weber and Osborn (1969). For immunological detection of WS/DGAT homologous proteins, the proteins in the polyacrylamide gels were transferred onto a nitrocellulose membrane (Towbin et al. 1979). Proteins on the membrane were analyzed immunologically employing 2 μg polyclonal rabbit anti-WS/DGAT IgGs ml^{-1} (Stöveken et al. 2005). IgGs were visualized on immunoblots using goat antirabbit IgG–alkaline phosphatase conjugates (diluted 1:20,000 in 10 mM potassium phosphate buffer, pH 7.2; 0.5%, *w*/*v*, NaCl; 0.1%, *v*/*v*, Tween 20).

PCR and RT-PCR analysis PCR was performed as described by Sambrook et al. (1989) using Plantinum *Pfx* polymerase (GIBCO BRL Life Technologies). For RT-PCR, the OneStep RT-PCR kit (Qiagen) was used according to the manufacturer's instructions employing oligonucleotide primers listed in Table 2.

DNA sequencing, sequence analysis, and alignments DNA sequencing was performed by employing IRD800-labeled primers, a model 4000L DNA sequencer (Li-COR), and a SequiTherm EXCEL II Long-Read L-C kit-LC (Epicentre) according to the manufacturer's instructions. Obtained sequences were analyzed with Genamics Expression software (http://genamics.com/expression/index.htm). Sequence comparisons and alignments were performed using the BLAST online service available on National Center for Biotechnology Information (NCBI), and alignments were done with BioEdit (Hall 1998) and ClustalW (Thompson et al. 1994).

Transcription analysis of SAV7256 in S. avermitilis MA-4680 Cells were cultivated separately on modified MSM under growth as well as under storage conditions. After harvesting, the cells were resuspended in Tris–ethylenediaminetetraacetic acid buffer (pH 8.0) containing 1 mg lysozyme ml^{-1} and incubated for 20 min at 37°C. The isolation of RNA was performed by using the RNeasy RNA purification kit (Qiagen) according to the manufacturer's protocol. Additionally, 0.1 mm glass beads (Carl Roth) and a Silamat® S5 (Ivoclar Vivadent) were used for extraction of RNA. RT-PCR was performed using 1 μg RNA as template. A negative control for DNA contamination was done by adding the RNA template after the reverse transcriptase step.

Isolation, manipulation, and transfer of DNA Isolation of total DNA of *Streptomyces* cells was performed according to Marmur (1961). The isolation of plasmid and cosmid DNA was conducted according to the method of Birnboim and Doly (1979). DNA manipulations and other standard molecular biology techniques were performed according to Sambrook et al. (1989). DNA was introduced into *Streptomyces* cells either by protoplast transformation according to Hopwood et al. (2000) or by conjugation using *E. coli* ET12567 with the helper plasmid pUZ8002. Competent cells of *E. coli* were prepared and transformed by the $CaCl_2$ procedure as described by Hanahan (1983). Electrocompetent cells of *E. coli* DY378 were prepared according to the method of Yu et al. (2000).

Gene disruption and knock out constructs For inactivation of the SAV7256 gene, an internal 0.4-kbp *Dra*II fragment of SAV7256 was deleted and replaced by a ΩKm cassette yielding pGEM-T Easy::SAV7256ΩKm A 44.1-kbp fragment containing the gene SAV7256 from cosmid CL_222_G04 (obtained from the genomic library of *S. avermitilis* MA-4680; The Kitasato Institute, Japan, http://avermitilis.ls.kitasato-u.ac.jp/) was cloned into the singular *Bam*HI site of vector pKU402. For gene disruption mutagenesis, the intact gene SAV7256 in CL_222_G04 was replaced by the disrupted gene SAV7256ΩKm yielding pKU402::CL_222_G04::SAV7256ΩKm using the *E. coli* strain DY378 which is suitable for integration of short

Table 2 Oligonucleotides used in this study as primers for PCR, diagnostic PCR, RT-PCR, and sequencing

Restriction sites used for cloning purposes are underlined. Start and stop codons are indicated in bold

Primer	Sequence
SAV7256_5′-*Hin*dIII	5′-AAAAAGCTTAAGGAGGTAGCAAGCCA**ATG**ACCTCTGACCT-3′
SAV7256_3′-*Xho*I	5′-TTTCTCGAG**TCA**GGGGCCGCAGACAGTGAGCA-3′
ΩKm^r(*Dra*II)_5′-end	5′-TTTGGGCCCTCCGGAATTGCCAGCTGGGGCGCC-3′
ΩKm^r(*Dra*II)_3′-end	5′-TTTGGGCCCCTCAGAAGAACTCGTCAAGAAGGC-3′
T7 promoter	5′-TAATACGACTCACTATAGGG-3′
T7 terminator	5′-GCTAGTTATTGCTCAGCGG-3′

linear DNA (at least 35–50 bp) by homologous recombination (Yu et al. 2000).

Amino acid sequence data The primary amino acid sequences of proteins investigated in this study are deposited under following accession numbers: AtfA (AF529086) from *A. baylyi* strain ADP1, SAV7256 (NP_828432) from *S. avermitilis* MA-4680, SCO0958 (NP_625255), SCO0123 (NP_624462), and SCO1280 (NP_625567) from *S. coelicolor* A3(2).

Results

Identification of a WS/DGAT homologous protein in *S. avermitilis* MA-4680 and primary structure comparison

Since it is known that streptomycetes are capable of accumulating lipids in the cytoplasm (Olukoshi and Packter 1994; Packter and Olukoshi 1995), the genome sequence of *S. avermitilis* strain MA-4680 was searched for genes coding for homologs of the recently detected promiscuous acyltransferase of *A. baylyi* strain ADP1 (Kalscheuer and Steinbüchel 2003). AtfA is present in *A. baylyi* and supposably in most other bacteria that are capable of synthesizing TAG and WE (Wältermann et al. 2007).

Based on the amino acid sequence of AtfA, one homologous protein in *S. avermitilis* MA-4680 referred to as SAV7256 was identified. Up to now, no biological function could be assigned to this hypothetical protein. SAV7256 showed an amino acid homology of 25.3% to AtfA. The three WS/DGAT homologs SCO0958, SCO0123, and SCO1280 of strain *S. coelicolor* A3(2), which were investigated recently (Arabolaza et al. 2008), showed amino acid homologies ranging from 17.7% to 26.6% to AtfA (Table 3). SAV7256 from *S. avermitilis* MA-4680 and SCO0958 from *S. coelicolor* A3(2) exhibited the highest homology (81%) to each other, as well as to AtfA (25.3% and 26.6%, respectively). The other two homologs SCO0123 and SCO1280 from *S. coelicolor* A3 (2) exhibited only weak homologies to each other (29%), as well as to SAV7256 and SCO0958 (Table 4).

The HHAxxDG active site motif found in AtfA of *A. baylyi* strain ADP1, which has been shown to be essential for catalytic activity (Stöveken et al. 2009), was strictly conserved in the acyltransferase homolog of *S. avermitilis* MA-4680 (SAV7256) and in one AtfA homolog of *S. coelicolor* A3(2) (SCO0958; Fig. 1). In the two other acyltransferase homologs of *S. coelicolor* A3(2), this motif was less conserved ("HAxxDG" in SCO0123 and "HxxxDG" in SCO1280).

Although the amino acid sequences of SCO0958 and SAV7256 showed a relative weak overall homology to AtfA, several highly conserved amino acid residues were identified (Fig. 1). Furthermore, the proteins exhibited highly similar hydrophobicity profiles (Fig. 2), which might be indicative for a similar structure and function of SAV7256, SCO0958, and AtfA.

Occurrence and composition of intracellular storage lipid inclusions

S. avermitilis MA-4680 was cultivated in modified MSM medium containing 0.01% (*w*/*v*) NH_4Cl and 1% (*w*/*v*) glucose, which are conditions known to induce TAG and WE biosynthesis in other bacteria. Transmission electron micrographs of thin sections revealed a large number of inclusions in the cells occupying the major part of the cytoplasm (about 70–80%; Fig. 3a, b). Each cell contained between ten and 30 well-separated granules with diameters between 100 and 500 nm exhibiting no coalescence. All granules stained positive with the lipophilic fluorescent dye Nile red (Spiekermann et al. 1999), which indicates the accumulation of abundant amounts of hydrophobic compounds in the granules (Fig. 3a).

Thin layer chromatography of whole cell extracts revealed the presence of TAG, while WE were not visible. Combined GC/MS analyses of the methyl esters obtained from whole cells, cultivated under storage conditions, as well as from purified TAG, confirmed a similar fatty acid composition with a high amount of *iso*- and *anteiso*-methyl-branched fatty acids. The cellular fatty acid composition is listed in Table 5, showing that *anteiso*-pentadecanoic acid was the predominant constituent. Only when *S. avermitilis* was cultivated on a mixture of 1% (*w*/*v*) glucose and

Table 3 Genes for WS/DGAT homologs detected in the genomes of *S. avermitilis* MA-4680 and *S. coelicolor* A3(2)

Species	Protein	Length (aa)	Molecular weight (kDa)	Homology (% identical aa)	Accession number
A. baylyi strain ADP1	AtfA	458	51.8	100	AF529086
S. avermitilis MA-4680	SAV7256	447	47.4	25.3	NP_828432
S. coelicolor A3(2)	SCO0958	446	47.1	26.6	NP_625255
	SCO0123	448	48.6	21.0	NP_624462
	SCO1280	413	44.5	17.7	NP_625567

Accession numbers in the NCBI protein database are shown. Identities to the AtfA from *A. baylyi* strain ADP1 were based on BLAST search results and calculated for full-length sequences

aa amino acids

0.3% (*v*/*v*) hexadecanol, very small amounts of WE (probably less than 5%) could be detected in total lipid extracts by TLC (data not shown). These WE represented a mixture of hexadecanoic acid and various alcohols. GC/MS analysis of TAG, accumulated under the same conditions, revealed only small differences in the fatty acid spectrum compared to those TAG obtained under storage conditions. Cultivation on hexadecanol or hexadecane alone did not result in the accumulation of any WE or TAG (data not shown). Evidence for the presence of poly(3-hydroxybutyrate) or other polyhydroxyalkanoates in the cells was not obtained. Therefore, the inclusions visible in Fig. 3 consist most probably of TAG.

Enzymatic detection of DGAT and WS activities and transcription analysis in *S. avermitilis* MA-4680

In crude extracts obtained from cells of *S. avermitilis* MA-4680 grown under storage conditions, high DGAT activity was detected (specific activity 143 pmol (mg min)$^{-1}$). In contrast, very low WS activity was measured (specific activity 1.3 pmol (mg min)$^{-1}$). DGAT activity increased in the cells during cultivation under storage conditions, which correlated with the accumulation of TAG (Fig. 4). These data are consistent with the high TAG and nondetectable WE content of the cells. The increase of DGAT activity under lipid storage conditions indicates the induction of gene expression of one or more DGAT enzymes. To examine whether SAV7256 from *S. avermitilis* MA-4680 is expressed under different cultivation conditions, RT-PCR was performed. For this, cells were grown in MSM containing 1% (*w*/*v*) glucose and 0.1% (*w*/*v*) NH_4Cl or 0.01% (*w*/*v*) NH_4Cl, allowing accumulation of TAGs. Transcripts of the SAV7256 gene were detected under both tested cultivation conditions. Thus, SAV7256 is potentially responsible for the basal TAG accumulation in strain *S. avermitilis* MA-4680 (Fig. 5).

Next, it was examined whether the existing polyclonal antibodies produced against AtfA from *A. baylyi* strain ADP1 (Stöveken et al. 2005) can be used to detect SAV7256 heterologously expressed in *E. coli*. The Western blot analysis on SAV7256 from recombinant *E. coli* as well as on crude extracts of *S. avermitilis* grown under normal growth as well as under lipid storage conditions resulted in the immunological detection of SAV7256 based on a cross reaction with the AtfA antibodies (data not shown). The expression level of SAV7256 isolated from crude extracts was similar under both cultivation conditions and in agreement with the results of transcription analysis.

To provide a final proof that SAV7256 was the only or at least major enzyme responsible for the storage lipid accumulation in the investigated *S. avermitilis* strain, we tried to generate a knock out mutant defective in the gene. However, even after several attempts with different variations of the constructs and transfer methods, a SAV7256 knock out mutant could not be obtained.

Table 4 Homologies between different WS/DGAT homologous proteins

	AtfA	SAV7256	SCO0958	SCO0123	SCO1280
AtfA	100	25	27	21	18
SAV7256	25	100	81	24	23
SCO0958	27	81	100	26	28
SCO0123	21	24	26	100	29
SCO1280	18	23	28	29	100

Data were obtained using BLAST search. Identities were calculated for full-length sequences and are presented as identical amino acids (%). AtfA derived from *A. baylyi* ADP1, SAV7256 from *S. avermitilis* MA-4680, and SCO0958, SCO0123, and SCO1280 from *S. coelicolor* A3(2)

Heterologous expression in *E. coli* and biochemical characterization of the acyltransferase

The identified WS/DGAT homologous gene SAV7256 was heterologously expressed in *E. coli* BL21(DE3) as described in the "Materials and methods" section. Protein

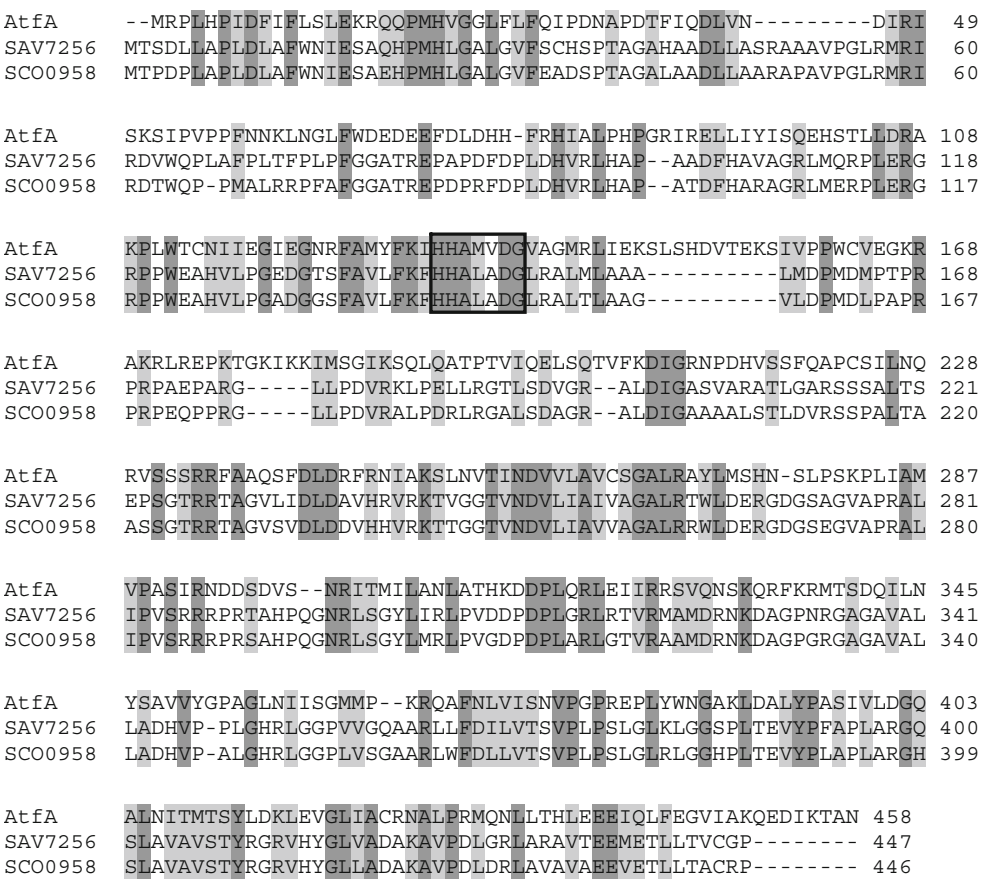

Fig. 1 Amino acid sequence alignment of the WS/DGAT enzymes AtfA from *A. baylyi* strain ADP1, SAV7256 from *S. avermitilis* MA-4680, and SCO0958 from *S. coelicolor* A3 (2). Analysis was done using the ClustalW program (Thompson et al. 1994). Residues identical in all sequences are shaded in *dark gray*; similar residues are shaded in *light gray*. A putative active site motif is *boxed*

expression was confirmed by SDS-polyacrylamide gel electrophoresis, and enzyme activity was determined by a radiometric assay using linearly saturated long-chain $C_{16:0}$-CoA ([1-^{14}C]palmitoyl-CoA) as acyl donor. If not stated otherwise below, 1-hexadecanol and 1,2-dipalmitoylglycerol were used as standard substrates for assaying WS and DGAT activity, respectively. SAV7256 surprisingly showed a high WS but low DGAT activity of 71.3 pmol (mg min)$^{-1}$ and 16.8 pmol (mg min)$^{-1}$, respectively. These values corresponded to 46% and 31%, respectively, of the activities of AtfA from *A. baylyi* ADP1 (positive control) and therefore, SAV7256 could be designated as an acyltranferase. As expected, the negative vector control *E. coli* BL21(DE3) (pET23a) proved low WS (0.27 pmol (mg min)$^{-1}$) as well as low DGAT (0.21 pmol (mg min)$^{-1}$) activity. It is noteworthy that the ratio of WS to DGAT activity is in stark contrast to enzyme activities detected in crude extract of *S. avermitilis* MA-4680 grown under storage conditions with high DGAT and low WS activity.

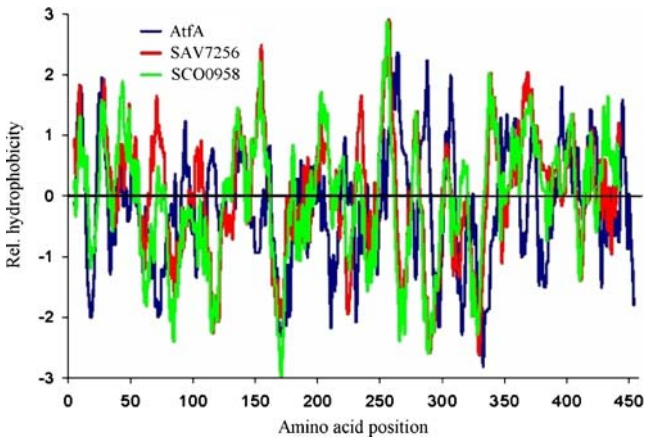

Fig. 2 Hydrophobicity plot of the WS/DGAT enzymes SAV7256 and SCO0958 compared to AtfA. Calculation was done as described by Kyte and Doolittle (1982). Hydrophobic regions show values >0

The substrate specificity of the *S. avermitilis* acyltransferase SAV7256 was measured in crude extracts of *E. coli* BL21(DE3) expressing pET23a::SAV7256. Since *E. coli* does not possess endogenous WS and DGAT activity, the results of such measurements allowed a general view on the substrate range of the enzyme from *S. avermitilis*, regardless of its purity. Using [1-^{14}C]palmitoyl-CoA as acyl donor and 1-hexadecanol as reference acyl acceptor (100%), the highest activity of SAV7256 was measured with the medium-chain-length alcohol 1-decanol (110.9%) as substrate. The short-chain-length C_4 alcohol as well as the long-chain-length C_{24} alcohol was utilized by this enzyme with lower activity. Lower activities were also measured for the acyl-glycerols 1,2- and 1,3-dipalmitoylglycerol (6.6 and 1.5%, respectively) as well as with 1-, 2-, or 3-monopalmitoylglycerol (5.8%, 14.3%, and 12.7%, respectively). The substrate range of

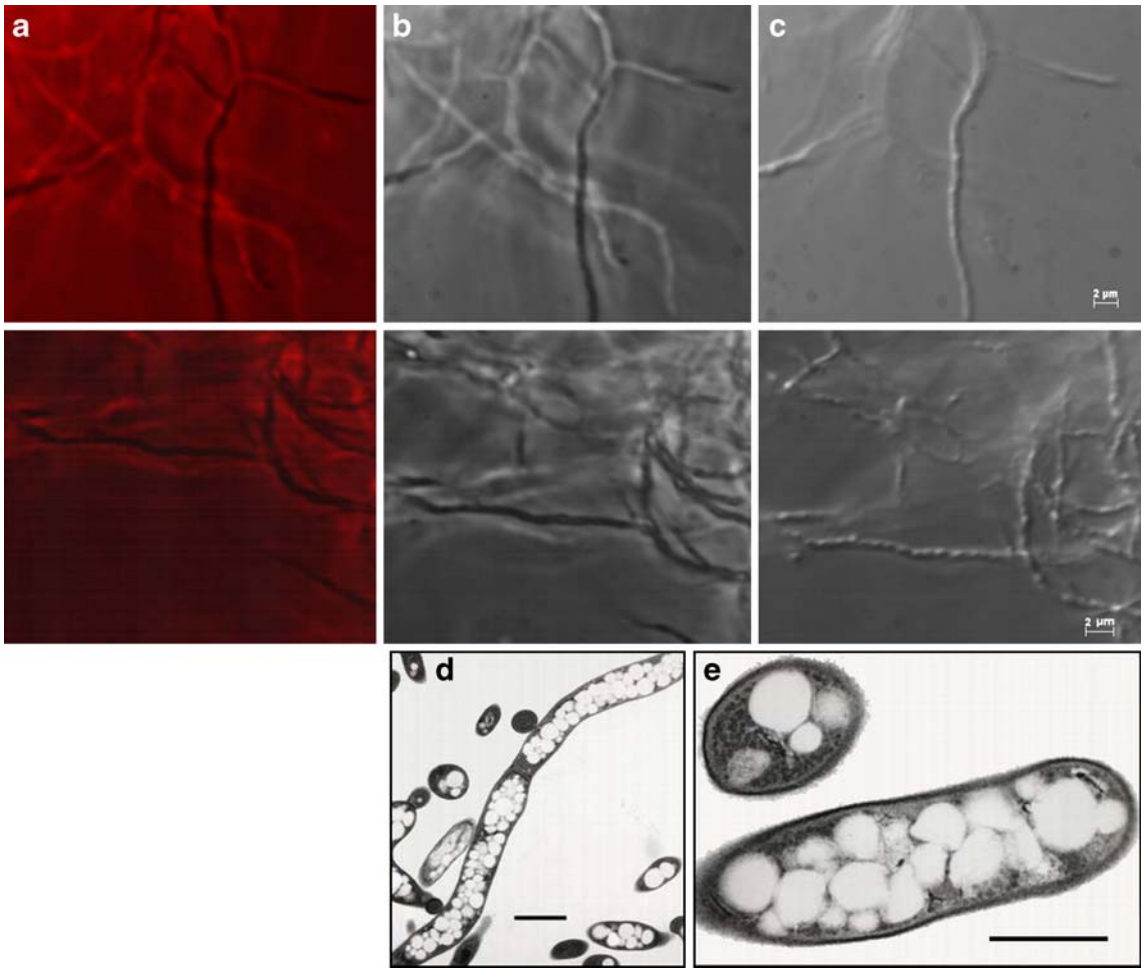

Fig. 3 Structure and accumulation of neutral lipid inclusions in *S. avermitilis* MA-4680 grown under growth (*upper line*) and storage (*lower lines*) conditions for 72 h at 30°C. Fluorescence images after staining with Nile red (**a**) as described in "Materials and methods" section, corresponding phase-contrast (**b**), and differential interference contrast images (**c**). Ultrathin sections were analyzed by TEM (**d**, **e**) as described in "Materials and methods" section. Scale bars: **a**–**c** 2, **d** 1, and **e** 0.5 µm

SAV7256 was further tested for cyclic and aromatic alcohols. 2-Cyclohexylethanol was acylated with relative high specific activity (24.9%), whereas the activity with cyclohexanol was rather low (1.2%). Phenylethanol, the only aromatic compound tested in this study, could also serve as substrate for SAV7256. In summary, enzyme SAV7256 from *S. avermitilis* showed a preference for medium-chain-length linear fatty alcohols and exhibited generally a narrower substrate range than that of AtfA of *A. baylyi* strain ADP1 (Stöveken et al. 2005; Table 6).

Discussion

Detailed investigations on the TAG accumulation in *S. coelicolor* were already done by Olukoshi and Packter

Table 5 Fatty acid composition of whole cells of *S. avermitilis* MA-4680 after cultivation of cells in modified MSM containing 0.01% (*w*/*v*) NH_4Cl and 1% (*w*/*v*) glucose for 72 h

Fatty acid	*iso*-C14:0	*iso*-C15:0	*anteiso*-C15:0	C15:0	*iso*-C16:0	C16:0	*iso*-C16:1	*anteiso*-C17:0
S. avermitilis MA-4680	5.1	7.2	26.1	6.3	14.4	15.5	1.2	5.5

Fatty acid analysis of 3–5-mg lyophilized cells was done by GC after derivatization to fatty acid methyl esters by sulfuric acid-catalyzed methanolysis. Values are percentages of total fatty acids

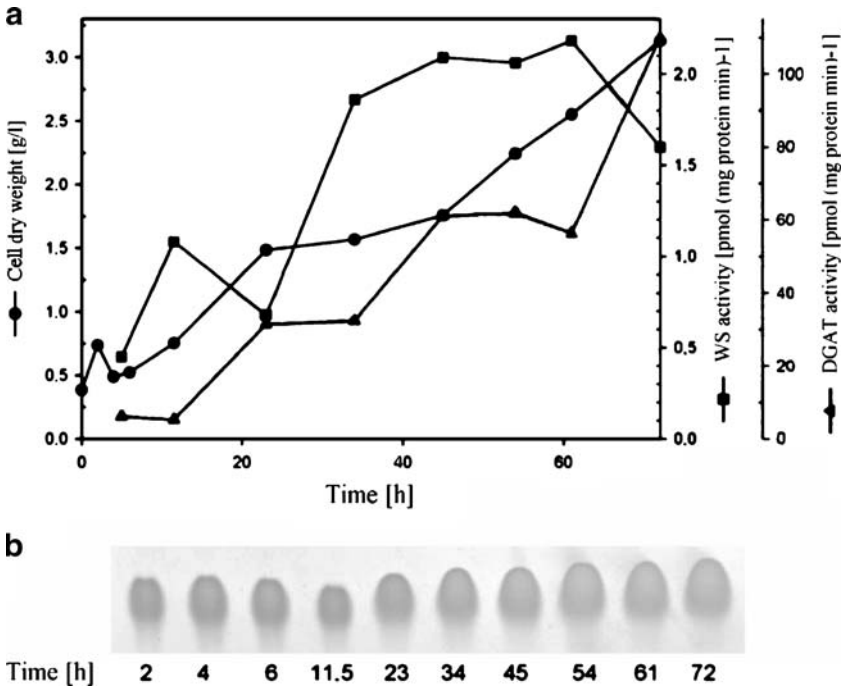

Fig. 4 Time courses of WS and DGAT activities related to cell density (CDW; **a**) and TAG production (**b**) in *S. avermitilis* MA-4680. Cells were cultivated under storage conditions for 72 h at 30°C. TAGs were extracted each from 1.5-mg lyophilized cell material with 200 µl chloroform/methanol (1:1, *v*/*v*) for 30 min. The supernatant was subjected to silica TLC using the solvent system hexane/diethylether/acetic acid (80:20:1, *v*/*v*/*v*) for TAG analysis

(1994), Shim et al. (1997), and Arabolaza et al. (2008), whereas investigations in *S. avermitilis* MA-4680 were only carried out on whole cell lipid extracts so far (Cropp et al. 2000). In this study, we have identified one protein in *S. avermitilis* MA-4680, which is homologous to the promiscuous AtfA, the key enzyme for neutral lipid biosynthesis in *A. baylyi* ADP1. The high degree of homology between SAV7256 from *S. avermitilis* and SCO0958 from *S. coelicolor* A3(2), their verified acyltransferase activities, as well as the same organization of the gene loci, strongly suggest a similar function and orthologous origin. Moreover, the low homologies between the *S. coelicolor* genes SCO0958, SCO0123, and SCO1280 (Arabolaza et al. 2008) indicate that they may have been developed through gene duplication in the region of the chromosome arm leading to paralogous genes.

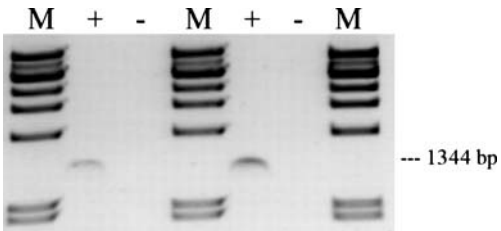

Fig. 5 Agarose gel electrophoresis (1%, *w*/*v*) of RT-PCR products of SAV7256 in *S. avermitilis* MA-4680. Expression of SAV7256 was analyzed in samples derived from cells cultivated under growth as well as under lipid storage conditions for 72 h at 30°C. The *left side* shows the 1,344 bp transcript of SAV7256 amplified from RNA from *S. avermitilis* under growth conditions, the *right side* under lipid storage conditions accordingly. *M PstI*-digested λ DNA, + RT-PCR assay, − control for DNA contamination

In this study, the accumulated storage lipids of *S. avermitilis* were biochemically analyzed in detail. The results shown in Fig. 3 are in accordance with the amount, structure, and size of accumulated TAG grana (76–87% of the CDW, 100–600 nm) described for *R. opacus* strain PD630 (Alvarez et al. 1996). TAG and WE inclusions in lipid accumulating bacteria are usually organized in form of spherical lipophilic grana (Wältermann and Steinbüchel 2005); it was recently that also rectangular, disk-shape, needle-like, or irregularly shaped forms were observed in the *n*-hexadecane-utilizing bacterium *Acinetobacter* sp. strain M-1 and in the hydrocarbonoclastic marine bacterium *A. borkumensis* SK2 (Ishige et al. 2002; Kalscheuer et al. 2007). Such untypical forms are probably caused by a biased fatty acid composition of the lipids with a high content of saturated fatty acids. A pecularity of streptomycetes is the presence of *iso*- and *anteiso*-methyl-branched fatty acids, representing more than 60% of total cellular fatty acids which are rarely found in other bacteria. The fatty acid profile of TAG purified from *S. avermitilis* MA-4680 was identical to that of total cells, which is in accordance with observations in *S. coelicolor* M145 (Arabolaza et al. 2008). Furthermore, TAG from *S. avermitilis* contained a substantial portion of odd chain fatty acids. This was similar to TAG from *R. opacus*, whereas odd chain fatty acids normally occur only in small quantities in bacteria (Fulco 1983; Alvarez et al. 1996; Kalscheuer et al. 2001).

Lack of de novo biosynthesis of WE despite the presence of endogenous WS activity and the intracellular availability of acyl-CoA for TAG biosynthesis indicate that,

Table 6 Specific activities of SAV7256 from *S. avermitilis* MA-4680 and AtfA from *A. baylyi* strain ADP1 in use with different acyl acceptors

Acyl acceptor	Rel. specific activity (in % of hexadecanol control)	
	SAV7256	AtfA
1-Butanol	2.15±0.61	46.60±8.13
1-Decanol	110.90±6.66	48.00±5.60
1-Hexadecanol	100.00±11.36	100.00±11.01
1-Tetracosanol	4.09±0.67	18.41±6.24
Cyclohexanol	1.15±0.02	32.00±6.20
2-Cyclohexylethanol	24.93±3.77	44.80±14.40
Phenylethanol	11.12±2.34	99.00±32.80
1,2-Dipalmitoylglycerol	6.57±0.46	77.44±3.67
1,3-Dipalmitoylglycerol	1.46±0.14	43.31±8.07
1-Monopalmitoylglycerol[a]	5.84±0.32	158.18±7.34
2-Monopalmitoylglycerol[a]	14.26±0.55	80.74±8.44
3-Monopalmitoylglycerol[a]	12.65±4.17	110.47±4.04

Measurements were done on crude extracts of *E. coli* BL21(DE3) (pET23a::SAV7256) as described in "Materials and methods" section. Values are means ± standard deviation of two independent experiments. The data from *A. baylyi* strain ADP1 were obtained from purified AtfA as reported in Stöveken et al. (2005)

[a] Both possible products, 1,2- and 1,3- or 1,2- and 2,3- as well as 1,3- and 2,3-dipalmitoylglycerol were each added for determination of activity

unlike *A. baylyi*, *S. avermitilis* is unable to reduce acyl-CoA to fatty alcohols and therefore could not form WEs. This is in agreement with reports on *Mycobacterium smegmatis* (Kalscheuer and Steinbüchel 2003). The presence of an acyl-CoA-reductase (*acr1*) required for fatty alcohol formation was described for *A. baylyi* ADP1 (Reiser and Somerville 1997); however, no homologous gene to *acr1* was identified in the genome of *S. avermitilis* MA-4680.

In crude extracts of *S. avermitilis*, the increase in DGAT activity correlated with TAG accumulation. Thus, it can be assumed that TAG accumulation in this strain occurred via the classical acyl-CoA-dependent DGAT pathway. Due to the contrary ratio of DGAT and WS activities found in crude extracts of *S. avermitilis* and recombinant *E. coli* with high DGAT activity in cells of *S. avermitilis* and only relatively low DGAT activity of SAV7256, it is likely that SAV7256 is only in part responsible for TAG synthesis in this strain. However, neither further WS/DGAT homologous genes nor genes homologous to the eukaryotic acyltransferases DGAT1 and DGAT2 (Cases et al. 1998, 2001) could be identified in the genome of *S. avermitilis* MA-4680. Therefore, this suggests the presence of yet unidentified DGAT isoforms, as it has been proposed for some other TAG accumulating bacteria (Kalscheuer et al. 2007). The definite role of SAV7256 for neutral lipid production in *S. avermitilis* can only be determined by a knockout mutant which became unfortunately not available.

Due to the inability of *S. avermitilis* to synthesize WE de novo, synthesis of linear WE may not be the main function

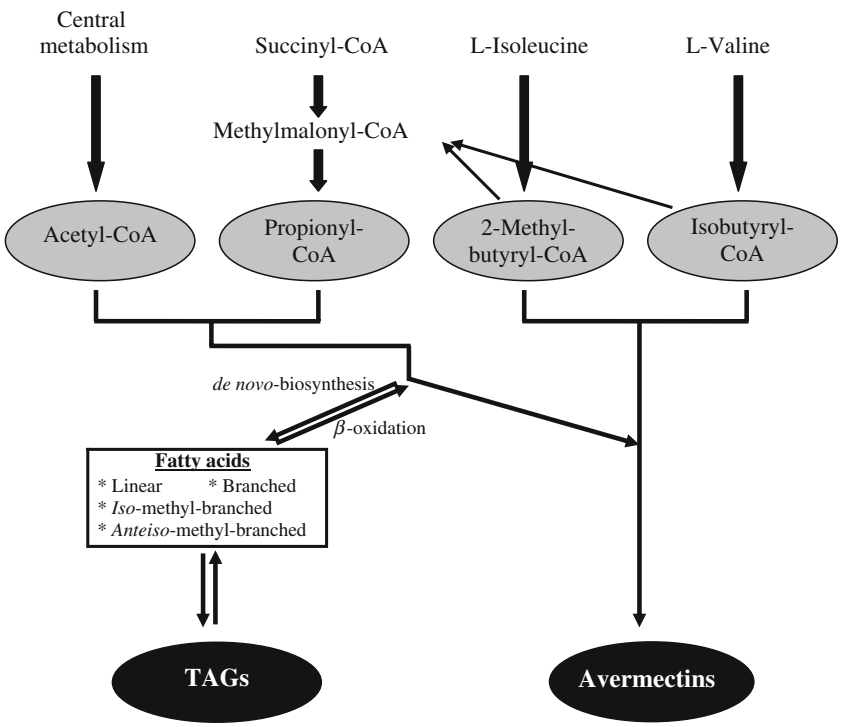

Fig. 6 Correlation between biosynthesis of TAGs and avermectins in *S. avermitilis* MA-4680. TAG and avermectin production share the same precursor substrates. In contrast to avermectins, TAGs can be mobilized and the released fatty acids can be degraded by β-oxidation

of SAV7256. The involvement of this acyltransferase in synthesis of yet unidentified fatty acid ester substances, which could not be detected by the applied methods, is also conceivable.

Arabolaza et al. (2008) recently generated disruption mutants of *S. coelicolor* M145 defective in the three AtfA homologs SCO0958, SCO0123, and SCO1280. Deletion of SCO0958 led to a reduced de novo TAG biosynthesis of approximately 70%. Mutations in the genes for the other two proteins showed no influence on neutral lipid accumulation. In addition, overexpression of SCO0958 resulted in increased TAG levels showing that this enzyme is the major acyltransferase for TAG accumulation in *S. coelicolor* M145. Remarkably, even in the triple mutant of strain M145, a reduced but substantial level of TAG accumulation remained, indicating the existence of an alternative non-WS/DGAT-dependent TAG biosynthesis pathway. It seems obvious that not WS/DGAT implemented production of storage lipids is mediated, at least in part, by an acyl-CoA-dependent pathway. Moreover, a substantial phospholipid: diacylglycerol acyltransferase (PDAT) activity was described for *S. coelicolor* (Arabolaza et al. 2008). In this acyl-CoA-independent reaction phospholipids act as acyl donors and DAG as acyl acceptor. However, it is unclear to what extend PDAT, which mediates TAG synthesis in plants and yeast (Dahlqvist et al. 2000), contributes to TAG production in *S. coelicolor*. PDAT activity was not determined in *S. avermitilis* in the current work. Trace amounts of TAGs in an *atfA* deletion mutant of *A. baylyi* strain ADP1 (Kalscheuer and Steinbüchel 2003) and in an *atfA1* and *atfA2* double deletion mutant of *A. borkumensis* SK2 (Kalscheuer et al. 2007), as well as in the *atf1* disruption mutant of *R. opacus* strain PD630 (Alvarez et al. 2008), support the speculation about alternative pathways.

S. avermitilis MA-4680 is a bacterium with high industrial potential due to the production of the secondary metabolite avermectin, which is used in agriculture, veterinary, and human medicine as antiparasitic agent (Yoon et al. 2004). During fatty acid de novo synthesis, the central intermediates acetyl-CoA and propionyl-CoA are used for the synthesis of linear and branched fatty acids. Avermectin biosynthesis additionally requires the precursors isobutyryl-CoA and 2-methylbutyryl-CoA, which are synthesized by the degradation of L-isoleucine and L-valine (Ikeda et al. 1999). Based on these pathways, TAG and avermectin biosynthesis share the same metabolic intermediates (Fig. 6). A quantitative correlation between avermectin and lipid production was observed (Novák et al. 1990). In contrast to avermectins, the fatty acid moieties of TAG can be mobilized by intracellular lipases and catabolized through β-oxidation into the precursor substrates, which in turn can serve for biosynthesis of avermectins (Fig. 6). In literature, it is discussed that TAG accumulated in the early stationary phase of *Streptomyces* represented a depot for the synthesis of secondary metabolites during the late stationary phase (Olukoshi and Packter 1994; Shim et al. 1997). Thus, the accumulated TAG in *S. avermitilis* may fulfill the function of a mobilizable depot for precursors of the avermectin biosynthesis, or TAG and avermectin synthesis are in competition to the limited supply of the same precursors. The hypothesis of a competition between TAG and antibiotic production was corroborated by a TAG-deficient mutant of *S. coelicolor*. It was found that the SCO0958 gene disruption mutant stored approximately 70% less TAGs but produced 20% more actinorhodin in comparison to the wild-type strain (Arabolaza et al. 2008). These facts support the assumption of competition for the same precursors. In the future, the generation of a SAV7256 mutant of *S. avermitilis* MA-4680 will be necessary to understand and explain the competition behavior between TAGs and avermectins, particularly with regard to industrial applications. A better understanding of the lipid metabolism in *S. avermitilis* and its engineering may lead to improved production strains.

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