

# Cloning and characterization of a gene involved in triacylglycerol biosynthesis and identification of additional homologous genes in the oleaginous bacterium *Rhodococcus opacus* PD630

Adrian F. Alvarez,<sup>2</sup> Héctor M. Alvarez,<sup>1</sup> Rainer Kalscheuer,<sup>2</sup>  
Marc Wältermann<sup>2</sup> and Alexander Steinbüchel<sup>2</sup>

## Correspondence

Alexander Steinbüchel  
steinbu@uni-muenster.de

<sup>1</sup>Centro Regional de Investigación y Desarrollo Científico Tecnológico (CRIDECIT), Facultad de Ciencias Naturales, Universidad Nacional de la Patagonia San Juan Bosco, Comodoro Rivadavia, Argentina

<sup>2</sup>Institut für Molekulare Mikrobiologie und Biotechnologie der Westfälischen Wilhelms-Universität Münster, Corrensstr. 3, 48149 Münster, Germany

The oleaginous bacterium *Rhodococcus opacus* strain PD630 serves as a model organism to investigate the metabolism of storage triacylglycerols (TAGs) in bacteria. The key enzyme catalysing the last step of TAG biosynthesis in bacteria is a promiscuous acyltransferase (Atf), exhibiting acyl-CoA acyltransferase activity to both diacylglycerols (DGAT activity) and fatty alcohols (wax ester synthase, WS activity). An 800 bp PCR product was obtained from chromosomal DNA of strain PD630 by using degenerate primers designed from conserved stretches of Atf proteins of *Acinetobacter baylyi* strain ADP1 and *Mycobacterium smegmatis* mc<sup>2</sup>155. The *atf* fragment was used as a probe on a strain PD630 gene library, resulting in the identification of a 3948 bp chromosomal DNA fragment containing the complete *atf1* gene. An *atf1* disruption mutant of strain PD630 exhibited a TAG-leaky phenotype and accumulated up to 50% less fatty acids than the wild-type, with significantly reduced oleic acid content when cultivated in the presence of gluconate or oleic acid. Whereas DGAT activity was drastically reduced in comparison to the wild-type, WS activity remained almost unchanged in the mutant. RT-PCR analysis of gluconate-grown cells of strain PD630 showed that there is expression of *atf1* under conditions of TAG synthesis. To identify additional Atfs in strain PD630, PCR employing non-degenerate primers deduced from *Rhodococcus jostii* RHA1 sequence data was used. This yielded nine additional *atf*-homologous genes exhibiting 88–99% sequence identity to the corresponding strain RHA1 enzymes. Besides Atf1 only Atf2 exhibited high DGAT and/or WS activity when heterologously expressed in *Escherichia coli*.

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## INTRODUCTION

Biosynthesis and intracellular accumulation of lipids is widely distributed among animals, plants and micro-organisms. Triacylglycerols (TAGs) are the most frequent storage lipids in higher eukaryotes and also in eukaryotic micro-organisms such as yeast, moulds and algae (Christiansen, 1978; Leman, 1997; Ratledge, 1989; Stahmann *et al.*, 1994). In contrast, bacteria usually accumulate specialized lipids such as polyhydroxyalkanoates (Anderson & Dawes, 1990; Steinbüchel, 1991;

Steinbüchel & Valentin, 1995; Alvarez *et al.*, 1997b). The accumulation of large amounts of TAGs in bacteria has been frequently reported in strains belonging to the order Actinomycetales such as *Mycobacterium* (Barksdale & Kim, 1977), *Streptomyces* (Olukoshi & Packter, 1994; Packter & Olukoshi, 1995), *Nocardia* and *Rhodococcus* (Alvarez *et al.*, 1996, 1997a). Moreover, biosynthesis of wax esters (WEs) has often been reported for *Acinetobacter* species (Fixter *et al.*, 1986). Biosynthesis of WEs has also been sporadically reported in species of the genera *Moraxella* (Bryn *et al.*, 1977), *Micrococcus* (Russell & Volkman, 1980) and *Alcanivorax* (Bredemeier *et al.*, 2003). In general, TAGs and WEs are stored as insoluble inclusions of different shapes and sizes inside the cytoplasm, depending on the lipid, strain and culture conditions (Kalscheuer *et al.*, 2001; Wältermann & Steinbüchel, 2005, 2006)

**Abbreviations:** Atf, acyltransferase; DGAT, diacylglycerol acyltransferase; TAG, triacylglycerol; WE, wax ester; WS, wax ester synthase.

A table of primers and a figure showing a multiple alignment of deduced Atf sequences are available with the online version of this paper.

*Rhodococcus opacus* strain PD630 is able to accumulate large amounts of TAGs and under certain culture conditions also minor amounts of WEs; it is one of the best-studied bacteria regarding biosynthesis and accumulation of lipids. Physiological studies analysing the accumulation and mobilization of storage lipids in *Rhodococcus* sp. showed that TAGs serve as a depot for carbon and energy in these bacteria (Alvarez *et al.*, 2000, 2004; Alvarez & Steinbüchel, 2002). Strain PD630 can be considered as an oleaginous micro-organism, since TAGs can account for up to 76 or 87 % of the cellular dry weight in gluconate- or olive-oil-grown cells, respectively (Alvarez *et al.*, 1996; Voss & Steinbüchel, 2001).

Condensation of acyl-CoA and diacylglycerol catalysed by a diacylglycerol-acyltransferase (DGAT or Atf) is the key enzymic step in TAG biosynthesis, since reactions involved in the formation of the diacylglycerol substrate are also part of the phospholipid biosynthesis routes. The first Atf enzyme was described in *Acinetobacter baylyi* strain ADP1, and it exhibited WE synthase (WS) and DGAT activities (Kalscheuer & Steinbüchel, 2003). *A. baylyi* ADP1 possesses only a single Atf enzyme, which is responsible for synthesis of WEs as the main lipid storage compound in addition to minor amounts of TAGs (Kalscheuer & Steinbüchel, 2003). This enzyme exhibits a high promiscuity with respect to acyl acceptor molecules *in vitro* (Kalscheuer *et al.*, 2003; Stöveken *et al.*, 2005; Uthoff *et al.*, 2005). This is the reason why it is attracting increasing interest for biotechnological processes, for example 'MicroDiesel' production by recombinant *Escherichia coli* strains (Kalscheuer *et al.*, 2006a, b). Atf enzymes constitute a heterogeneous family of bacterial acyl-CoA-acyltransferases which do not have significant sequence similarities to eukaryotic DGAT or WS enzymes (Wältermann *et al.*, 2006). The widespread occurrence of *atf* genes in the genomes of actinomycetes and different Gram-negative bacteria suggests that they play an important role in the life cycle of these organisms (Wältermann & Steinbüchel, 2006). As an example, Daniel *et al.* (2004) identified 15 *atf*-homologous genes in *Mycobacterium tuberculosis*, which exhibited variable DGAT and WS activity when heterologously expressed in *E. coli*. Sirakova *et al.* (2006) suggested that the different sets of *atf* genes in *M. tuberculosis* are induced in response to different environmental stresses, to enable the organism to synthesize TAGs with maximum efficiency.

Although *R. opacus* PD630 is considered as a model oleaginous prokaryote, the genes involved in TAG biosynthesis and accumulation had not been identified prior to this study. Here we report the characterization of one *atf* gene and the identification of nine additional putative *atf* genes from this micro-organism.

## METHODS

**Strains, plasmids and growth conditions.** *R. opacus* strain PD630 (Alvarez *et al.*, 1996; DSM 44193) and the *atf1* mutant derived from it were grown aerobically at 30 °C in Luria-Bertani (LB) medium

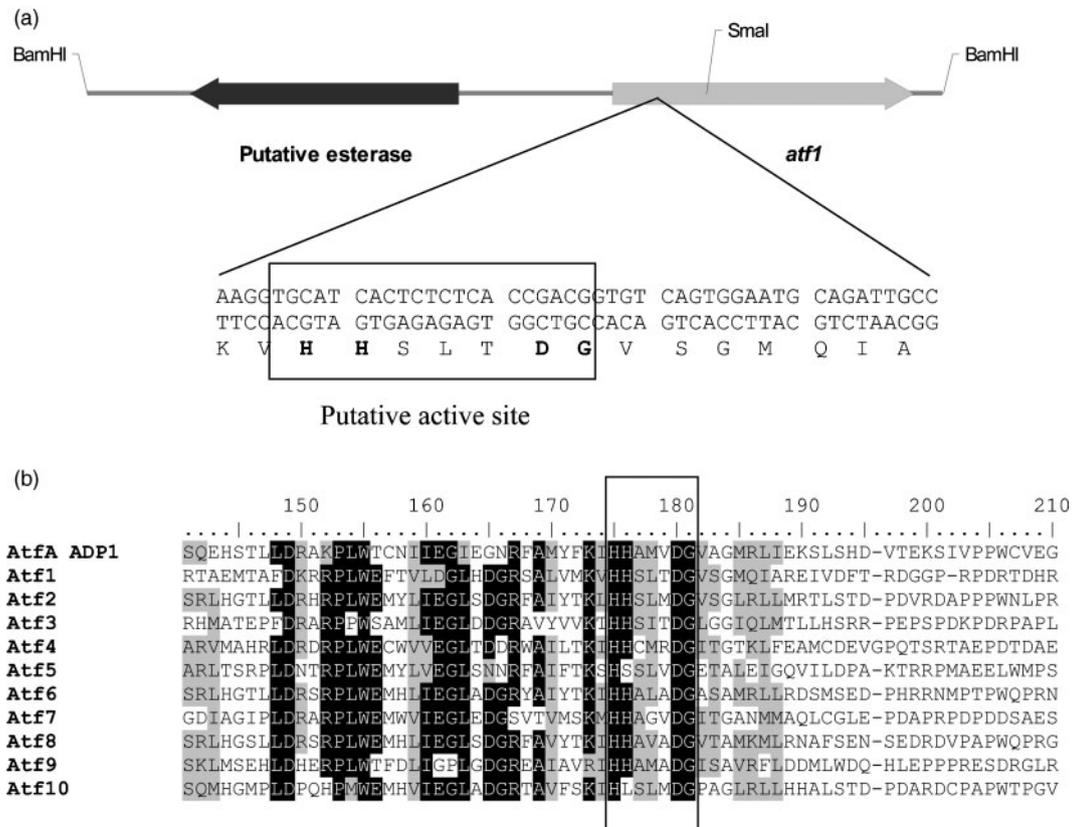
(Sambrook *et al.*, 1989) or in mineral salt medium (MSM) according to Schlegel *et al.* (1961) with 1 g NH<sub>4</sub>Cl l<sup>-1</sup> and 1 g sodium gluconate l<sup>-1</sup> or 0.1 % (v/v) oleic acid as carbon source. These conditions are referred to as growth conditions. To promote accumulation of TAGs, NH<sub>4</sub>Cl in MSM was reduced to 0.1 g l<sup>-1</sup> (storage conditions). *E. coli* strains were cultivated in LB medium at 37 °C. *E. coli* strains XL1 Blue (Bullock *et al.*, 1987) and S17-1 (Simon *et al.*, 1983) were used for cloning and for conjugational transfer of plasmids to *R. opacus*, respectively. Solid media were prepared by addition of 1.8 % (w/v) agar-agar. Sucrose sensitivity of *R. opacus* mediated by the *sacB* gene was tested on LB agar supplemented with 10 % (w/v) sucrose (LBS). Ampicillin, kanamycin and gentamicin were used at final concentrations of 75, 50 or 15 µg ml<sup>-1</sup>, respectively, for recombinant *E. coli* strains. For *Rhodococcus* strains, kanamycin and gentamicin were used at final concentrations of 75 µg ml<sup>-1</sup> or 15 µg ml<sup>-1</sup>, respectively. Plasmids used in this study were pBluescript SK<sup>-</sup> (Stratagene), pGEMT-Easy (Promega), pJQ200mp18 (Quandt & Hynes, 1993), pHC79 (Hohn & Collins, 1980) and pSKsymΩKm (Overhage *et al.*, 1999).

**Isolation, analysis and transfer of DNA.** Chromosomal DNA, plasmid DNA and DNA restriction fragments were isolated and analysed by standard methods (Marmur, 1961; Sambrook *et al.*, 1989). Conjugations of *E. coli* S17-1 harbouring hybrid plasmids (donors) and of *Rhodococcus* strains (recipients) were performed on solidified LB medium as described by Friedrich *et al.* (1981). Transformation of *R. opacus* strain PD630 was carried out as described by Kalscheuer *et al.* (1999).

**DNA amplification, cloning and sequencing.** For amplification of an 800 bp internal fragment of *atf1* from strain PD630, degenerate oligonucleotide primers *tgs-int1* (5'-TCSCGCCCGCTSTGGGAG-3') and *tgs-int2* (5'-SGGGCCSGGSACGTTCCGA-3') were used (Supplementary Fig. S1). The thermocycling parameters were as follows: 5 min at 94 °C, 30 cycles of 0.5 min at 94 °C, 0.5 min at 45 °C and 1 min at 72 °C, and finally 10 min at 72 °C. The PCR product was cloned into pGEMT-Easy and subjected to DNA sequencing. Subsequently, the *atf1* fragment was DIG-labelled for hybridization analysis and used as a probe on a pBluescript SK-based *Bam*HI-restricted PD630 gene library. For amplification of the complete coding regions of the paralogous *atf* genes from strain PD630, primers listed in Supplementary Table S1, which were designed based on the sequences of the orthologous genes from *Rhodococcus jostii* strain RHA1, were used. For cloning of PCR products collinear to the *lacZ* promoter of pBluescript SK<sup>-</sup> and effective gene expression, restriction enzyme recognition sites and suitable ribosome-binding sites were incorporated into the oligonucleotide sequences. DNA and deduced protein sequence similarity searches were carried out using BLAST (Altschul *et al.*, 1997). Multiple sequence alignments were done using BioEdit Sequence Alignment Editor (Ibis Biosciences).

### Inactivation of *atf1* in *R. opacus* PD630 by gene disruption.

Plasmid pJQ200mp18, containing the RP4 origin of transfer for conjugative mobilization and *sacB* of *Bacillus subtilis* for positive selection of double recombination events, was used to construct a mutagenic plasmid for the insertion of a ΩKm gene cassette into *atf1* of the strain PD630 chromosome. The ΩKm gene cassette, recovered from *Sma*I-digested pSKsymΩKm, was cloned into a unique *Sma*I restriction site in the F11 fragment (Fig. 1a) yielding F11-*atf1*ΩKm. The mutagenic plasmid was constructed by cloning of the *Bam*HI-*Bam*HI F11-*atf1*ΩKm fragment into pJQ200mp18. The resulting plasmid pJQ200mp18::F11-*atf1*ΩKm was introduced into *E. coli* S17-1 and mobilized to *R. opacus* PD630 by conjugation. All resulting Gm<sup>R</sup> and Km<sup>R</sup> *R. opacus* transconjugants were unable to grow after replica plating on LBS agar supplemented with kanamycin and gentamicin (Suc<sup>S</sup>). The single recombination event was confirmed by



**Fig. 1.** (a) Molecular organization of the 3948 bp *Bam*HI restriction fragment of *R. opacus* strain PD630 chromosomal DNA (fragment F11) harbouring the *atf1* gene. The sequence of the region of *atf1* that contains the putative active site is shown. (b) Multiple sequence alignment of the regions constituting the suggested active sites of Atf proteins from *R. opacus* PD630 with the AtfA protein from *A. baylyi* strain ADP1. Conserved amino acids are shaded in black, and homologous residues in grey. The putative active site is boxed.

Southern blot analysis (not shown). Gene inactivation of *atf1* was achieved by overnight culture of transconjugants under non-selective conditions and subsequent plating on LBS containing 75 µg kanamycin ml<sup>-1</sup>. The Suc<sup>R</sup>/Km<sup>R</sup> colonies were replica plated on LBS plates containing gentamicin plus kanamycin and on LBS plates with only kanamycin. Gm<sup>S</sup>/Km<sup>R</sup> colonies were taken for subsequent PCR assays, using the primers listed in Supplementary Table S1 for *atf1*, and Southern blot analysis was done to confirm disruption of the *atf1* gene (data not shown).

**Lipid analysis.** The qualitative and semiquantitative analyses of intracellular lipids in *R. opacus* PD630 were carried out by thin-layer chromatography (TLC). For this, 1 mg of lyophilized cells was extracted with 200 µl chloroform/methanol (2:1, v/v) for 30 min. The supernatant was then concentrated and subjected to silica G TLC as previously described, using hexane/diethyl ether/acetic acid (80:20:1, by vol.) as solvent for TAG analysis (Wältermann *et al.*, 2000). Triolein and oleyl oleate were used as TAG and WE reference substances, respectively.

Fatty acid analysis of whole cells or purified TAGs was done by gas chromatography (GC) according to Kalscheuer *et al.* (2004). For this, 3–5 mg of lyophilized cells was subjected for 4 h to methanolysis at 100 °C in the presence of 15% (v/v) sulphuric acid suspended in

methanol. The resulting fatty acid methyl esters were analysed on an Agilent 6850 gas chromatograph equipped with a BP21 capillary column (50 m × 0.22 mm; film thickness 250 nm) (SGE, Darmstadt, Germany) and a flame-ionization detector (Agilent Technologies). A 2 µl portion of the organic phase was analysed after split injection; hydrogen (constant flow of 0.6 ml min<sup>-1</sup>) was used as a carrier gas. The temperatures of the injector and detector were 250 and 275 °C, respectively. The following temperature programme was applied: 120 °C for 5 min, increase of 3 °C min<sup>-1</sup> to 180 °C, increase of 10 °C min<sup>-1</sup> to 220 °C, and 220 °C for 31 min. Substances were identified by comparison of their retention times with those of standard fatty acid methyl esters.

**Determination of enzyme activities.** Cells of *R. opacus* were incubated overnight under storage conditions, whereas for heterologous expression, recombinant *E. coli* were cultivated for 6 h in the presence of 1 mM IPTG. Cells were then harvested and broken using a French press, and the resulting crude extracts were used for enzyme activity determination. WS and DGAT activities were determined in a total volume of 250 µl containing 12.5 µg BSA ml<sup>-1</sup>, 4.72 µM [1-<sup>14</sup>C]palmitoyl-CoA (specific activity, 1.961 Bq pmol<sup>-1</sup>), 125 mM sodium phosphate buffer (pH 7.4), and 3.75 mM 1-hexadecanol or 1,2-dipalmitoylglycerol. Water-insoluble substrates and BSA were applied as double-concentrated stock solutions emulsified by

ultrasonification. The assay mixtures were incubated for 30 min at 35 °C, and the reactions were stopped by extraction with 500 µl chloroform/methanol (1:1, v/v). After centrifugation, the chloroform phase was withdrawn and evaporated to dryness, and 40 µg of unlabelled reference substances was added. The reaction products were separated by TLC using the solvent systems hexane/diethyl ether/acetic acid (90:7.5:1, by vol.) and hexane/diethyl ether/acetic acid (80:20:1, by vol.). After separation of lipids and their staining with iodine vapour, spots corresponding to the reaction products were scraped from the plates, and radioactivity was measured by scintillation counting.

**RNA isolation and RT-PCR.** Total RNA was extracted from cells of *R. opacus* PD630 at different growth phases by three breaking cycles (1 min of breaking at maximal speed, 1 min on ice) on a Mini-Bead Beater in the presence of 0.1 mm glass beads. RNA was purified using the RNeasy Miniprep kit (Qiagen) and treated with DNase I (RNase-Free DNase Set, Qiagen). Total RNA (1 µg) served as template in a one-step RT-PCR using the following internal primers: 5'-AGATCGTGGATTTCACCCGCGATG-3' (*Tag1-RT-PCRup*) and 5'-CGTCATCCGCAGTTCGCTGAT-3' (*Tag1-RT-PCRdown*) for *atf1*; 5'-GATGCCGACGCTGTCGACCGA-3' (*Tag2-RT-PCRup*) and 5'-GGCGTCGGCCTTCTGCTCGTC-3' (*Tag2-RT-PCRdown*) for *atf2*. The thermocycling programme of the one-step PCR consisted of a reverse transcription at 50 °C for 30 min, an initial denaturation at 95 °C for 15 min, 30–35 cycles (denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 60 s), and a final 10 min elongation at 72 °C. PCR products were subjected to electrophoresis on a 1.2% agarose gel.

## RESULTS

### Identification of a DNA fragment containing a gene involved in TAG synthesis in *R. opacus* PD630

The sequences of the AtfA protein from *A. baylyi* ADP1 (accession number AAO17391) and seven homologous proteins from *Mycobacterium smegmatis* mc<sup>2</sup>155 (accession numbers ABK74273, ABK70911, ABK69671, ABK70404, ABK69832, ABK75217 and ABK71433) were aligned, and two short conserved sequences were selected for design of degenerate primers according to the codon usage of *R. opacus*. An 800 bp fragment exhibiting regions with high similarities to *atfA* of *A. baylyi* ADP1 was amplified by PCR

from chromosomal DNA of *R. opacus* PD630 as a template. To obtain the whole putative *R. opacus atf* gene, the fragment was used as a probe in a Southern blot hybridization assay employing *Bam*H-restricted chromosomal DNA from *R. opacus*, yielding a single hybridizing fragment of approx. 4 kbp (not shown). A partial library of *Bam*HI-restricted *R. opacus* chromosomal DNA in plasmid pBluescriptSK<sup>-</sup> was constructed in *E. coli* and investigated by dot-blot hybridization assays. One clone (F11), harbouring the desired 3948 bp fragment, was isolated. Fragment F11 contained two complete ORFs in opposite directions: a TAG-synthase-homologous gene (*atf1*) and a putative esterase gene (Fig. 1a). The amino acid sequence deduced from *atf1* shared conserved sequences, including the putative active-site motif HHxxxDG, with the AtfA protein from *A. baylyi* strain ADP1 and other reported Atf enzymes. However, the Atf1 amino acid sequence showed the highest similarity (89% identity) to a predicted protein of *R. jostii* strain RHA1 (accession number YP\_700033) (Table 1).

### Construction and characterization of the disruption mutant *R. opacus atf1*ΩKm

An *atf1* knockout mutant was constructed by a double recombination strategy using the counter-selectable marker *sacB* as described in Methods. To compare the *atf1* knockout mutant and the wild-type for their abilities to accumulate TAGs, the two strains were cultivated under both storage and growth conditions. As revealed by TLC, the disrupted *atf1* mutant was still able to synthesize TAGs after incubation under storage conditions; however, diminished amounts of TAGs were accumulated in the cells (Fig. 2a). Quantitative and qualitative GC analysis of cells cultivated under growth conditions revealed that the mutant strain *atf1*ΩKm contained up to 50% less total fatty acids during stationary phase (after 36 h) compared to the wild-type (Fig. 2b). Interestingly, the relative proportion of oleic acid (C18:1) in crude cell masses and also in TAG samples purified from the cells was generally lower in the mutant (Fig. 3a–c). The relative amount of C18:1 in the cell mass and in TAG samples of mutant cells decreased to as low

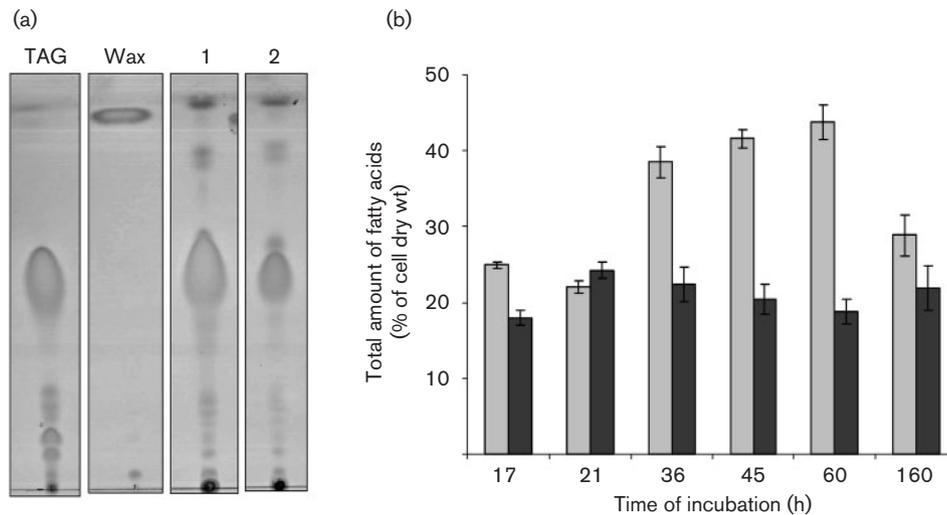
**Table 1.** Identities of Atf1 from *R. opacus* PD630 to different Atf-homologous proteins

Strain	No. of Atf1 homologues	Protein*	Length (aa)	Identity to Atf1 (%)†	Accession no.‡
<i>Acinetobacter baylyi</i> ADP1	1	AtfA	458	24	AAO17391
<i>Mycobacterium smegmatis</i> mc <sup>2</sup> 155	8	MSMEG_3933	451	35	YP_888223
<i>Mycobacterium tuberculosis</i> H37Rv	15	Rv2484c	491	29	NP_217000
<i>Rhodococcus jostii</i> RHA1	14	RO00039	473	89	YP_700033
<i>Streptomyces coelicolor</i> A3(2)	3	SCO1280	413	23	NP_625567
<i>Alcanivorax borkumensis</i> SK2	2	AtfA1	451	20	YP_693524

\*Protein with the highest identity to the Atf1 protein from *R. opacus* PD630.

†Identities were based on alignments of primary protein structure derived from full-length gene sequences.

‡Accession numbers in the NCBI protein database.



**Fig. 2.** Lipid analysis of whole-cell extracts of *R. opacus* strains grown with gluconate as carbon source. (a) TLC. Lanes: TAG, TAG standard; Wax, wax standard; 1, wild-type *R. opacus* PD630; 2, mutant *R. opacus* *atf1*ΔKm; the cells were grown in LB medium for 24 h, harvested, washed and then incubated for 17 h under storage conditions. (b) Total amounts of fatty acids of *atf1* mutant strain (dark grey bars) in comparison to the wild-type (light grey bars) in different growth stages; the cells were grown in MSM (growth conditions). The data represent the means of two independent experiments. Error bars indicate the range of results.

as 50 % of that of the wild-type cells grown in presence of oleic acid. The relative amounts of palmitoleic acid (C16:1) in cell dry mass and in TAG samples of the mutant cells grown under these conditions were also significantly lower in comparison to the wild-type (Fig. 3b, c).

The WS and DGAT activities in wild-type and mutant cells incubated under storage conditions with sodium gluconate as carbon source were measured. Whereas the DGAT activity in the mutant decreased to about one-third [ $62.2 \pm 8.6$  pmol (mg min)<sup>-1</sup>] of the respective activity in the parental strain [ $169.0 \pm 10.4$  pmol (mg min)<sup>-1</sup>], the WS activity in the knock-out mutant did not show a significant decrease [from  $79.9 \pm 13.6$  pmol (mg min)<sup>-1</sup> in the wild-type to  $65.0 \pm 2.3$  pmol (mg min)<sup>-1</sup> in the mutant].

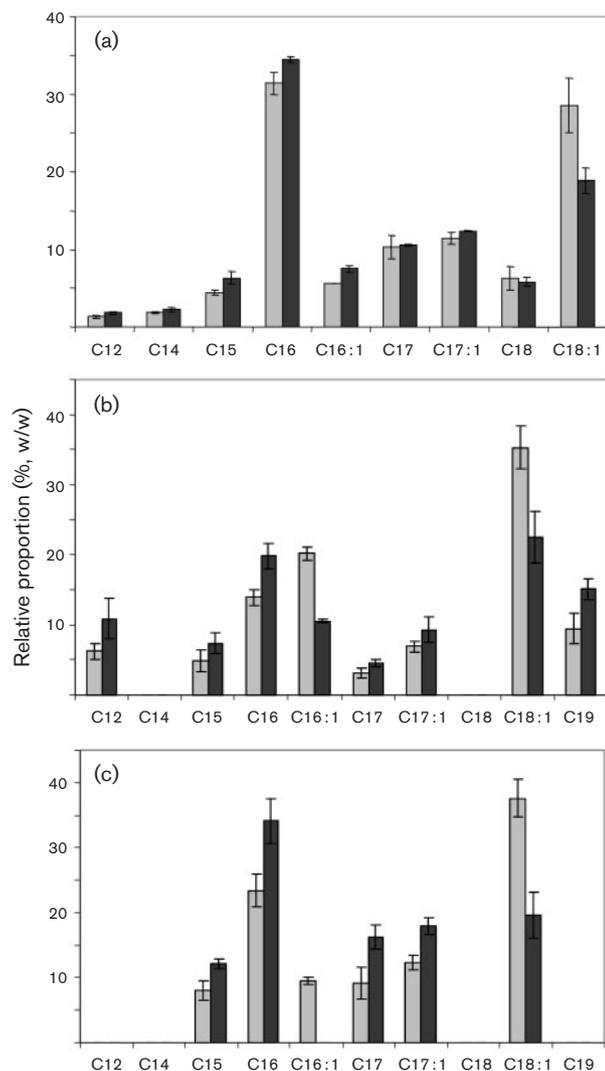
### Identification of other *atf1*-homologous genes in *R. jostii* strain RHA1 and *R. opacus* strain PD630

The only sequenced genome of a species of the genus *Rhodococcus* is that of *R. jostii* strain RHA1 (McLeod *et al.*, 2006). The *R. jostii* RHA1 genome data at Canada's Michael Smith Genome Sciences Centre (<http://www.bcgsc.ca>) were analysed for the occurrence of *atf1*-homologous genes. Fourteen genes whose products have significant amino acid identity (>22 %) to the Atf1 protein from *R. opacus* PD630 were identified in this strain. Three of these genes were located on the linear megaplasmid pRHL1 (YP\_707847, YP\_707571, YP\_707862); the third of these three genes was a truncated form of the first one. The deduced amino acid sequences of all 11 chromosomal *atf1*-homologous genes exhibited the conservative active-site

motif HHxxxDG and also other consensus sequences. One of these genes (YP\_700033) and its flanking regions shared about 90 % nucleotide identity with the *atf1* gene of strain PD630 and with the complete F11 fragment.

The analysis of the *atf1* mutant provided clear evidence that further Atfs are present in *R. opacus* strain PD630. To amplify as many of the additional *atf* genes as possible from *R. opacus* PD630, and taking the high sequence homology and the phylogenetic relationship between strains RHA1 and PD630 into account, non-degenerate primers, according to the 10 remaining chromosomal *atf*-homologous genes found in the RHA1 genome, were constructed (Supplementary Table S1). By applying these primers, nine further *atf*-homologous genes were successfully amplified (*atf2* to *atf10*). All deduced amino acid sequences possess the complete putative active-site motif HHxxxDG described for bacterial Atf proteins. Those of Atf5 and Atf10 exhibited a modified active-site motif, in which the second histidine was replaced by serine or lysine, respectively (Fig. 1b and Supplementary Fig. S1). As a particular case, the *atf2* gene exhibited a premature stop-codon due to a point mutation in position 1107, thereby yielding a protein of only 374 instead of 453 amino acids in the RHA1 protein. This gene was amplified twice to confirm this observation and to exclude a mistake during PCR amplification.

The deduced amino acid sequences of the 10 putative Atf proteins of strain PD630 were analysed with the transmembrane prediction program DAS (Cserző *et al.*, 1997). Eight Atf proteins of strain PD630 possessed at least one putative transmembrane region which may permit proteins



**Fig. 3.** Fatty acid composition of *R. opacus* PD630 (light grey bars) and *R. opacus att1*ΔKm (dark grey bars) incubated under storage conditions. (a) Whole-cell extracts from cells incubated in MSM containing gluconate as carbon source; (b) whole-cell extracts from cells incubated in MSM containing oleic acid as carbon source; (c) TAG fraction from extracts of cells incubated as in (b). The data represent the means of two independent experiments. Error bars indicate the range of results.

to anchor to a phospholipid membrane. Atf1 possesses two putative transmembrane domains, as shown in Fig. 4(a). Only Atf2 and Atf6 seemed to be cytoplasmic enzymes without a predicted membrane-spanning region (Fig. 4b).

### Heterologous expression of *atf* genes from *R. opacus* PD630

The 10 putative *atf* genes from *R. opacus* PD630 were amplified by tailored PCR introducing a ribosome-binding site and were cloned into pBluescript SK<sup>-</sup> collinear to the *lacZ* promoter. After transfer of plasmids to *E. coli* XL1

Blue, expression of all putative *atf* genes was demonstrated by SDS-PAGE (data not shown). To reveal whether the synthesized proteins exhibit WS and DGAT activities, crude protein extracts of the recombinant strains were analysed for their acyltransferase activities (Table 2). In general, all crude extracts of recombinant *E. coli* strains exhibited only low enzymic activities compared to those obtained from the *R. opacus* strains used in this study. Although *atf1* seemed to be responsible for a significant part of the TAG accumulation in strain PD630, as was concluded from the gene-disruption experiment, the *atf1* gene product exhibited almost no DGAT activity when expressed in recombinant *E. coli*. Interestingly, Atf1 exhibited significant WS activity. In contrast, recombinant *E. coli* harbouring plasmid pBluescriptSK<sup>-</sup>::*atf2* exhibited WS as well as significant DGAT activities. However, crude protein extracts of *E. coli* strains expressing *atf3* to *atf10* exhibited no or only slightly increased WS/DGAT activities in comparison to the vector control cultivated under conditions used in this study (Table 2).

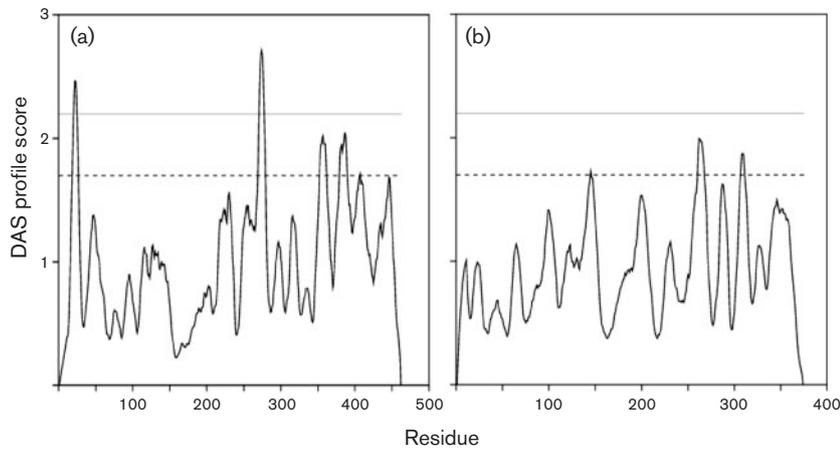
### *In vivo* expression of *atf* genes in *R. opacus* PD630

Since the *atf1* and *atf2* gene products showed the highest acyltransferase activities when expressed in recombinant *E. coli*, their expression in cells of *R. opacus* PD630 from different stages of growth and TAG accumulation was investigated by RT-PCR. For this, cells were grown in MSM containing gluconate, allowing accumulation of TAGs, and cell samples for RNA isolation were withdrawn in the early (14 h) and the late (21 h) exponential growth phase. In addition, samples from cells of the stationary growth phase (45 h), which had accumulated large amounts of TAGs, were taken and subjected to RNA isolation. Transcripts of both *atf1* and *atf2* genes were detected in all cells harvested at the indicated growth phases (Fig. 5).

## DISCUSSION

The present study was undertaken to identify genes involved in the key step of TAG biosynthesis in the oleaginous bacterium *R. opacus* strain PD630. A 3948 bp chromosomal DNA fragment of strain PD630 harbouring the *atf1* gene was identified after degenerate PCR and dot-blot hybridization experiments. The Atf1 protein contained the putative active-site motif HHxxxDG as well as other conserved regions which are typically also present in related proteins from *A. baylyi* ADP1, *M. smegmatis* mc<sup>2</sup>155 and *M. tuberculosis* (Kalscheuer & Steinbüchel, 2003; Daniel *et al.*, 2004).

An *atf1* knockout mutant was constructed using the *sacB* gene as a counter-selectable marker against single crossover events (Jäger *et al.*, 1995; Pelicic *et al.*, 1996; Ramakrishnan *et al.*, 1997). The disruption of the *atf1* gene resulted in a significant decrease of the total fatty acids (up to 50%) as well as of the cellular TAG content, as shown by TLC



**Fig. 4.** Hydrophobicity plot 'DAS' transmembrane (TM) segment prediction of (a) Atf1 and (b) Atf2. The dashed line indicates a loose cutoff, whereas the line at 2.2 DAS score corresponds to a strict cutoff for TM segment prediction. Only Atf6 showed a plot similar to Atf2 without putative TM regions. All other Atf paralogues of PD630 possessed at least one putative TM region as shown in the DAS plot of Atf1.

analysis, and also in a substantial reduction of DGAT activity in crude extracts; on this basis, Atf1 could be considered as a major enzyme for lipid biosynthesis in *R. opacus* PD630. However, this must be further confirmed by analysis of a complemented mutant strain. Furthermore, the mutant exhibited a modified fatty acid profile, with reduced relative amounts of oleic and palmitoleic acids. The same pattern was observed with fatty acids from both whole-cell extracts and TAG fractions. These results indicate that other TAG-synthesizing enzymes beside Atf1, with different substrate specificities, contribute to the total DGAT activity and TAG accumulation in strain PD630, as was also shown in the pathogenic *M. tuberculosis* (Daniel *et al.*, 2004). The *M. tuberculosis* genome encodes 15 putative TAG synthases (Tgs), and disruption of the *tgs1* gene in this bacterium reduced TAG accumulation drastically under conditions of hypoxia or acidic environment, or upon treatment with NO (Sirakova *et al.*, 2006). These results suggest that *tgs1* in *M. tuberculosis* is a major contributor to TAG synthesis under the mentioned

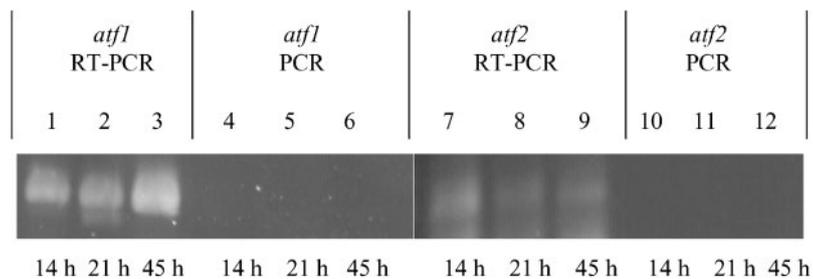
conditions. Interestingly, the authors demonstrated that Tgs1 preferred C26:0-CoA for TAG biosynthesis (Sirakova *et al.*, 2006). C26:0 was the major fatty acid in the TAGs stored under stress; however, this fatty acid was absent even in the very small amounts of TAG produced by the *tgs1*-deficient mutant. A change in the fatty acid pattern was also described in a DGAT mutant (AS11) of the higher plant *Arabidopsis thaliana*; this mutant, which is characterized by a reduced amount of storage lipid in mature seeds, showed an increased accumulation of linoleic acid (C18:3) as the major fatty acid and reduced levels of C18:1 incorporation in comparison to the wild-type (Katavic *et al.*, 1995). Changes in the fatty acid profiles of accumulated TAGs in Atf mutants may therefore indicate that in actinomycetes Atf isoenzymes are specialized for the selective incorporation of different fatty acyl residues into TAGs.

Enzymes which accomplish the last step of TAG or WE biosynthesis in bacteria exhibit high sequence variability. The *R. opacus* Atf1 exhibits only around 30% sequence similarity to other Atf proteins known so far. Based on sequence alignments, we have identified 11 additional homologous genes in the chromosome of *R. jostii* strain RHA1, and one of these showed an unusually high sequence identity. By using oligonucleotides designed on the basis of strain RHA1 homologous genes, nine further genes (*atf2* to *atf10*) encoding putative Atf enzymes exhibiting 88–99% sequence identity to strain RHA1 genes were amplified from *R. opacus* PD630 DNA and were subsequently cloned. However, only *atf1* and *atf2* exhibited high WS and/or DGAT activity *in vitro*, indicating that these genes encode functional WS/DGAT enzymes. Similar results were reported by Daniel *et al.* (2004) for *M. tuberculosis*. Among the 15 Tgs proteins in this bacterium, only four exhibited high DGAT activity when expressed in *E. coli*. Since the sequence data do not provide evidence that these genes encode functionally inactive enzymes, the substrate range of these enzymes may be quite different and they may not react with the routinely used substrates of C<sub>16</sub> chain length. In addition, heterologous expression of genes

**Table 2.** WS and DGAT activities in crude extracts of recombinant *E. coli* XL1 Blue containing *atf* genes from *R. opacus* PD630

Strain	DGAT activity* [pmol (mg min) <sup>-1</sup> ]	WS activity* [pmol (mg min) <sup>-1</sup> ]
XL1 Blue pSK <sup>-</sup>	0.22 ± 0.04	0.19 ± 0.01
XL1Blue pSK:: <i>atf1</i>	0.37 ± 0.09	4.65 ± 0.04
XL1Blue pSK:: <i>atf2</i>	7.19 ± 0.15	4.02 ± 0.44
XL1Blue pSK:: <i>atf3</i>	0.07 ± 0.01	0.08 ± 0.03
XL1Blue pSK:: <i>atf4</i>	0.48 ± 0.09	0.31 ± 0.08
XL1Blue pSK:: <i>atf5</i>	0.42 ± 0.01	0.39 ± 0.08
XL1Blue pSK:: <i>atf6</i>	0.44 ± 0.04	0.42 ± 0.07
XL1Blue pSK:: <i>atf7</i>	0.19 ± 0.05	0.08 ± 0.03
XL1Blue pSK:: <i>atf8</i>	0.08 ± 0.01	0.10 ± 0.02
XL1Blue pSK:: <i>atf9</i>	0.10 ± 0.04	0.08 ± 0.02
XL1Blue pSK:: <i>atf10</i>	0.09 ± 0.01	0.08 ± 0.01

\*Values are means ± SD of at least two independent experiments.



**Fig. 5.** Agarose gel electrophoresis of RT-PCR products of *atf1* and *atf2*. Lanes 1–3, 450 bp fragments of *atf1* amplified from RNA from strain PD630 at different incubation times; lanes 4–6, negative control. Lanes 7–9, 460 bp fragments of *atf2* amplified from RNA from strain PD630 at different incubation times; lanes 10–12, negative control.

from *R. opacus* in *E. coli* could yield incorrectly folded proteins that do not reflect their activities *in vivo*.

Most of the 10 Atfs from *R. opacus* seem to be transmembrane proteins, as revealed by the TM prediction program. By applying immunogold labelling, Stöveken *et al.* (2005) showed AtfA from *A. baylyi* ADP1 to be associated with the cytoplasmic membrane as well as with lipid inclusions and to occur also in soluble form in the cytoplasm. Nevertheless, as strain PD630 possesses multiple paralogous proteins, the exact cellular distribution of the Atf proteins must be determined by further studies.

RT-PCR analysis revealed that the *atf1* and *atf2* genes were transcribed during all stages of growth and TAG accumulation under the conditions used in this study. The occurrence of at least 10 genes encoding putative Atf enzymes in *R. opacus* PD630 suggests a key role of these enzymes for the physiology of this soil micro-organism and probably for its ability to cope with diverse environmental stresses. However, evidence for the existence of an as yet unknown alternative TAG biosynthesis route was recently found in *Alcanivorax borkumensis* (Kalscheuer *et al.*, 2007), and as yet unknown types of TAG-synthesizing enzymes may also occur in other TAG-accumulating bacteria such as *R. opacus* PD630.

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