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

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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* mc²155. 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*.

INTRODUCTION

Biosynthesis and intracellular accumulation of lipids is widely distributed among animals, plants and microorganisms. 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; Wälttermann & Steinbüchel, 2005, 2006).
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 glucose- 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 synthise (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; Stövenken et al., 2005; Utthoff 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 NH₄Cl l⁻¹ and 1 g sodium glutamate l⁻¹ or 0.1% (v/v) oleic acid as carbon source. These conditions are referred to as growth conditions. To promote accumulation of TAGs, NH₄Cl in MSM was reduced to 0.1 g l⁻¹ (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 conjugal 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⁻¹, respectively, for recombinant E. coli strains. For Rhodococcus strains, kanamycin and gentamicin were used at final concentrations of 75 μg ml⁻¹ or 15 μg ml⁻¹, respectively. Plasmids used in this study were pBluescript SK (Stratagene), pGEMT-Easy (Promega), pJQ200mp18 (Quandt & Hynes, 1993), pHC79 (Hohn & Collins, 1980) and pSKsymtKm (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 3g-int1 (5’-TSCGGCGGCGCTAGGAG-3’) and 3g-int2 (5’-GGGCCTCGGGAGCATTGCGA-3’) were used (see 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 BamHI-restricted PD630 genome 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 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 βKm gene cassette into atf1 of the strain PD630 chromosome. The βKm gene cassette, recovered from Smal-digested pSKsymtKm, was cloned into a unique Smal restriction site in the F11 fragment (Fig. 1a) yielding F11-atf1βKm. The mutagenic plasmid was constructed by cloning of the BamHI–BamHI F11-atf1βKm fragment into pJQ200mp18. The resulting plasmid pJQ200mp18::F11-atf1βKm was introduced into E. coli S17-1 and mobilized to R. opacus PD630 by conjugation. All resulting Gm⁻ and Km⁻ R. opacus transconjugants were unable to grow after replica plating on LBS agar supplemented with kanamycin and gentamicin (Suc²). The single recombination event was confirmed by

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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⁻¹. The Suc R/KmR colonies were replica plated on LBS plates containing gentamicin plus kanamycin and on LBS plates with only kanamycin. Gm S/KmR 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 6890 gas chromatograph equipped with a BP21 capillary column (50 m × 0.22 mm; film thickness 250 μm) (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⁻¹) 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⁻¹ to 180 °C, increase of 10 °C min⁻¹ 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⁻¹, 4.72 μM [1-¹⁴C]palmitoyl-CoA (specific activity, 1.961 Bq pmol⁻¹), 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 RNasy 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'-AGATCGTGATTTCTACCCGGCAGATG-3' (Tag1-RT-PCRup) and 5'-CGTCATCCGCAGTTCGCTGAT-3' (Tag1-RTPCRdown) for *atf1*; 5'-GATCGGGACGGCTGACGCCA-3' (Tag2-RT-PCRup) and 5'-GGCCCTCGCCCTCTCCTGCTGTC-3' (Tag2-RTPCRdown) 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* mc²155 (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 *BamH*I-restricted chromosomal DNA from *R. opacus*, yielding a single hybridizing fragment of approx. 4 kbp (not shown). A partial library of *BamH*I-restricted *R. opacus* chromosomal DNA in plasmid pBluescriptSK⁻ 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::Km***

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::Km* 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>
<thead>
<tr>
<th>Strain</th>
<th>No. of <em>Atf1</em> homologues</th>
<th>Protein*</th>
<th>Length (aa)</th>
<th>Identity to <em>Atf1</em> (%)†</th>
<th>Accession no.‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter baylyi ADP1</td>
<td>1</td>
<td>AtfA</td>
<td>458</td>
<td>24</td>
<td>AAO17391</td>
</tr>
<tr>
<td>Mycobacterium smegmatis mc²155</td>
<td>8</td>
<td>MSMEG_3933</td>
<td>451</td>
<td>35</td>
<td>YP_888223</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis H37Rv</td>
<td>15</td>
<td>Rv2484c</td>
<td>491</td>
<td>29</td>
<td>NP_217000</td>
</tr>
<tr>
<td>Rhodococcus jostii RHA1</td>
<td>14</td>
<td>RO00039</td>
<td>473</td>
<td>89</td>
<td>YP_700033</td>
</tr>
<tr>
<td>Streptomyces coelicolor A3(2)</td>
<td>3</td>
<td>SCO1280</td>
<td>413</td>
<td>23</td>
<td>NP_625567</td>
</tr>
<tr>
<td>Alcanivorax borkumensis SK2</td>
<td>2</td>
<td>AtfA1</td>
<td>451</td>
<td>20</td>
<td>YP_693524</td>
</tr>
</tbody>
</table>

*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.
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 \[62.2 \pm 8.6 \text{ pmol (mg min)}^{-1}\] of the respective activity in the parental strain \[169.0 \pm 10.4 \text{ pmol (mg min)}^{-1}\], the WS activity in the knockout mutant did not show a significant decrease \[from \ 79.9 \pm 13.6 \text{ pmol (mg min)}^{-1}\] in the wild-type to \[65.0 \pm 2.3 \text{ pmol (mg min)}^{-1}\] 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 \textit{Rhodococcus} is that of \textit{R. jostii} strain RHA1 (McLeod et al., 2006). The \textit{R. jostii} RHA1 genome data at Canada’s Michael Smith Genome Sciences Centre (http://www.bcgsc.ca) were analysed for the occurrence of \textit{atf1}-homologous genes. Fourteen genes whose products have significant amino acid identity (\(>22\%\)) to the Atf1 protein from \textit{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 \textit{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 \textit{atf1} gene of strain PD630 and with the complete F11 fragment.

The analysis of the \textit{atf1} mutant provided clear evidence that further Atfs are present in \textit{R. opacus} strain PD630. To amplify as many of the additional \textit{atf} genes as possible from \textit{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 \textit{atf}-homologous genes found in the RHA1 genome, were constructed (Supplementary Table S1). By applying these primers, nine further \textit{atf}-homologous genes were successfully amplified (\textit{atf2} to \textit{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 \textit{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 (Cserzö et al., 1997). Eight Atf proteins of strain PD630 possessed at least one putative transmembrane region which may permit proteins

![Fig. 2](http://mic.sgmjournals.org) Lipid analysis of whole-cell extracts of \textit{R. opacus} strains grown with gluconate as carbon source. (a) TLC. Lanes: TAG, TAG standard; Wax, wax standard; 1, wild-type \textit{R. opacus} PD630; 2, mutant \textit{R. opacus} \textit{atf1}Km; 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 \textit{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.
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+ 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::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^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.
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 C16 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

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

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