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The *atf*2 gene is involved in triacylglycerol biosynthesis and accumulation in the oleaginous *Rhodococcus opacus* PD630

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Abstract *Rhodococcus opacus* PD630 is an oleaginous bacterium able to accumulate large amounts of triacylglycerols (TAG) in different carbon sources. The last reaction for TAG biosynthesis is catalyzed by the bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT) enzymes encoded by *atf* genes. *R. opacus* PD630 possesses at least 17 putative *atf* homologous genes in its genome, but only *atf*1 and *atf*2 exhibited a significant DGAT activity when expressed in *E. coli*, as revealed in a previous study. The contribution of *atf*1 gene to TAG accumulation by strain PD630 has been demonstrated previously, although additional Atfs may also contribute to lipid accumulation, since the *atf*1-disrupted mutant is still able to produce significant amounts of TAG (Alvarez et al., Microbiology 154:2327–2335, 2008). In this study, we investigated the in vivo role of *atf*2 gene in TAG accumulation by *R. opacus* PD630 by using different genetic strategies. The *atf*2-disrupted mutant exhibited a decrease in TAG accumulation (up to 25–30 %, w/w) and an approximately tenfold increase in glycogen formation in comparison with the wild-type strain. Surprisingly, in contrast to single mutants, a double mutant generated by the disruption of *atf*1 and *atf*2 genes only showed a very low effect in TAG and in glycogen accumulation under lipid storage conditions. Overexpression of *atf*1 and *atf*2 genes in strain PD630 promoted an increase of approximately 10 % (w/w) in TAG accumulation, while heterologous expression of *atf*2 gene in *Mycobacterium smegmatis* caused an increase in TAG accumulation during cultivation in nitrogen-rich media. This study demonstrated that, in addition to *atf*1 gene, *atf*2 is actively involved in TAG accumulation by the oleaginous *R. opacus* PD630.

Keywords *Rhodococcus* · Triacylglycerols · *atf* · DGAT

Introduction

Triacylglycerols (TAG) are a common form of storage compounds in eukaryotic organisms such as plants, animals, microalga, fungi, and yeast (Leman 1997, Ratledge 1989). However, it is now known that there is a wide variety of bacterial genera able to accumulate these compounds (Alvarez and Steinbüchel 2002, Alvarez 2010). With a few exceptions, the ability to accumulate TAG is common in bacteria belonging to the group of actinomycetes such as *Rhodococcus*, *Nocardia*, *Mycobacterium*, and *Streptomyces* (Barksdale and Kim 1977, Olukoshi and Packter 1994, Alvarez et al. 1996, 1997).

In recent years, these lipids have attracted great interest as potential sources for biotechnological purposes (Gouda et al. 2008, Li et al. 2008). The potential application of bacterial TAG may be similar to that of vegetable sources, including use as feed additives, cosmetics, oleochemicals, lubricants, and other manufactured products. In addition, bacterial oils have been proposed as a source for biofuel production (Alvarez 2010). The biosynthesis and accumulation of TAG is a complex process that involves several catalytic enzymes that participate

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at different metabolic levels. One of the key reactions is the condensation of acyl-CoA with diacylglycerol to form TAG. This reaction is the last step of Kennedy pathway and is catalyzed by the bifunctional wax ester synthase/acyl-CoA: diacylglycerol-acyltransferase (WS/DGAT) enzymes encoded by *atf* genes. The first bacterial *atf* gene was reported for *Acinetobacter baylyi* strain ADP1 (Kalscheuer and Steinbüchel 2003); since then several studies have reported the existence of such enzymes in other Gram-negative bacteria (Kalscheuer et al. 2007, Holtzapple and Schmidt-Dannert 2007). Curiously, in the Gram-positive actinomycetes group, there is a high genetic redundancy of these enzymes, including 15 putative *atf* genes in *Mycobacterium tuberculosis* (Daniel et al. 2004), 14 *atf* genes in *Rhodococcus jostii* RHA1 (Hernández et al. 2008), three in *Streptomyces coelicolor* (Arabolaza et al. 2008), and 17 in *Rhodococcus opacus* PD630 (Holder et al. 2011). Before any rhodococcal genome was available, Alvarez et al. (2008) identified and cloned the first *atf* gene (called *atf*1) in *R. opacus* PD630. An *atf*1 mutant strain of this bacterium showed a significant reduction of DGAT activity and a concomitant reduction of total fatty acids accumulated, 50 % less fatty acids in comparison with the wild type after cultivation on gluconate under nitrogen-limiting conditions (Alvarez et al. 2008). TAG accumulated by the *atf*1-disrupted mutant showed a significant reduction in the oleic acid content in comparison with the TAG produced by the wild-type strain, suggesting a selective incorporation of fatty acyl residues into TAG (Alvarez et al. 2008). In addition, this mutant showed a twofold increase of glycogen formation compared with the wild-type strain, suggesting that TAG and glycogen biosyntheses compete for a common carbon flux in cell metabolism (Hernández and Alvarez 2010). From the ten *atf* genes initially identified in *R. opacus* PD630 (*atf*1 to *atf*10), only *atf*1 and *atf*2 showed significant wax ester synthase (WS) and diacylglycerol acyltransferase (DGAT) activities when heterologously expressed in *E. coli* (Alvarez et al. 2008). For this reason, we decided to investigate the role of *atf*2 in TAG accumulation in this study. *R. opacus* strain PD630 is an oleaginous bacterium able to accumulate large amounts of TAG (up to 70 % of the cellular dry weight, CDW). This bacterium is one of the best-studied regarding TAG biosynthesis and accumulation and has become a model microorganism in this field (Alvarez and Steinbüchel 2010). A better knowledge of lipid metabolism of this microorganism will be relevant for the development of further biotechnological processes.

Materials and methods

Strains, culture conditions, and plasmids

The strains and plasmids used in this work are listed in Table 1. *E. coli* strains were grown on solid or liquid Luria-Bertani (LB) medium at 37 °C. *Rhodococcus* strains were cultivated aerobically at 28 °C in LB medium or minimal salt medium (MSM) according to Schlegel et al. (1961). Sodium gluconate or glucose was used in MSM media as the sole carbon source at a final concentration of 1 % (w/v). In some experiments, hexadecane 0.1 % (v/v) was used. For nitrogen-limiting conditions, the concentration of ammonium chloride in MSM was reduced to 0.1 gl^{-1} (MSM0.1) to allow lipid accumulation (storage conditions). Cells were harvested at specific timepoints and washed with a NaCl solution (0.85 %, w/v) and dried at 37 °C to a constant weight for chemical analyses. If necessary, antibiotics were used at the following final concentrations: 100 $\mu g ml^{-1}$ ampicillin (Ap), 50 $\mu g ml^{-1}$ kanamycin (Km), 5 $\mu g ml^{-1}$ gentamycin (Gm), 34 $\mu g ml^{-1}$ chloramphenicol (Cm), 100 $\mu g ml^{-1}$ apramycin (Am), and 100 $\mu g ml^{-1}$ spectinomycin (Spc) for both *E. coli* and *Rhodococcus* strains. Induction of the acetamidase (P*ace*) promoter of pJAM2 and its derivatives was routinely achieved by addition of 0.5 % (w/v) acetamide to the respective cultures. Induction of the thiostrepton promoter (P*tipA*) of pTip-QC2 and its derivatives was routinely achieved by addition of 1 $\mu g ml^{-1}$ of thiostrepton to the respective cultures.

DNA analysis, amplification, cloning, and sequencing

Chromosomal DNA, plasmids, and DNA fragments were isolated and analyzed by standard methods. For DNA amplification of internal DNA fragments or complete ORFs, PCR was performed using specific primers (Table 2). The general thermocycler parameters used were as follows: 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 30 s at 60 °C, 1 min at 72 °C, and finally 5 min at 72 °C. The PCR products were cloned into pGEM-T-easy vector and subjected to DNA sequencing.

DNA transfer via conjugation and electroporation into *Rhodococcus* cells

Plasmids and derivatives were introduced into *R. opacus* strains via conjugation or electroporation. For conjugation experiments, *E. coli* DH5α harboring pKOS111-47 helper plasmid and a mobilizable plasmid was used as donor and *Rhodococcus* strains as recipients. Both strains, donor and recipients, were grown in LB until cultures reached an $OD_{600\ nm}$ of 0.5, and 100 μl of each strain was plated on LB agar medium. The plates were incubated for 16 h at 30 °C and the transconjugants were then replated on LB agar medium containing Km (50 $\mu g ml^{-1}$) and nalidixic acid (30 $\mu g ml^{-1}$). The plates were incubated for 3–5 days at 30 °C. Electroporation assays were performed using a Model 2510 electroporator (Eppendorf-Netheler-Hinz

Table 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
E. coli		
DH5α	*E. coli K-12 F–lacU169* (*Φ80lacZΔM15*) *endA1 recA1 hsdR17 deoR supE44 thi-1-l2 gyrA96 relA1*	Hanahan (1983)
JM109	*endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB*$^+$ Δ(*lac-proAB*) *e14-*[*F' traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*] *hsdR17*($r_K^- m_K^+$)	Promega
DH5α pKOS111-47	*E. coli* DH5α carrying pKOS111-47 as conjugation donor	Arabolaza et al. (2010)
Rhodococcus opacus		
PD630	Parental strain	DSM 44193.
PDTag1	*atf1* disruption mutant, Km^R; derivative of PD630	Alvarez et al. (2008)
PDTag2	*atf2* disruption mutant, Km^R; derivative of PD630	This study
PDTag2C	PDTag2 mutant, Km^R, complemented with pPR27/*atf2*; Gm^R	This study
PDTag1/2 clone 3	*atf1*/*atf2* disruption mutant, Km^R , Am^R; derivative of PD630	This study
PDTag1/2 clone C	*atf1*/*atf2* disruption mutant, Km^R, Spc^R; derivative of PD630	This study
PD630 pJAM2	PD630 derivative carrying pJAM2 plasmid, used as control strain; Km^R	This study
PD630 pJAM2/ATF1	PD630 derivative carrying pJAM2/*atf*1; Km^R	This study
PD630 pPR27	PD630 derivative carrying pPR27 plasmid, used as control strain; Gm^R	This study
PD630 pPR27ace/ATF2	PD630 derivative carrying pPR27/*atf2*; Gm^R	This study
PD630 pTip-QC2	PD630 derivative carrying pTip-QC2 plasmid, used as control strain; Cm^R	This study
PD630 pTip-QC2/ATF2	PD630 derivative carrying pTip-QC2/*atf2*; Cm^R; Thio^R	This study
Mycobacterium smegmatis		
mc^{2}155/pVV2	mc^{2}155 carrying pVV2; Km^R	This study
mc^{2}155 pVV2/ATF2	mc^{2}155 derivative carrying pVV2/*atf2*; Km^R	This study
Plasmids		
pGEM-T-easy vector	Linear plasmid used for cloning PCR products; Ap^R	Promega
pGEM-T-easy/"*atf2*" ΩSp	pGEM-T-easy derivative carrying 702 bp of *atf2*; Spc^R	This study
pGEM-T-easy/"*atf2*" ΩAm	pGEM-T-easy derivative carrying 702 bp of *atf2*; Am^R	This study
pGEM-T-easy/"*atf2*"-*ori*T-ΩKm	pGEM-T-easy derivative carrying 702 bp of *atf2* and *ori*T fragment; Km^R	This study
pGEM-T-easy/*atf*1	pGEM-T-easy derivative carrying intact copy of *atf*1; Ap^R	This study
pGEM-T-easy/*atf2*	pGEM-T-easy derivative carrying intact copy of *atf2*; Ap^R	This study
pRT801	Integrative vector derived from the ΦBT1 *attP-int* locus for *Streptomyces*	Gregory et al. (2003)
pUC4K	Cloning vector carrying a cassette of Kanamycin resistance	Amersham Biosciencies
pKOS111-47	RK2 derivative with defective *ori*T; Ap^R	Arabolaza et al. (2010)
pJAM2	Shuttle *E. coli–Mycobacterium–Rhodococcus* with P*ace* promoter; Km^R	Triccas et al. (1998)
pJAM2/*atf*1	pJAM2 derivatives carrying *atf*1 gene under control of P*ace*; Km^R	This study
pMVace (pMR22)	Integrative pMV306 plasmid of *Mycobacterium* with P*ace* promoter; Km^R	Salzman et al. (2010)
pMVace/*atf2*	pMVace carrying *atf2*-Hys tag fusion gene under control of P*ace*; Km^R	This study
pPR27	*E. coli–Mycobacterium* shuttle vector, *ori*M temps, *sacB*, *Xyl*E; Gm^R	Pelicic et al. (1997)
pPR27ace/*atf2*	pPR27 derivative carrying *atf2* gene under control of P*ace*; Gm^R	This study
pVV2	Expression plasmid for Mycobacteria with P*hsp*60 constitutive promoter; Km^R	Dhiman et al. (2004)
pVV2/*atf2*	pVV2 derivative carrying *atf2* gene under control of P*hsp*60; Km^R	This study
pTip-QC2	Expression vector fo*r Rhodococcus* with P*tipA* promoter, *repAB* (pRE2895); Cm^R	Nakashima and Tamura (2004)
pTip-QC2/*atf2*	pTip-QC2 carrying *atf2* gene under control of P*tipA*; Cm^R	This study

GmbH). Transformation of *R. opacus* strain PD630 was performed as described by Kalscheuer et al. (1999) with minimal modifications. Immediately before electroporation, 400 μl of competent cells was mixed with DNA and preincubated at 40 °C for 5 min followed by 10 min of incubation on ice and then electroporated.

Table 2 Oligonucleotides used as PCR primers

Primers	Sequence[a]
atf2mhF	5′TCGCGAGAGGATCCGATGCACG 3′
atf2mhR	5′GCGTTCAAGCTTCCAGGACTGC 3′
atf2MHF	5′TGGGGCATATGCCGGTTACCGATTC 3′
atf2MHR	5′AAGCTTTCAGAGCAATGCCGCCTCGA 3′
M13F	5′CGCCAGGGTTTTCCCAGTCACGAC 3′
M13R	5′TCACACAGGAAACAGCTATGAC 3′
atf1MHF	5′ACGAATCAGGGATCCATGACCCAGACGGA 3′
atf1MHR	5′GTGCGTGTGTCTAGAGCACGAGG 3′
aceF	5′TCGCAGCGCCGTCAGTCACCAA 3′

[a] Restriction sites used for cloning purposes are underlined

Generation of single *atf*2 and double *atf*1–2 disrupted mutants of *R. opacus* PD630

To disrupt the *atf*2 gene, we used a derivate of pGEM-T-easy vector as a suicide plasmid and then followed a strategy previously reported for *Rhodococcus erythropolis* SQ1 (Van der Geize et al. 2000). For this, an internal 700-bp fragment of *atf*2 gene was amplified by PCR using the primers atf2mhF and atf2mhR and cloned in the pGEM-T-easy vector to get the plasmid pGEM-T-easy/"*atf*2". Then, a kanamycin-resistant cassette obtained from the pUC4K plasmid was cloned into a *Pst*I restriction site of pGEM-T-easy/"*atf*2" to get pGEM-T-easy/"*atf*2"-ΩKm. Finally, a 800-bp *Hinc*II fragment containing an *ori*T site from plasmid pRT801 (Gregory et al. 2003) was cloned into a *Sca*I site to get pGEM-T-easy/"*atf*2"-*ori*T-ΩKm. The resulting suicide plasmid was transferred in *R. opacus* PD630 by conjugation using *E. coli* DH5α-pKOS111-47 as donor. After 4–5 days, transconjugants were obtained, isolated, and analyzed by colony PCR using primers M13F and atf2MHR. A single Km-resistant colony named PDTag2 was selected for further analysis after its genotype was confirmed by PCR from chromosomal DNA.

For complementation of the *atf*2 mutant, a *Nde*I/*Hind*III fragment of pGEMT-easy/*atf*2 (Table 1, see below) was subcloned into a *Nde*I/*Hind*III site of the integrative *Mycobacterium* plasmid pMVace (pMR22, Table 1) which contains an inducible acetamide promoter (P*ace*) and six histidine codons upstream of the *Nde*I site yielding pMVace/*atf*2. To obtain a replicative version of the plasmid, a *Not*I/*Bam*HI fragment of pMVace/*atf*2 containing the his_6-*atf*2 gene under the control of P*ace* was subcloned into *Not*I/*Bam*HI of pPR27 vector yielding pPR27ace/*atf*2. The resulting plasmid was electroporated into PDTag2 mutant cells and transformants were selected on solid LB plates with Gm and Km.

To obtain the *atf*1–*atf*2 double mutant, we used a similar strategy as for the generation of the *atf*2 single mutant. The pGEM-T-easy/"*atf*2" vector (containing the internal fragment of *atf*2) was cut in the *Bam*HI site provided by the sequence of the forward primer used for its amplification (Table 2), dephosphorylated, and ligated with a Spc or Am resistance cassette. The plasmids pGEM-T-easy/"*atf*2"-ΩAm and pGEM-T-easy/"*atf*2"-ΩSpc were transferred by electroporation in PDTag1 strain. After 3–5 days, the obtained Spc- or Am-resistant colonies were analyzed to confirm the single homologous recombination event by PCR using primer M13R and atf2MHF. Two independent colonies being Spc-Km or Am-Km resistant, respectively, were selected, confirmed genotypically, and used for further analysis.

Cloning and overexpression of *atf*1 and *atf*2 genes from *R. opacus* PD630

*atf*1 and *atf*2 genes were amplified from total genomic DNA of *R. opacus* PD630 by PCR using the primers *atf*1MHF and *atf*1MHR, and *atf*2MHF and *atf*2MHR, respectively (Table 2). The resulting PCR products were cloned in pGEMT-easy vector (pGEMT-easy/*atf*1 and pGEMT-easy/*atf*2, Table 1) and verified by DNA sequencing. For overexpression of *atf*1 gene, a *Bam*HI/*Xba*I digest from pGEMT-easy/*atf*1 was subcloned into the *Bam*HI/*Xba*I site of the shuttle *E. coli–Mycobacterium–Rhodococcus* vector pJAM2, which contains an inducible acetamide promoter (P*ace*) and six histidine codons downstream of the *Xba*I site yielding pJAM2/*atf*1.

For overexpression of *atf*2 gene, the same strategy as for the complementation of PDTag2 mutant was used, but this time, the plasmid (pPR27ace/*atf*2) was transferred into wild-type cells. Overexpression of *atf*1 and *atf*2 was analyzed by western blotting according to standard methods using monoclonal anti-his_6 antibodies. On the other hand, we used an alternative strategy for *atf*2 overexpression in *Rhodococcus* cells. A *Nde*I/*Hind*III fragment from pMVace/*atf*2 was subcloned into the *Nde*I/*Hind*III site of the expression vector pTip-QC2 to obtain pTip-QC2/*atf*2. This time, *atf*2 is expressed without the six histidine codons and under an inducible thiostrepton promoter (P*tipA*).

In the case of *atf*2 gene, a third strategy was tested. A *Nde*I/*Hind*III digest from pMVace/*atf*2 was subcloned into the *Nde*I/*Hind*III site of the expression vector pVV2 and transferred into *Mycobacterium smegmatis* cells.

All constructions were transferred by electroporation. The transformants containing *atf*1 or *atf*2 genes under *ace*-promoter were checked using primers *ace*F/*atf*1MHR or *ace*F/*atf*2MHR, respectively.

Lipid analysis

The qualitative and semiquantitative analyses of total intracellular lipids in *R. opacus* PD630 were carried out by thin layer chromatography (TLC). For this, 5 mg of lyophilized cells was extracted by means of vigorous shaking with 100

or 200 μl chloroform/methanol (2:1, v/v) for 90–120 min at 4 °C. Fifteen to 30 μl of chloroformic phase (depending on the strain or culture conditions) was then subjected to TLC on silica Gel 60 F254 plates (Merck) using hexane/diethyl ether/acetic acid (80:20:1, by vol.) as mobile phase (Wältermann et al. 2000). Tripalmitin (Merck) was used as TAG reference substance. Lipid fractions were revealed using iodine vapor.

Since in *Rhodococcus* cells TAG account for over 98 % of the total lipids, the quantification of total fatty acids is a good estimation of TAG content. For qualitative and quantitative determination of total fatty acids, 5–8 mg of dry cells was subjected to methanolysis in the presence of 15 % (v/v) sulfuric acid as described by Brandl et al. (1988), and the resulting acyl-methylesters were analyzed by gas chromatography (GC) using an HP 5890 A gas chromatograph equipped with an InnoWAX capillary column (30 m × 0.53 mm × 1 μm) and a flame ionization detector. The injection volume was 0.2 ml, and helium (13 ml min^{-1}) was used as a carrier gas. A temperature program was used for efficient separation of the methyl esters (90 °C for 5 min, temperature increase of 6 °C min^{-1}, 220 °C for 10 min). For quantitative analysis, tridecanoic acid was used as internal standard.

In addition, a copper method for total fatty acid quantification established in previous works (Wawrik and Harriman 2010, Duncombe 1963) was performed with some modifications. For this, 5 mg of dry cells was hydrolyzed with alkaline reagent (25 % methanol in NaOH 1 N) at 95–100 °C for 3 h and the resultant sodium salts of fatty acids were treated with concentrated acetic acid. Then, copper reagent, chloroform for lipid extraction, and revealing reagent were sequentially added. Finally, samples were analyzed spectrophotometrically at 440 nm wavelength. The standard curve was performed with oleic acid as representative fatty acid of TAG in *R. opacus* cells.

Polysaccharide analysis in mutant strains

Qualitative and quantitative analyses of the intracellular polysaccharide content in mutant strains were performed according to Hernández and Alvarez (2010). Wild-type cells of *R. opacus* were used as control.

Results

Occurrence of additional putative *atf* genes in *R. opacus* PD630 and analysis of *atf*2 gene sequence

The recently published complete genome of *R. opacus* PD630 indicates the occurrence of at least 17 *atf* homologous genes. Some of these annotated genes, such as OPAG_06690 and OPAG_07212, do not present the putative active site motif HHxxxDG usually described for WS/DGAT proteins (Kalscheuer and Steinbüchel 2003), or as in the case of OPAG_07837, the canonical catalytic active site motif is modified (SHVLADA). Extending this analysis to other related *Rhodococcus* species (*R. opacus* B4 and *R. jostii* RHA1), we identified all putative *atf* genes in these bacteria and built a phylogenetic tree with all annotated putative Atf homologous proteins containing an evident HHxxxDG-like active site motif (Fig. S.1). Considering this clustering, we built Table 3 that summarizes the occurrence of these *atf* genes in *R. opacus* PD630 and their gene orthologs in *R. opacus* B4 and *R. jostii* RHA1. Fourteen of the *R. opacus* PD630 genes possess a corresponding *atf* ortholog in *R. jostii* RHA1. In contrast, OPAG_06225 and OPAG_06690 genes do not possess their orthologs in *R. jostii* RHA1 and in *R. opacus* B4; thus, they may be considered as specifics for the *R. opacus* PD630 strain. On the other hand, a genomewide bioinformatic analysis of the *atf* genes present in all the *Rhodococcus* species which genomes have been sequenced (including *R. opacus* PD630, *R. jostii* RHA1, *R. opacus* B4, *R. erythropolis* PR4, *R. erythropolis* SK121 and *R. equi* ATCC33707) revealed that *atf*2 (OPAG_00138) of *R. opacus* PD630 is conserved in all these species, unlike other rhodococcal *atf* genes which are absent in some of them. Despite the fact that *atf*2 gene seems to be highly conserved in rhodococcal genomes, its genomic context is not conserved among the analyzed species. A multiple amino acid sequence alignment for Aft2 of *R. opacus* PD630 and its protein orthologs in other species is shown in Fig. 1. The Atf2 sequence shares high similarity with its orthologs from *R. jostii* RHA1 (98 % identity) and *R. opacus* B4 (98 %) and lower similarity with its orthologs from *R. erythropolis* PR4 (52 %), *R. erythropolis* SK121 (52 %), and *R. equi* ATCC33707 (48 %).

Disruption of *atf*2 and *atf*2–*atf*1 genes in *R. opacus* PD630 and their effect on TAG accumulation

An *atf*2 knockout mutant was constructed by a single recombination strategy and the genotype confirmed by PCR (Fig. 2). Growth of the mutant, called PDTag2, was comparable to that of the wild type in both solid and liquid LB or MSM media (data not shown). As expected, and according to the predicted DGAT activity of Atf2, the mutant strain revealed a decrease in TAG content compared with the wild-type strain (Fig. 3a). Quantitative analyses revealed a decrease of the total fatty acids of the mutant PDTag2 of 10–30 % (w/w of the CDW) in comparison to the wild type. The highest differences in total fatty acid content were found after 48 and 72 h of cultivation in MSM0.1 medium with gluconate as the sole carbon source (Fig. 3b), which correlates well with the differences found in TAG contents (Fig. 3a).

Table 3 Ocurrence of acyltransferases (Atfs) in *R. opacus* PD630 and their orthologs in *R. jostii* RHA1 and *R. opacus* B4

Atfs of *R. opacus* PD630 NCBI database[a]	Orthologs in *R. jostti* RHA1	Orthologs in *R. opacus* B4	PD630 HHxxxDG putative active site motif
OPAG_03922 (Atf10)[a]	RO_00087	ROP_02100	HLSLTDG
OPAG_03918	RO_08645	–	HHALADG
OPAG_03334 (Atf5)	RO_06332	ROP_63930	HSSLVDG
OPAG_03807 (Atf7)	RO_06855	ROP_68400	HHAGVDG
OPAG_04307 (Atf3)	RO_05649	ROP_57120	HHSITDG
OPAG_07837	RO_05565	ROP_56340	SHVLADA
OPAG_07648 (Atf8)	RO_05356	ROP_54550	HHAVADG
OPAG_05286 (Atf9)	RO_00583	ROP_04650	HHAMADG
OPAG_05208	RO_08660	–	HPAVIDG
OPAG_06690	–	–	–
OPAG_07212 (low quality protein)	C-terminal of RO_00087	C-terminal of ROP_02100	–
OPAG_07257 (Atf1)	RO_00039	–	HHSLTDG
OPAG_07266 (Atf4)	RO_00024	–	HHCMADG
OPAG_07267	RO_00023	–	HHVVADG
OPAG_06225	–	–	HHSVGDG
OPAG_00138 (Atf2)	RO_01601	ROP_13050	HHSLMDG
OPAG_01553 (Atf6)	RO_02966	ROP_26950	HHALADG

[a] Equivalent nomenclature previously reported in Alvarez et al. (2008)

To confirm that the phenotype of the PDTag2 mutant was exclusively related to the absence of *atf*2, we complemented this mutant strain with a replicative vector pPR27ace/*atf*2 containing the *his*$_6$-*atf*2 gene under P*ace* to yield PDTag2C and analyzed its lipid content. PDTag2C accumulated higher amounts of TAG in comparison with the PDTag2 mutant, but lesser amounts than wild-type cells (Table 4, Fig. 3). The partial complementation of PDTag2 may be explained by the low expression rates of recombinant genes induced by *ace* promoters when cloned in *Rhodococcus* species (Hänisch et al. 2006a, b).

In a previous study, it was demonstrated that Atf1 and Atf2 were the unique proteins, among the ten Atfs studied in strain PD630, which exhibited high DGAT and/or WS activity when heterologously expressed in *E. coli* (Alvarez et al. 2008). Based on these results, we decided to construct a double *atf*1–*atf*2 knockout mutant of *R. opacus* PD630 and determine the effect on TAG biosynthesis. For this purpose, we disrupted the *atf*2 gene in the PDTag1 mutant (*atf*1ΩKm in Alvarez et al. 2008) by a single recombination strategy, obtaining the PDTag1/2 double mutant strain (Fig. S.2). Attempts to obtain the double mutant by electroporation showed a relatively high frequency of illegitimate recombination, in contrast to the DNA transfer methodology based in conjugation for PDTag2 construction (data not shown). After checking several colonies and confirming the correct genotype by PCR (Fig. S.2), we analyzed the TAG-related phenotype of the resulting double mutant in comparison with the wild-type and the single *atf* mutants (PDTag1 and PDTag2). When cells of the double mutant PDTag1/2 (clone C) were cultivated in nitrogen-rich media, such as LB or MSM1 with gluconate as the sole carbon source, they accumulated lower amounts of TAG compared with the wild-type and the single mutant PDTag2 (Table 4 and Fig. 4a). In contrast, after cultivation of cells under storage conditions (MSM0.1) with gluconate as the sole carbon source, the double mutant PDTag1/2 (clone C) showed TAG content similar to the wild-type strain (Fig. 4b). As shown in Table 4, the same phenotype was observed in a second clone (clone 3) harboring a double knockout of *atf*1 and *atf*2 and that was isolated independently from the first one (clone C).

Analysis of fatty acid composition revealed only slight differences between the wild-type and the single *atf*2 or the double *atf*1–*atf*2 mutant strains. In this context, reduced oleic acid content and a slight increase of medium chain and the 17:1 unsaturated fatty acids were observed in the double mutant strain (Fig. 4c). Interestingly, the cell morphology of the double mutant PDTag1/2 was similar to that of the wild-type PD630, with short and wide cells showing lipid inclusion bodies, in contrast to the more elongated and thinner cells observed for the PDTag2 mutant (Fig. 5).

Overexpression analyses of *atf*1 and *atf*2 genes in *R. opacus* PD630

In order to analyze the in vivo effect of *atf*1 and *atf*2 overexpression in *R. opacus* PD630, we constructed the strains PD630 pJAM2/ATF1 and PD630 pPR27ace/ATF2. In both

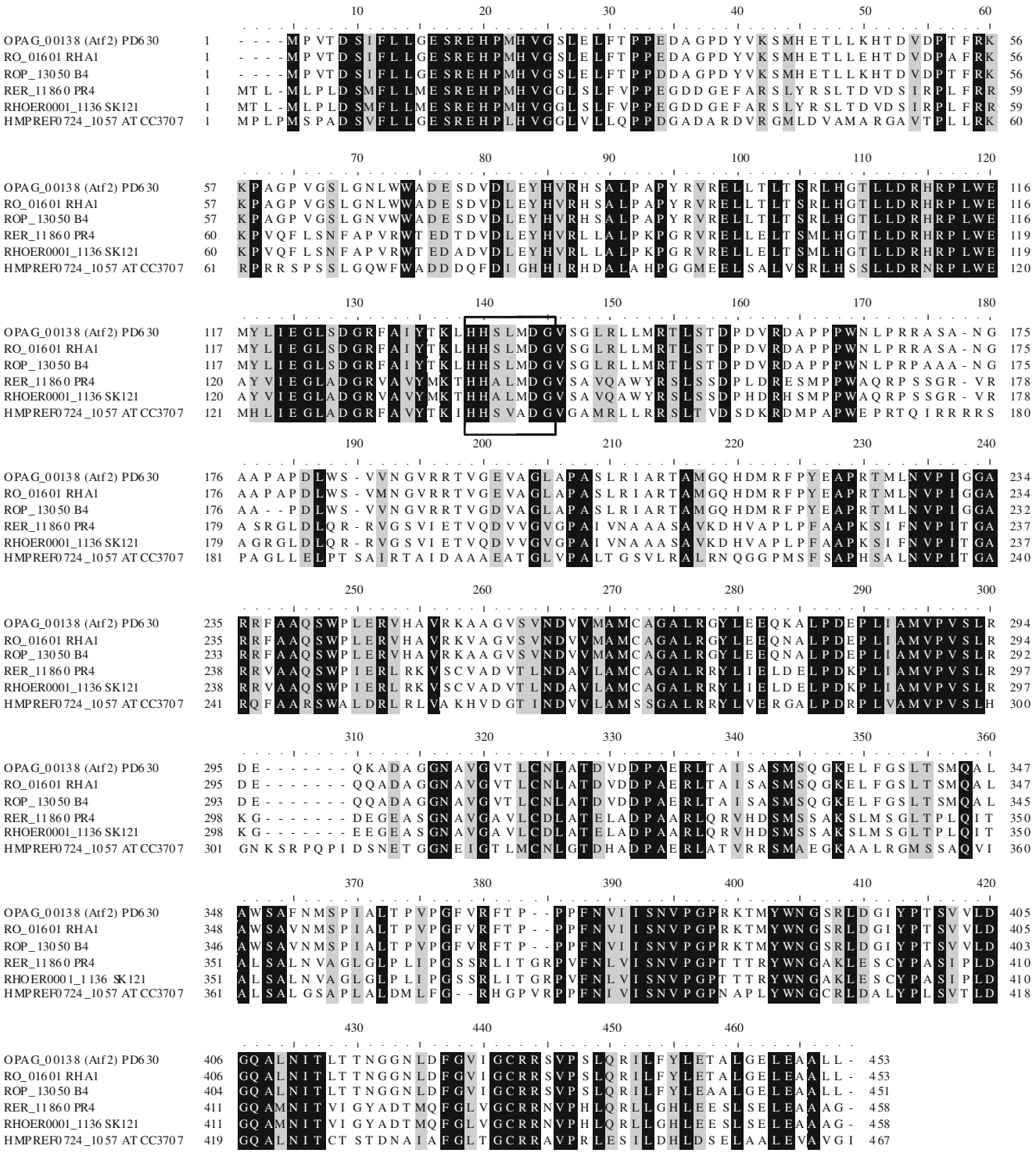

Fig. 1 Multiple sequence alignment between Atf2 of *R. opacus* PD630 and the orthologs of other *Rhodococcus* species. Conserved amino acids are shaded in *black* and homologous residues in *grey*. The putative active site is *boxed*. Ref: ro01601, *R. jostii* RHA1 (98 %); ROP_130150, *R. opacus* B4 (98 %); RER_11860, *R. erythropolis* PR4 (52 %); RHOER0001_1136, *R. erythropolis* SK 121 (52 %); HMPREF0724_10657, *R. equi* ATCC 33707 (48 %). Percentages of identities were calculated relative to *R. opacus* PD630 Atf2 protein sequence

plasmids, the corresponding *atf* genes were cloned under the acetamide-inducible promoter (Table 1). Expression of *atf*1 and *aft*2 from P*ace* was confirmed by western blot experiments using anti-His_6 antibodies (Fig. S.3). TLC analyses of the recombinant strains revealed an increase in TAG content after the induction of *atf*1 or *atf*2 expression in strain

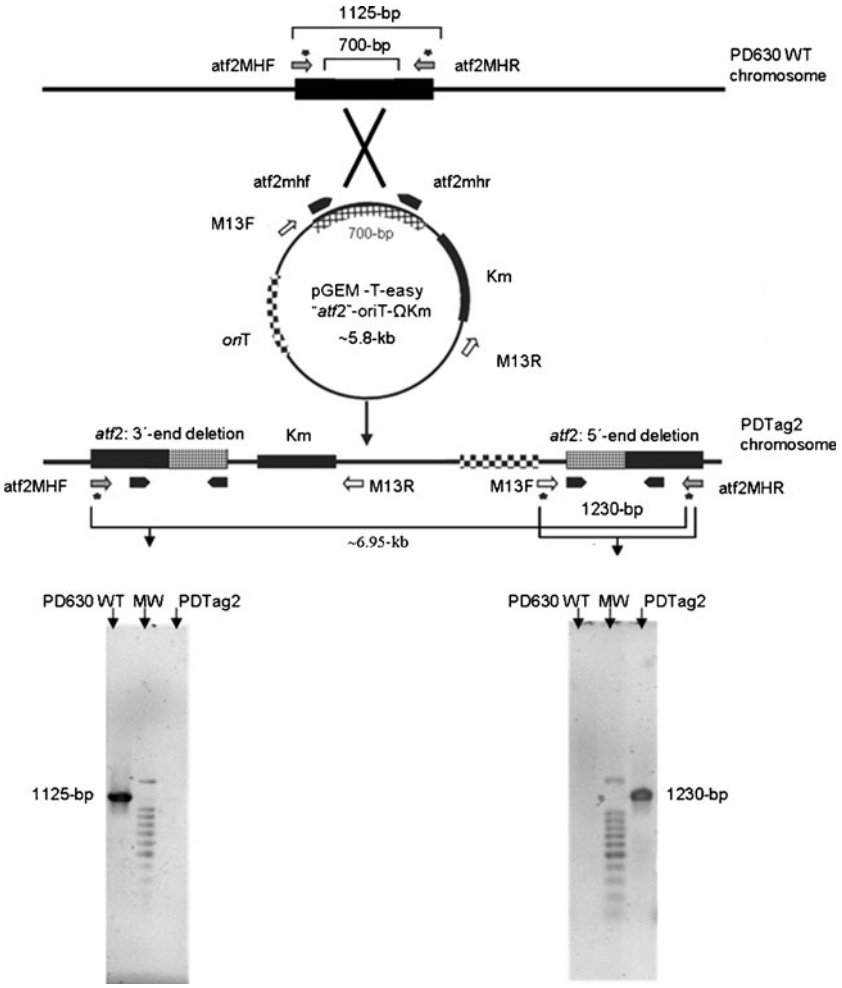

Fig. 2 Schematic overview of targeted gene disruption of *atf2* by integration of pGEM-T-"*atf2*"-*ori*T-ΩKm into the *R. opacus* PD630 chromosome. Single homologous recombination occurs between wild-type *atf2* gene on the *R. opacus* PD630 chromosome and a 700-bp internal "*atf2*" fragment located in the plasmid. Individual transconjugants (PDTag2) were checked for loss of the wild-type *atf2* PCR fragment (~1,125 bp) using atf2MHF/atf2MHR primers and by identification of a new PCR fragment (~1,230 bp, including a portion of suicide plasmid) using M13F/atf2MHR primers. All primers used are indicated with an *asterisk*. *MW* 1,500-bp molecular marker (Promega, USA)

PD630, when they were cultivated in MSM0.1 with glucose or hexadecane as sole carbon sources (Fig. 6a and b). Quantification of lipids showed an increase in TAG content of approximately 10 % (CDW) in comparison with the wild type (Table 5). Alternatively, *atf2* was subcloned into pTip-QC2 vector and expressed in strain PD630. TAG analyses

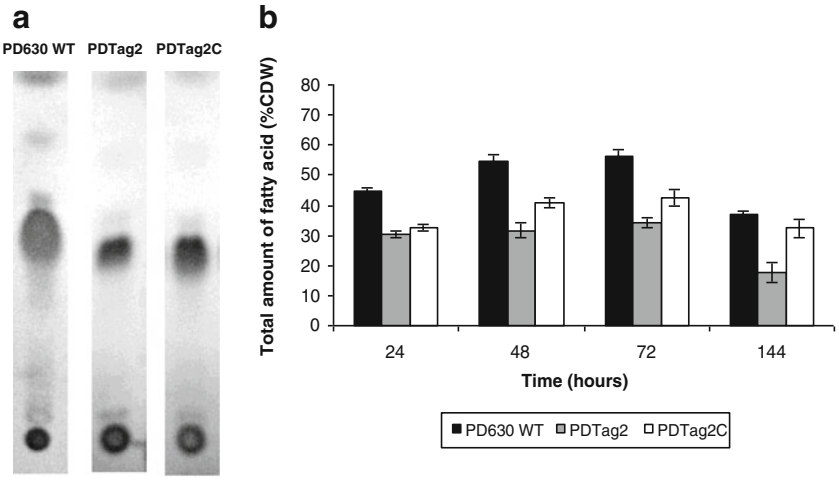

Fig. 3 Lipid analysis of whole-cell extracts of *R. opacus* strains grown in MSM0.1 with gluconate as carbon source. Cells were grown in LB medium for 24 h, harvested, washed and then incubated under storage conditions. **a** TLC analysis of wild-type *R. opacus* PD630, PDTag2, and PDTag2C after 48 h. **b** Total amounts of fatty acids in PD630 WT, PDTag2, and PDTag2C at different growth stages. Because acetamide can affect nitrogen levels of media and the concomitant TAG accumulation, it was added not only to PDTag2C but also to PD630 wild type and PDTag2 at the same final concentration

Table 4 Total fatty acid and glycogen content in mutant strains

Strain	Culture conditions	% total fatty acid content (CDW)	% glycogen (CDW)
PD630	MSM0.1/gluconate 1 % 48 h	62.5	0.9
PDTag1	MSM0.1/gluconate 1 % 48 h	48.0	–
PDTag2	MSM0.1/gluconate 1 % 48 h	35.2	8.2
PDTag2C	MSM0.1/gluconate 1 % 48 h	47.0	–
PDTag1/2 clone 3	MSM0.1/gluconate 1 % 48 h	59.2	–
PDTag1/2 clone C	MSM0.1/gluconate 1 % 48 h	60.1	1.3
PD630	MSM1/gluconate 1 % 48 h	25.4	–
PDTag2	MSM1/gluconate 1 % 48 h	19.6	–
PDTag1/2 clone 3	MSM1/gluconate 1 % 48 h	16.0	–
PDTag1/2 clone C	MSM1/gluconate 1 % 48 h	15.4	–
PD630	LB 24 h	16.1	–
PDTag2	LB 24 h	9.8	–
PDTag1/2 clone 3	LB 24 h	8.6	–
PDTag1/2 clone C	LB 24 h	8.2	–

– not analyzed

also revealed an increase of the TAG content in the recombinant cells in a similar proportion to that of the PD630 pPR27ace/ATF2 strain (Fig. 6c and Table 5).

Furthermore, we subcloned *atf*2 of *R. opacus* PD630 in pVV2 vector under the constitutive *Hsp*60 promoter and introduced this plasmid into *M. smegmatis* cells. *Mycobacterium* strains are able to accumulate TAG as storage lipids, but they usually produce lower amounts of TAG than *R. opacus* PD630 (Daniel et al. 2004, Hänisch et al. 2006a, b). The heterologous expression of *atf*2 gene from *R. opacus* PD630 in *M. smegmatis* promoted an increase in the TAG content as revealed by TLC analysis (Fig. 6d), when cells were cultivated in nitrogen-rich media, such as LB and MSM1 with glucose as the sole carbon source. Curiously, during cultivation under nitrogen-limiting conditions (MSM0.1), *atf*2-expressing cells of *M. smegmatis* produced lesser amounts of TAG than wild-type cells (Table 5). In contrast to *M. smegmatis* wild-type cells, recombinant cells formed clumps in liquid medium and the colony morphology on solid agar was modified under storage condition.

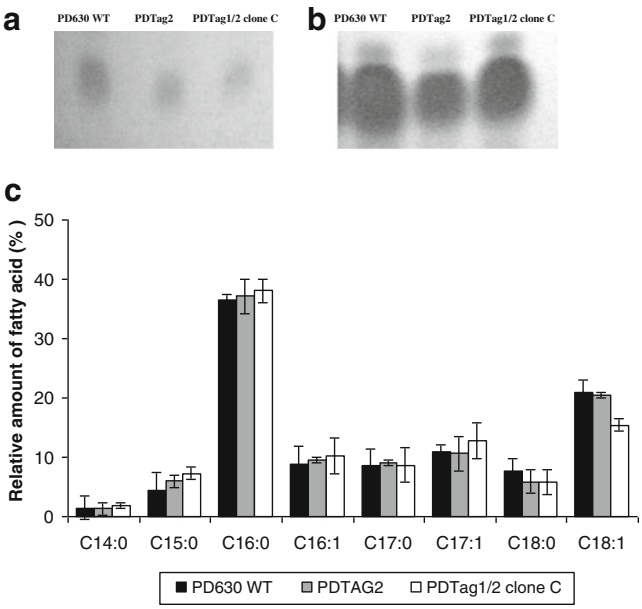

Fig. 4 Analysis of *R. opacus* wild-type and mutants strains. **a** TLC analysis of cells grown on LB medium at 24 h. **b** TLC analysis of cells grown under storage conditions MSM0.1 with gluconate as carbon source after 48 h. **c** Fatty acid composition of whole cells of *R. opacus* PD630 WT, PDTag2, and PDTag1/2 clone C after cultivation of cells in MSM0.1 and 1 % (w/v) of glucose for 48 h. The data represent the means of three independent experiments

Glycogen formation by *atf* mutants of *R. opacus* PD630

In a previous study, we observed a metabolic relationship between TAG accumulation and glycogen formation in different strains of the *Rhodococcus* genus (Hernández and Alvarez 2010). We demonstrated that the *atf*1 mutant of strain PD630 showed a twofold increase in glycogen content in comparison with the wild type during cultivation on gluconate (Hernández and Alvarez 2010). Here, we analyzed the glycogen formation by the single mutant PDTag2 and the double mutant PDTag1/2 after cultivation on gluconate as the sole carbon source. The *atf*2 disruption in *R. opacus* PD630 resulted in an approximately tenfold increase in the glycogen content in comparison with the wild type (Table 4). In contrast, the double mutant PDTag1/2 (clone C), which in gluconate accumulates similar amounts of TAG than the wild type, exhibited a similar content of glycogen in comparison with the wild-type strain (Table 4).

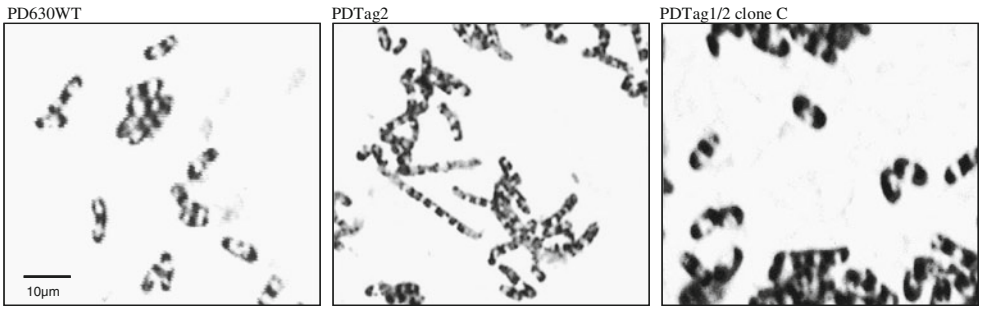

Fig. 5 Cell morphology on MSM0.1 plus gluconate 1 % at 48 h. The photographs were obtained with Motic Images Plus 2 ML software and have been digitally processed to increase magnification (original magnification 1,000×)

Discussion

A previous study demonstrated that *R. opacus* PD630 possesses at least ten genes coding for putative WS/DGAT or Atf enzymes, which may be involved in TAG biosynthesis and accumulation by this strain (Alvarez et al. 2008). Since *atf*2, in addition to *atf*1, was the unique enzyme that exhibited WS as well as significant DGAT activities when expressed in *E. coli* (Alvarez et al. 2008), we decided to analyze the influence of *atf*2 on TAG accumulation in *R. opacus* PD630. According to our study, the predicted Atf2 protein has a length of 453 amino acid residues instead of 374 as has been reported in a previous work (Alvarez et al. 2008). This result was definitely confirmed with the recent publication of the complete *R. opacus* PD630 genome. In addition, a thorough genome analysis showed that *R. opacus* PD630 possesses at least 17 putative *atf* genes. Further studies are necessary to prove the functionality of these putative *atf* genes and their role in TAG and/or wax biosynthesis and accumulation.

The disruption of *atf*2 promoted a decrease in TAG accumulation ranging from 10 to 30 % in comparison with the wild-type strain PD630. However, PDTag2 was still able to accumulate significant amounts of TAG, which confirmed the contribution of additional Atfs to TAG biosynthesis and accumulation in *R. opacus* PD630. Bioinformatic analysis of genomic databases shows that rhodococcal genomes usually contain a high redundancy of *atf* genes (Alvarez and Steinbüchel 2010), as has been also observed for other actinomycetes (Daniel et al. 2004, Wältermann et al. 2000). The number of *atf* genes found in the rhodococcal genomes seems to be a strain-dependent feature. The diversity of WS/DGAT isoenzymes in *Rhodococcus* may permit cells to incorporate fatty acids into TAG with different specificity or to activate lipid accumulation under different conditions. In this context, the disruption of *atf*1 resulted in a decrease of TAG accumulation with a reduced oleic acid content when cultivated in the presence of gluconate or oleic acid (Alvarez et al. 2008). A similar effect on fatty acid composition has been observed in the double mutant PDTag1/2 in this study. This result suggests that Atf1 isoenzyme possesses some preference for incorporation of oleic acid into TAG. In contrast, the disruption of *atf*2 in PD630 did not result in any evident modification in the fatty acid composition of TAG.

Since *atf*1 and *atf*2 of strain PD630 showed the highest enzymatic activities during expression in *E. coli* and the other eight tested *atf* genes exhibited none or only low WS or DGAT activities (Alvarez et al. 2008), a double *atf*1–*atf*2 mutant was constructed in this study in order to determine

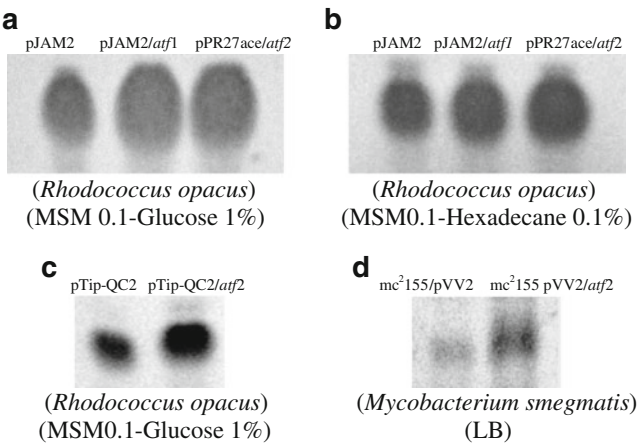

Fig. 6 Semiquantitative TLC analysis of TAG in parental and recombinant *Rhodococcus* strains grown with glucose 1 % (**a** and **c**) or hexadecane 0.1 % (**b**) as the sole carbon source in MSM0.1 medium and of parental and recombinant *Mycobacterium* strains in LB (growth conditions) (**d**)

Table 5 Total fatty acid content in recombinant strains and controls in MSM0.1 medium at 48 h

Strain	Substrate/s	% total fatty acid content (CDW)
PD630 pJAM2	Glucose/hexadecane	38.3/22.0
PD630 pJAM2/ATF1	Glucose/hexadecane	45.5/29.3
PD630 pPR27	Glucose/hexadecane	33.8/21.0
PD630 pPR27ace/ATF2	Glucose/hexadecane	45.8/30.4
PD630 pTip-QC2	Glucose	43.8
PD630 pTip-QC2/ATF2	Glucose	54.1
mc^2155/pVV2	Glucose	19.7
mc^2155 pVV2/ATF2	Glucose	7.6

the contribution of both isoenzymes in TAG accumulation in *R. opacus* PD630. We expected to see a significant reduction in the TAG content in the double *atf*1–*atf*2 mutant, since the disruption of each of these genes individually leads to a decrease of 30–50 % on the cellular TAG. However, this effect was not additive in PDTag1/2. For instance, PDTag1/2 only accumulated reduced amounts of TAG when cultivated in nitrogen-rich media, while in nitrogen-limiting conditions, which promote lipid accumulation, the TAG content was similar to the wild-type strain. This curious effect was confirmed for two independent double mutant clones. In addition, this effect of the double disruption of *atf*1–*atf*2 on the TAG content correlated well with the formation of glycogen by cells. In a previous study, we showed the metabolic relationship between TAG accumulation and glycogen formation in rhodococcal cells (Hernández and Alvarez 2010). We demonstrated that a reduction of TAG accumulation by mutagenesis or the use of lipid inhibitors promoted an increase in glycogen content in *R. opacus* PD630. In this context, the disruption of *atf*2 in PD630 produced a significant decrease in the content of TAG and an increase of glycogen formation by the mutant cells, whereas the simultaneous disruption of *atf*1 and *atf*2 produced only a slight decrease in TAG content and an increase of glycogen formation in comparison with the wild type (Table 4). It is difficult to explain the phenotype of the double mutant regarding lipid accumulation. An alternative, hitherto unknown, mechanism to compensate the negative effect of the disruption of both *atf* genes might be activated during TAG accumulation, but not when those genes are disrupted individually. These results reflect the complexity of TAG metabolism and its regulation in the oleaginous *R. opacus* strain PD630.

Overexpression of *atf*1 and *atf*2 in strain PD630 promoted an increase of TAG content in both cases, confirming the role of both genes in TAG biosynthesis in this oleaginous microorganism. The overexpression of *atf*1 or *atf*2 both led to an increase of approximately 10 % (CDW) on the TAG content in comparison with the wild type, when cells were grown on glucose as well as on hexadecane, as sole carbon sources. The relatively low increase of TAG content observed in this study may be due to the low levels of expression of the *atf* genes cloned under the *ace* promoter (P*ace*) used in this study. In this regard, previous studies revealed that the expression of genes was limited in *R. opacus* when using the weak *ace* promoter (Hänisch et al. 2006a, b). This phenomenon could also explain the partial complementation of the PDTag2 mutant observed in this study when *aft*2 was expressed from the *ace* promoter (Fig. 3a). Furthermore, as the acetamide has a negative effect on TAG metabolism in *Mycobacterium* cells (Hänisch et al. 2006a, b), the pVV2 expression vector was used for the heterologous expression of *atf*2 in *M. smegmatis* cells. The Atf2 primary sequence of strain PD630 shares identities of 58 % and 57 % with its orthologs in *M. smegmatis* (MSMEG_6322) and *M. tuberculosis* (Rv3734c), respectively. The latter showed the second highest DGAT activity of all tested *M. tuberculosis* Atfs in *E. coli* (Daniel et al. 2004). The heterologous expression of *atf*2 of strain PD630 in *M. smegmatis* promoted an increase of TAG content in comparison with the wild type only when cells were cultivated in nitrogen-rich media, but not under nitrogen-limiting conditions. These results suggest that the expression of the rhodococcal *atf* genes may interfere with the complex lipid metabolism of *M. smegmatis* under conditions that promote accumulation of lipids.

The results of this study demonstrate an active role of Atf2 in the TAG accumulation process in the oleaginous model microorganism *R. opacus* PD630. In addition to *atf*2 and *atf*1, other *atf* genes are actively contributing to TAG accumulation in strain PD630. Some of them may be able to compensate and maintain TAG biosynthesis and accumulation when *atf*1, *atf*2, or both simultaneously are disrupted in strain PD630. The differential function of each isoenzyme and their regulation remain to be investigated.

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