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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

# Overexpression of a phosphatidic acid phosphatase type 2 leads to an increase in triacylglycerol production in oleaginous *Rhodococcus* strains

**Martín A. Hernández · Santiago Comba · Ana Arabolaza · Hugo Gramajo · Héctor M. Alvarez**

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**Abstract** Oleaginous *Rhodococcus* strains are able to accumulate large amounts of triacylglycerol (TAG). Phosphatidic acid phosphatase (PAP) enzyme catalyzes the dephosphorylation of phosphatidic acid (PA) to yield diacylglycerol (DAG), a key precursor for TAG biosynthesis. Studies to establish its role in lipid metabolism have been mainly focused in eukaryotes but not in bacteria. In this work, we identified and characterized a putative PAP type 2 (PAP2) encoded by the *ro00075* gene in *Rhodococcus jostii* RHA1. Heterologous expression of *ro00075* in *Escherichia coli* resulted in a fourfold increase in PAP activity and twofold in DAG content. The conditional deletion of *ro00075* in RHA1 led to a decrease in the content of DAG and TAG, whereas its overexpression in both RHA1 and *Rhodococcus opacus* PD630 promoted an increase up to 10 to 15 % by cellular dry weight in TAG content. On the other hand, expression of *ro00075* in the non-oleaginous strain *Rhodococcus fascians* F7 promoted an increase in total fatty acid content up to 7 % at the expense of free fatty acid (FFA), DAG, and TAG fractions. Moreover, co-expression of *ro00075*/*atf2* genes resulted in a fourfold increase in total fatty acid content by a further increase of the FFA and TAG fractions. The results of this study suggest that *ro00075* encodes for a PAP2 enzyme actively involved in TAG biosynthesis. Overexpression of this gene, as single one or with an *atf* gene, provides an alternative approach to increase the biosynthesis and accumulation of bacterial oils as a potential source of raw material for biofuel production.

**Keywords** *Rhodococcus* · Triacylglycerols · PAP · Lipid metabolism · Biofuels

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## Introduction

Triacylglycerol (TAG) is a storage compound commonly found in eukaryotic organisms such as plants, animals, microalgae, fungi, and yeast (Leman 1997; Ratledge 1989). However, it is now known that some members of prokaryotes are also able to accumulate such a compound (Alvarez 2010; Alvarez and Steinbüchel 2010; Alvarez and Steinbüchel 2002). With a few exceptions, the ability to accumulate TAG is common in actinobacteria belonging to the *Rhodococcus*, *Nocardia*, *Mycobacterium*, and *Streptomyces* genera (Alvarez et al. 1997; 1996; Barksdale and Kim 1977; Daniel et al. 2004; Olukoshi and Packter 1994). In recent years, these lipids have attracted great interest as potential resources for biotechnological purposes (Wältermann et al. 2000; Li et al. 2008). The potential of bacterial TAG may be similar to that of vegetable sources, including their 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; Holder et al. 2011).

The biosynthesis and accumulation of TAG is a complex process that involves several catalytic enzymes that participate at different metabolic levels. In this context, Holder et al. (2011) reported at least 261 genes implicated in the *Rhodococcus opacus* PD630 TAG cycle based on metabolic reconstruction and gene family analysis. On the other hand,

Chen et al. (2013) described at least 177 genes associated with lipid metabolism in the same strain based on transcriptome and lipid-droplet proteome analyses.

The main biosynthetic pathway for TAG biosynthesis in rhodococci, known as the Kennedy pathway (Kennedy 1961), involves the sequential esterification of glycerol-3-phosphate producing phosphatidic acid (PA) (Fig. 1). PA is a key molecule for the biosynthesis of membrane glycerophospholipids through the synthesis of the liponucleotide intermediate CDP-diacylglycerol (CDP-DAG) which is a precursor of phosphatidylinositol, phosphatidylglycerol, and phosphatidylserine in bacteria (Parsons and Rock 2013; Zhang and Rock 2008). PA can also be dephosphorylated by a phosphatidic acid phosphatase (PAP, EC 3.1.3.4) to yield diacylglycerol (DAG), which is the direct precursor for TAG synthesis through an additional step of acylation catalyzed by the wax ester/diacylglycerol acyltransferase (WS/DGAT) enzymes encoded by the *atf* genes in rhodococci (Fig. 1). Because PAP and WS/DGAT might catalyze the rate-limiting steps in the TAG formation in oleaginous actinobacteria, the identification of the genes encoding for both types of enzymes constitutes an important issue for a better understanding of the glycerolipid metabolism in such microorganisms. Rhodococci exhibit a high redundancy of genes encoding for WS/DGAT enzymes (Alvarez et al. 2008; Hernández et al. 2008, 2013; Holder et al. 2011; Villalba et al. 2013). Two *atf* genes were previously cloned and widely characterized in *R. opacus* PD630 (Alvarez et al. 2008; Hernández et al. 2013). This strain has been one of the best oleaginous bacterium studied to date regarding TAG metabolism and its biotechnological potential (Alvarez et al. 1996, 1997, 2000; Voss and Steinbüchel 2001; Holder et al. 2011; MacEachran et al. 2010; Wältermann et al. 2000; Hetzler and Steinbüchel 2013). Similar results have been reported in *Rhodococcus jostii* RHA1, an oleaginous strain able to accumulate high levels of TAG under culture conditions reported for PD630 strain (Hernández et al. 2008; Ding et al. 2012; Villalba and Alvarez 2014). According to these studies, it is clear that several proteins, including the WS/DGAT enzymes, as well as the cellular culture conditions are involved in the biosynthesis and accumulation of TAG in *R. opacus* PD630 and probably in other oleaginous *Rhodococcus* strains. In contrast, the role of PAP enzymes in bacterial TAG accumulation is barely known, and so far, no PAP has been identified in rhodococci. This enzyme has been well characterized in higher eukaryotic cells and microorganisms like yeasts, microalgae, and fungi (Kocsis and Weselake 1996). Two families of PAP enzymes, $Mg^{2+}$-dependent (PAP type 1, PAP1) and $Mg^{2+}$-independent (PAP type 2, PAP2), have been described in those organisms. The PAP1 family utilizes PA as a unique substrate and is localized in the soluble fraction of the cell (Carman and Han 2006). In contrast, the PAP2 enzymes, currently known as a family of lipid phosphate phosphatases (LPPs), can utilize a broad range of substrates such as PA, lysophosphatidic acid (LPA), sphingosine-1-phosphate, and diacylglycerol pyrophosphate (DGPP), among others, and are localized in the cells as integral membrane proteins. In eukaryotic cells, PAP enzymes have been associated with lipid metabolism and lipid signaling (Carman and Han 2006).

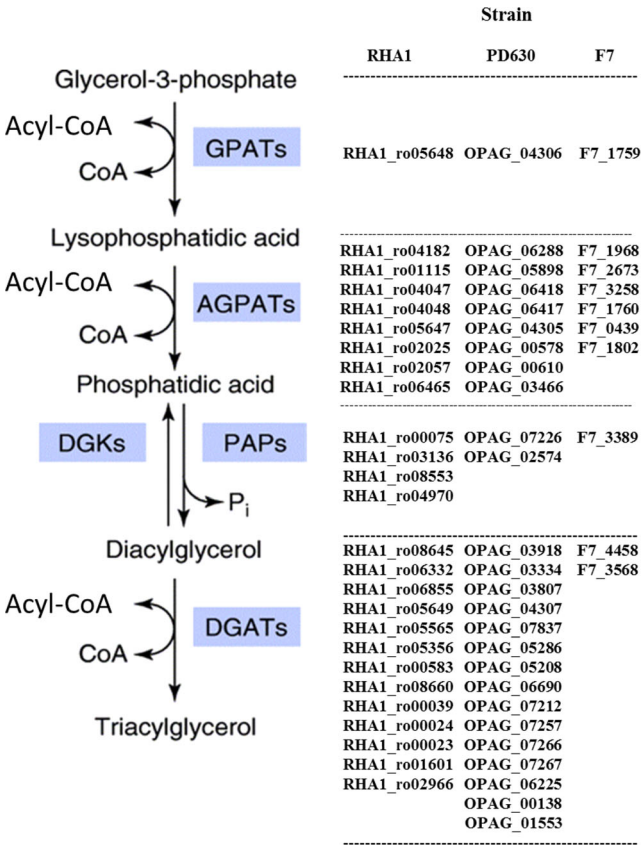


**Fig. 1** Kennedy pathway reactions for the biosynthesis and accumulation of TAG. Putative proteins for each reaction of the Kennedy pathway (*left panel*) and their locus numbers for selected *Rhodococcus* strains (*right panel*) are showed. *GPATs* glycerol-3-phosphate acyltransferases, *AGPATs* acyl-glycerol-3-phosphate acyltransferases, *PAPs* phosphatidic acid phosphatases, *DGKs* diacylglycerol kinases, *DGATs* diacylglycerol acyltransferases

The first member of the PAP1 family of enzymes (Pah1) has been purified and characterized from membrane and cytosolic fractions in yeast cells (Carman 1997). Pah1 has been associated with DAG biosynthesis, since a *pah1* mutant accumulated PA and produced reduced amounts of DAG and TAG (Han et al. 2006). Although the genes encoding for the family of PAP1 enzymes are highly conserved among eukaryotes, they are not in prokaryotes. In contrast, the PAP2 superfamily (pfam 01569) includes a high number of enzymes (including the LPP enzymes) occurring in eukaryotic and prokaryotic cells. In yeasts, for example, PAP2 enzymes DPP1 and LPP1 are integral membrane proteins with six transmembrane spanning regions and are localized in the vacuole and Golgi compartments of the cell, respectively (Toke et al. 1998a, b).

Because prokaryotes do not possess any homologs of PAP1, the study of PAP2-like enzymes in lipid metabolism and, potentially, in lipid signaling becomes a crucial issue in these organisms. Only a few PAP2-like enzymes have been characterized in prokaryotes (Dillon et al. 1996; Carman 1997; Zhang et al. 2008; Comba et al. 2013). Interestingly, Nakamura et al. (2007) identified and characterized five plastidic enzymes (LPPs β, γ, δ, ε1, and ε2) with PAP activity in *Arabidopsis thaliana*, all of which were evolutionarily associated with a LPP of the cyanobacterium *Synechocystis* sp. PCC6803 (synLPP) forming, thereby, a subfamily of PAP2 enzymes with "prokaryotic" origin.

Within the members of the PAP2 superfamily, the enzyme PgpB of *Escherichia coli* was the only enzyme known to display PAP activity (Dillon et al. 1996). Enzymatic activities in vitro have demonstrated that PgpB has a broad substrate spectrum being able to use phosphatidylglycerol phosphate (PGP), PA, LPA, DGPP, and undecaprenyl pyrophosphate (C55-PP) as substrates (Dillon et al. 1996 and Touze et al. 2008). Recently, Rucker et al. (2013) co-expressed the native PgpB enzyme and a WS/DGAT enzyme of *Acinetobacter baylyi* to produce TAG in *E. coli*.

On the other hand, Comba et al. (2013) reported two PAP2-like enzymes named Lppα and Lppβ in the actinobacterium *Streptomyces coelicolor*. Both enzymes showed a significant PAP activity when expressed in *E. coli*. In addition, overexpression of these enzymes in *S. coelicolor* resulted in an enhanced proportion of TAG in total intracellular lipids.

The identification and characterization of PAP2 enzymes in rhodococci are essential for enhancing our understanding in lipid metabolism in these oleaginous bacteria. Moreover, the manipulation of such genes may provide an interesting strategy for optimizing the production of bacterial oils for the biofuel industry. In this work, we performed a thorough bioinformatic analysis to identify putative PAP2 enzyme(s) in rhodococcal strains. Based on this information, we identified and characterized the *ro00075* gene of *R. jostii* RHA1 which encodes for a putative PAP2 protein (GenPept accession no. YP_700069). Additionally, we explored the effect of *ro00075* overexpression on TAG accumulation in both oleaginous strains (RHA1 and PD630) and in non-oleaginous *Rhodococcus fascians* F7.

## 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 in 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). Glucose was used in MSM as the sole carbon source at a final concentration of 1 % (*w/v*). For nitrogen-limiting conditions, to allow lipid accumulation (storage conditions), the concentration of ammonium chloride was reduced to 0.1 g $L^{-1}$ (MSM0.1). In MSM0 culture medium, the addition of ammonium chloride was omitted. Cells were harvested at specific time-points, washed with sterile NaCl solution (0.85 %, *w/v*), and dried at 37 °C for chemicals analyses. When LBS medium was used, 10 % (*w/v*) of sucrose was added to LB medium. Antibiotics were used at the following final concentrations: 100 μg/mL ampicillin (Ap), 50 μg/mL kanamycin (Km), 30 μg/mL nalidixic acid (Na), 5 μg/mL gentamycin (Gm), and 34 μg/mL chloramphenicol (Cm) in both *E. coli* and *Rhodococcus* strains. For overexpression analysis of genes under the acetamidase promoter (P*ace*) of pJAM2 and the thiostrepton promoter (P*tipA*) of pTip-QC2, 0.5 % (*w/v*) of acetamide and 1–3 μg/mL of thiostrepton were respectively added to cell cultures at time zero.

### DNA analysis, amplification, cloning, and sequencing

Chromosomal DNA, plasmids, and DNA fragments were isolated and analyzed by standard methods. For specific DNA amplification, the PCR assay was performed with different specific primers listed in 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.

### Strategy to delete the *ro00075* gene in RHA1 strain

Mutagenic plasmid pK18*mobsacB*-Δ*ro00075*$_{RHA1}$ was constructed to delete *ro00075*, as follows. An upstream region of *ro00075* was amplified by PCR using the MAH-F/ro00075a set of primers containing a *Bam*HI and *Xba*I restriction site, respectively (Table 2). The resulting 700-bp amplicon was cloned into pGEM-T-easy vector (pGEM-T-easy/*ro00075*-up), digested with *Bam*HI/*Xba*I, and subcloned into *Bam*HI/*Xba*I-digested pK18*mobsacB*, resulting in pK18*mobsacB-ro00075*-up. An 811-bp amplicon of the *ro00075* downstream flanking region including the *ro00075* stop codon was obtained using the ro00075b/MAH-R set of primers containing a *Xba*I and *Hin*dIII restriction site, respectively (Table 2). This amplicon was cloned into pGEM-T-easy vector (pGEM-T-easy/*ro00075*-down), digested with *Xba*I/*Hin*dIII, and subcloned into *Xba*I/*Hin*dIII linearized pK18*mobsacB-ro00075*up yielding in pK18*mobsacB*-Δ*ro00075*$_{RHA1}$ (Table 1). Then, the pK18*mobsacB*-Δ*ro00075*$_{RHA1}$ plasmid was transferred via conjugation into *R. jostii* RHA1 cells using the *E. coli* S17-1 as the donor. The first event of recombination was selected on LB plates supplemented with kanamycin and nalidixic acid. For the second recombination event, individual colonies were

**Table 1** Strains and plasmids used in this study

| Strain or plasmid | Description | 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+ lacIq lacZΔM15] hsdR17($r_K^-m_K^+$)* | Promega |
| C41(DE3) | BL21 derivated strain. *$F^-$ ompT gal dcm lon hsd$S_B$ ($r_B^-$ $m_B^-$) λ(DE3)* | Lucigen |
| C41(DE3) pET23/*ro00075* | C41(DE3) carrying pET23/*ro00075* | This study |
| S17-1 | *thi,pro,hsd$R^-$,hsd$M^+$,recA*; RP4-Tc::Mu-Kn::Tn7; used as the donor for conjugating plasmids into *Rhodococcus* | Simon et al. (1983) |
| *Rhodococcus* | | |
| *R. jostii*: | | |
| RHA1 | Parental strain | Seto et al. (1995) |
| RHA1 pJAM2 | RHA1 derivative carrying pJAM2 plasmid, used as control strain; $Km^R$ | This study |
| RHA1 pJAM2/RO00075 | RHA1 derivative carrying pJAM2/*ro00075*; $Km^R$ | This study |
| RHA1 pTip-QC2 | RHA1 derivative carrying pTip-QC2 plasmid, used as control strain; $Cm^R$ | This study |
| RHA1 pTip-QC2/RO00075 | RHA1 derivative carrying pTip-QC2 /*ro00075*; $Cm^R$; $Thio^R$ | This study |
| RHA1 Δ*ro00075*-pTip-QC2/RO00075 | RHA1 Δ*ro00075* mutant carrying pTip-QC2/*ro00075*; $Cm^R$; $Thio^R$ | This study |
| *R. opacus*: | | |
| PD630 | Parental strain | DSM 44193 |
| PD630 pJAM2 | PD630 derivative carrying pJAM2 plasmid, used as control strain; $Km^R$ | This study |
| PD630 pJAM2/RO00075 | PD630 derivative carrying pJAM2/*ro00075*; $Km^R$ | This study |
| PD630 pTip-QC2 | PD630 derivative carrying pTip-QC2 plasmid, used as control strain; $Cm^R$ | This study |
| PD630 pTip-QC2/RO00075 | PD630 derivative carrying pTip-QC2 /*ro00075*; $Cm^R$, $Thio^R$ | This study |
| *R. fascians*: | | |
| F7 | Parental strain | BNM 542 |
| F7 pJAM2 | F7 derivative carrying pJAM2 plasmid, used as control strain; $Km^R$ | This study |
| F7 pJAM2/RO00075 | F7 derivative carrying pJAM2/*ro00075*; $Km^R$ | This study |
| F7 pTip-QC2 | F7 derivative carrying pTip-QC2 plasmid, used as control strain; $Cm^R$ | This study |
| F7 pTip-QC2/RO00075 | F7 derivative carrying pTip-QC2 /*ro00075*; $Cm^R$, $Thio^R$ | This study |
| F7 pTip-QC2/RO00075- pJAM2/Atf1 | F7 derivative carrying pTip-QC2 /*ro00075* and pJAM2/*atf*1 $Cm^R$, $Thio^R$, $Km^R$ | This study |
| F7 pTip-QC2/RO00075-pPR27/Atf2 | F7 derivative carrying pTip-QC2 /*ro00075* and $pPR27_{ace}$/*atf*2 $Cm^R$, $Thio^R$, $Km^R$ | This study |
| Plasmids | | |
| pGEM-T-easy vector | Linear plasmid used for cloning PCR products; $Ap^R$ | Promega |
| pGEM-T-easy/*ro00075*-up | pGEM-T-easy derivative carrying a 700 bp upstream region of *ro00075*; $Ap^R$ | This study |
| pGEM-T-easy/*ro00075*-down | pGEM-T-easy derivative carrying a 811 bp downstream region of *ro00075*; $Ap^R$ | This study |
| pGEM-T-easy/*ro00075* | pGEM-T-easy derivative carrying complete 687 bp of *ro00075*; $Ap^R$ | This study |
| pJAM2 | Shuttle *E. coli-Mycobacterium-Rhodococcus* with P*ace* promoter; $Km^R$ | Triccas et al. (1998) |
| pJAM2/*ro00075* | pJAM2 derivatives carrying the *ro00075* gene under control of P*ace* ; $Km^R$ | This study |
| pTip-QC2 | Expression vector for *Rhodococcus* with P*tipA* promoter, *repAB* (pRE2895); $Cm^R$ | Nakashima and Tamura (2004) |
| pTip-QC2/*ro00075* | pTip-QC2 carrying *ro0075* gene under control of P*tipA*; $Cm^R$ | This study |
| pK18*mobSacB* | Mobilizable cloning vector used as suicide plasmid with *SacB* gene; $Km^R$ | Schäfer et al. (1994) |
| pK18*mobSacB*-Δ$ro00075_{RHA1}$ | pK18*mobSacB* carrying the deleted *ro00075* gene; $Km^R$ | This study |
| pJAM2/*atf*1 | pJAM2 derivatives carrying *atf*1 gene under control of P*ace* ; $Km^R$ | Hernández et al. (2013) |
| pPR27ace/*atf*2 | pPR27 derivatives carrying *atf*2 gene under control of P*ace* ; $Gm^R$ | Hernández et al. (2013) |

**Table 1** (continued)

| Strain or plasmid | Description | Source |
|---|---|---|
| pET23 | Vector for expression of genes under control of the T7 promoter; $Ap^R$ | Novagen |
| pET23/*ro00075* | pET23 carrying the *ro00075* gene; $Ap^R$ | This study |

cultivated in LB during 48 h without antibiotics and then several aliquots were plated on LBS plates. Alternatively, pK18*mobsacB*-Δ*ro00075*$_{RHA1}$ plasmid was transferred into RHA1 pTip-QC2/RO00075 strain. The first event of recombination was selected on LB plates supplemented with kanamycin, nalidixic acid, and chloramphenicol. For the second recombination event, individual colonies were cultivated during 48 h in LB in the presence of an inducer (thiostrepton) and then several aliquots were plated on LBS plates in the presence of the inducer. The genotype of the resulting mutant strain (RHA1 Δ*ro00075*-pTip-QC2/RO00075) was verified by PCR from chromosomal DNA with primers ro00075F/ro00075R (200 bp), MAH-F/ro00075R (800 bp), ro00075F/MAH-R (~900 bp), and MAH-F/MAH-R (1,500 bp) (see Table 2 and Fig. S1).

## Cloning of the *ro00075* gene

The *ro00075* gene was amplified from total genomic DNA of *R. jostii* RHA1 by PCR using the primers RO00075F and RO00075R (Table 2). The resulting PCR product was cloned in pGEMT-easy vector, replicated in *E. coli* JM109, and verified by DNA sequencing. To achieve overexpression of *ro00075* in *Rhodococcus* strains, a *Bam*HI/*Xba*I digest from pGEMT-easy/*ro00075* was subcloned into the *Bam*HI/*Xba*I site of the shuttle *E. coli-Mycobacterium-Rhodococcus* vector pJAM2, which contain an inducible acetamidase promoter (P*ace*), and six His codons downstream to the *Xba*I site, yielding pJAM2/*ro00075*.

Alternatively, a *Bam*HI/*Hin*dIII fragment from pJAM2/*ro00075* was subcloned into the *Bam*HI/*Hin*dIII site of the expression pTip-QC2 vector yielding pTip-QC2/*ro00075*.

In order to heterologously express *ro00075* in *E. coli* C41 (DE3), the *Bam*HI/*Hin*dIII fragment from the pJAM2/*ro00075* containing the *ro00075* gene with the C-terminal $His_6$-tag fusion was purified and subcloned into the *Bam*HI/*Hin*dIII sites of pET23a expression vector (Novagen), yielding the plasmid pET23/*ro00075*. Both pET23 (control) and pET23/*ro00075* plasmids were maintained in *E. coli* DH5α and transferred to *E. coli* C41 (DE3) to perform the membrane protein expression analysis. All the plasmids described in this section are listed in Table 1.

**Table 2** Oligonucleotides used as PCR primers

| Primers | Sequence[a] |
|---|---|
| MAH-F | 5′ GGATCCGGCGTTCCGCATCAAGAA 3′ |
| MAH-R | 5′ AAGCTTTCACCCGCACGCCCGCCCACAT 3′ |
| ro00075a | 5′TCTAGATCAGTGGGCCGCCGTCGCGGACCT 3′ |
| ro00075b | 5′ TCTAGACGGTAGGCGCCGCCACGTA 3′ |
| ro00075F | 5′TGGATCCATGCCCCACACCTCCATCGCCA 3′ |
| ro00075R | 5′GTTCCCCTCTAGAGCCTCCCACTCGGT 3′ |
| aceF | 5′TCGCAGCGCCGTCAGTCACCAA 3′ |
| thioF | 5′TACATATCGAGGCGGGCTCCCA 3′ |
| Atf2MHR | 5′AAGCTTTCAGAGCAATGCCGCCTCGA 3′ |
| Atf1MHR | 5′GTGCGTGTGTCTAGAGCACGAGG 3′ |

[a] Restriction sites used for cloning purposes are underlined

## DNA transfer and genotype screening in *Rhodococcus* cells

All replicative plasmids were transferred to *R. jostii* RHA1, *R. opacus* PD630, and *R. fascians* F7 by electroporation. Electroporation assays were carried out as described by Kalscheuer et al. (1999) using a Model 2510 electroporator (Eppendorf-Netheler-Hinz, Hamburg, Germany). The electrotransformants containing the *ro00075* gene under P*ace*-promoter and P*tip*A-promoter were checked using primers aceF/RO00075R and thioF/RO00075R, respectively (Table 2).

In F7 strain, the presence of the couples pTip-QC2/*ro00075*-pJAM2/*atf*1 or pTip-QC2/*ro00075*-pPR27ace/*atf*2 was checked by colony PCR using the primers thioF/ro00075R to detect the *ro00075* gene and the primers aceF/Atf1MHR or aceF/Atf2MHR to detect the *atf*1 and *atf*2 genes, respectively (Table 2).

## Membrane preparation of *E. coli* and PAP activity assay

*E. coli* C41 (DE3) strains harboring plasmids pET23 and pET23/*ro00075* were grown in LB at 37 °C until $OD_{600nm}$ 0.6. Gene expression was induced by addition of 0.1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) followed by overnight incubation at 23 °C and 120 rpm. Cells were

harvested by centrifugation at 4,000×*g* for 20 min at 4 °C, washed twice with buffer A (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol), and resuspended in the same buffer. The next steps were all done at 4 °C. Cell disruption was carried out by sonication (Vibra-Cell™, Sonics & Material, Inc.) in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF). The lysate was cleared by centrifugation at 15,000×*g* for 30 min to separate cell debris, and the supernatant was ultracentrifuged at 120,000×*g* for 2 h to pellet the membrane fraction. The resulting pellet was washed twice with buffer B (20 mM Tris-HCl pH 7.5, 10 mM β-mercaptoethanol, 0.5 mM PMSF) and resuspended in the same buffer. Protein concentration was quantified by the Lowry assay using BSA as the standard (Lowry et al. 1951).

To test the phosphatase activity of the putative PAP, phosphatidic acid was used as the enzyme substrate. The DAG generated in the reaction was measured by LC-MS/MS. Standard phosphatase assays were performed in a 100-μL reaction mixture containing 25 mM Tris-HCl, pH 7.5, 2.5 mM Triton X-100, and 0.25 mM dipalmitoylphosphatidic acid (DPPA; Avanti Polar Lipids, Alabama, USA) as the substrate. Aliquots of membrane fractions of the corresponding strains were added to initiate the reaction, and after incubation at 30 °C, the reactions were quenched by adding methanol/chloroform (2:1). Subsequent lipid extraction was performed by the addition of chloroform and distilled water. The organic phase was dried and solubilized in 50 μL of mobile phase (water/methanol), and 5-μL aliquots were injected for HPLC and LC-MS/MS analysis. The organic extracts were separated on a ZORBAX Eclipse XDB-C8 column (3.0×50 mm, particle size=1.8 μm; Agilent, USA) using a binary solvent system of water (solvent A) and methanol (solvent B). A linear gradient from 80 % B to 100 % B was applied between 0 and 25 min. Both solvents were supplemented with 5 mM ammonium acetate. The outlet of the liquid chromatograph was connected to a micrOTOF mass spectrometer (Bruker Daltonics, Bremen, Germany) operating in the positive-ion mode. The data was acquired online in the mass range *m*/*z* 35–1,000. Dipalmitoylglycerol (DPG) was detected as the transition $[M+NH_4]^+ \rightarrow [M\text{-}R\text{-}OH]^+$ ion (*m*/*z* 586.5 → *m*/*z* 313.3). A calibration curve was done using DPG as the standard (Avanti Polar Lipids, Alabama, USA), under the same conditions as the phosphatase reaction. DAG concentration in the samples was calculated by the linear regression equation obtained from the calibration curve. A unit of enzymatic activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product/min. Specific activity was defined as units per milligram of protein. PAP activity was linear-dependent to time and protein concentration within the range tested.

## Bioinformatical analyses

To analyze *ro00075* and its homologs, we used the available *Rhodococcus* genomes in the NCBI database (http://www.ncbi.nlm.nih.gov/) for *R. jostii* RHA1, *R. opacus* PD630, *R. opacus* B4, *R. erythropolis* PR4, and *Rhodococcus equi* 103S or in the Rapid Annotation Subsystem Technology (RAST) server (http://rast.nmpdr.org/) for *R. fascians* F7. Protein screening and alignments were carried out using BLAST 2.2.17 (Altschul et al. 1997) and ClustalW (Thompson et al. 1994, Bioedit software) algorithms. Reference protein sequences were retrieved from the NCBI database. Identities were determined from alignments of full-length sequences. Transmembrane domains were detected using the TMHMM server available at http://www.cbs.dtu.dk/services/TMHMM/. Predicted secondary structure and hydrophobicity profiles were obtained with Protean (from DNASTAR Lasergene) software. Phylogenetic analyses were carried out from curated sequences using the neighbor joining method with the program MEGA 5.1.

## Lipid analysis

*E. coli* strains harboring pET23a and pET23/*ro00075* were grown in LB media at 37 ° C until $OD_{600nm}$ 0.6. Then, protein expression was induced by addition of 0.1 mM IPTG and 3 μCi [$^{14}$C]acetate was also added at the same time and the culture was kept 12 h at 23 °C. Total lipids of *E. coli* strains were extracted as described by Bligh and Dyer (1959) directly from $^{14}$C-labeled cells. The lipid extracts were dried and analyzed by thin-layer chromatography (TLC) on silica gel 60 F254 plates (0±2 mm, Merck), using the solvent systems hexane/diethyl ether/acetic acid (70:30:1, *v*/*v*/*v*) (Rotering and Raetz 1983). After TLC, spots were visualized by autoradiography, which was carried out by exposure for 2 days at −80 °C in Carestream® Kodak® BioMax® MR films. Spots were quantified using the ImageJ software version 1.47v (http://rsbweb.nih.gov/ij/). Unlabeled tripalmitin (Sigma), oleic acid (Sigma), glycero-3-phosphoethanolamine (Sigma), and dipalmitin (Sigma) were used as reference substances for TAG, free fatty acid (FFA), phospholipid (PL), and DAG, respectively and visualized by Cu-phosphoric staining.

Semiquantitative analyses of total intracellular lipids in *Rhodococcus* cells were carried out by thin-layer chromatography (TLC). For this, 5 mg of dried cells was extracted with 300 μL chloroform/methanol (2:1, *v*/*v*) for 90–120 min. For neutral lipids analysis, 15 μL (strains RHA1 and PD630) or 30 μL (strain F7) of chloroformic phase was subjected to TLC on silica gel 60 F254 plates (Merck) using hexane/diethyl ether/acetic acid (80:20:1, *v*/*v*/*v*) as the solvent (Wältermann et al. 2000). Spots samples were compared with a mixture containing tripalmitin (Merck), dipalmitin (Sigma), DL-α-palmitin (Sigma), palmitic acid (Merck), and 1,2-dipalmitoyl-*sn*-

glycero-3-phosphoethanolamine (Sigma) used as TAG, DAG, MAG, FFA, and PL references substances, respectively. All lipid fractions were visualized using iodine vapor, and spots of samples were quantified using the ImageJ software.

A colorimetric method described in previous works (Duncombe 1963; Wawrik and Harriman 2010; Hernández et al. 2013) was performed for the quantitative determination of total fatty acids in *Rhodococcus* cells. For this, dry cells (5–10 mg, depending on the used strain) were hydrolyzed with alkaline reagent (1 N 25 % methanol in NaOH) at 95–100 °C for 3 h with vigorous agitation (each 30 min). The soaps of fatty acids were neutralized with concentrated acetic acid, and the resultant free fatty acids were treated with copper reagent, extracted with chloroform, and developed with revealing reagent (diethyldithiocarbamate in 2-propanol). The resultant colored samples were spectrophotometrically read at 440 nm. The standard curve was performed with oleic acid as representative fatty acid of TAG in rhodococcal cells.

## Results

### Occurrence of putative PAP2 proteins in *Rhodococcus* genus and RO00075 sequence analysis

Bioinformatic analysis of the *R. jostii* RHA1 genome showed the occurrence of several genes coding for proteins belonging to the PAP2 superfamily, but only *ro00075* was originally annotated as a gene encoding a putative PAP enzyme. The predicted RO00075 protein possesses three conserved domains reported for the PAP2 superfamily (cl00474, pfam_01569). These domains, which comprise the consensus sequences KxxxxxxRP (domain 1), PSGH (domain 2), and SRxxxxxHxxxD (domain 3), are shared by a superfamily of lipid phosphatases that do not require $Mg^{2+}$ ions for activity (Stukey and Carman 1997; Carman and Han 2006). An additional analysis of the primary sequence of RO00075 using the tool CD-Search, an interface from NCBI to search the conserved domains with protein or nucleotide query sequences, showed a high association between the RO00075 sequence and a specific region known as PAP2_like_2 single-domain (cd03392), which usually occurs in bacterial membrane lipid phosphatases. This specific region includes the amino acid residues reported as part of the active site in the domains described above (K and R in domain 1; SGH in domain 2; and R, H, and D in domain 3), and they are also present in RO00075 (Table 3). In order to identify additional putative PAP enzymes in rhodococci, several *Rhodococcus* genomes were analyzed based on the presence of this specific region and by homology analysis using the BLAST algorithm and the RO00075 sequence as query. The analysis revealed the occurrence of three additional hypothetical membrane proteins (RO03136, RO0497, and RO08553) in *R. jostii* RHA1 and a variable number of putative lipid phosphatases in the other *Rhodococcus* strains (Table 3). The highest redundancy was observed in *R. jostii* RHA1 (four putative PAPs), whereas only one putative PAP was found in *R. equi* 103S and *R. fascians* F7. In all cases, redundancy of PAP enzymes was lower in comparison with that of other enzymes of the Kennedy pathway such as the DGATs and AGPATs (Fig. 1).

The sequence alignment between RO00075 of *R. jostii* RHA1 and all PAP2-like proteins found in the different rhodococci used in this study showed the highest identity (*I*, 86 %) with the protein OPAG_07226 of *R. opacus* PD630, an oleaginous bacterium able to accumulate significant amounts of TAG (up to 60 % by cellular dry weight) with carbon sources such as gluconate, glucose, and fructose (Alvarez et al. 1996). On the other hand, the lowest identity (*I*, 29 %) was observed with the protein F7_3389 of *R. fascians* F7, a non-oleaginous strain, which accumulates low amounts of TAG after growth with those carbon sources. Figure 2a shows the full alignment of RO00075, OPAG_07226, and F7_3389. Both RO00075 and OPAG_07226 proteins exhibited not only a similar primary structure but also a similar secondary structure with a predicted molecular weight of 24.0 kDa. Further, both proteins were predicted to be integral membrane proteins containing a six-transmembrane topology structure according to the TMHMM server (data not shown) and their hydrophobicity profiles (Fig. 2b). On the other hand, the protein found in F7 strain showed a predicted five-transmembrane topology and significant differences in its primary and secondary structures compared to RO00075 (Fig. 2). Moreover, the first amino acid residues forming the first transmembrane domain in RO00075 were absent in F7_3389 (Fig. 2). The alignment of RO00075 with the second protein identified in PD630 strain (OPAG_02574) showed a low identity (*I*, 23 %) between them, confirming that the OPAG_07226 is the corresponding ortholog of RO00075. Interestingly, the two proteins found in *R. opacus* B4 showed low identities (48 % with ROP_pROB01-01880 and 41 % with ROP_50370) when comparing with RO00075. Similar identities were observed for proteins found in *R. erythropolis* PR4 (48 % for RER_10170 and 43 % for RER_46290). Finally, the second lowest identity was obtained with a PAP2-like protein found in the non-oleaginous strain *R. equi* 103S (REQ_09750; *I*, 34 %). These results show a high genetic variability of rhodococcal PAP2-like proteins, suggesting that the occurrence of these different enzymes may be not only species-dependent but also strain-dependent in rhodococci.

Phylogenetic analysis using curated sequences of LPPs from different organisms showed that RO00075 as well as the other rhodococcal proteins were evolutionary associated with the protein synLPP of the cyanobacteria *Synechocystis* sp. PCC6803 forming a group of "prokaryotic" LPPs. This

**Table 3** Putative PAP2 proteins containing the PAP2_like_2 specific hit (cd03392) and their three catalytic domains in several *Rhodococcus* species

| Strain | Protein annotation | Length (aa) | KxxxxxxRP | PSGH | SRxxxxxHxxxD |
|---|---|---|---|---|---|
| *R. jostii* RHA1 | | | | | |
| RO00075 | PAP2 | 228 | KLVVERSRP | PSGH | TRVYLGVHWMSD |
| RO03136 | HP | 376 | KSVVGRARP | PSGH | TRLYLGEHWLSD |
| RO04970 | HP | 184 | KNLFERQRP | PSGH | SRVYLAAHWMTD |
| RO08553 | MAP | 338 | KSVVGRARP | PSGH | TRLYLGEHWLTD |
| *R. opacus* PD630 | | | | | |
| OPAG_07226 | PAP | 228 | KLVVGRSRP | PSGH | TRVYLGVHWLTD |
| OPAG_02574 | HP | 184 | KNLFERQRP | PSGH | SRVYLAAHWTTD |
| *R. opacus* B4 | | | | | |
| ROP_ pROB01-01880 | HMP | 313 | KMIVARSRP | PSGH | TRLYLGVHWLTD |
| ROP_50370 | HP | 180 | KNLFERQRP | PSGH | SRVYLGAHWMTD |
| *R. erythropolis* PR4 | | | | | |
| RER_10170 | HP/HMP | 273 | KALVGRERP | PSGH | TRLYLGAHWLTD |
| RER_46290 | HP/HMP | 183 | KRIFTRTRP | PSGH | SRVYLAAHWMTD |
| *R. equi* 103S | | | | | |
| REQ_09750 | PE | 185 | KLFFGRQRP | PSGH | SRVYLAAHWLTD |
| *R. fascians* F7 | | | | | |
| F7_3389 | HP | 177 | KFAFARERP | PSGH | SRIYLGAHWTTD |

*PAP* phosphatidic acid phosphatase, *HP* hypothetical protein, *MAP* membrane-associated phosphatase, *PE* phosphoesterase

group was closely related to the clade of "plastidic LPPs with prokaryote origin" of *A. thaliana* reported by Nakamura et al. (2007) but not with eukaryotic type LPPs (data not shown). Further, phylogenetic analysis using only proteins found in selected *Rhodococcus* strains allowed us to separate all rhodococcal PAP2-like protein sequences into two groups: the first one (small PAPs) includes proteins with short (177–185 aa) amino acid length and the second group (medium/large PAPs) includes proteins with medium (228 aa) and long (273–376 aa) amino acid length (Fig. 3). While the F7_3389 protein of *R. fascians* F7 forms a subgroup within the small proteins of rhodococci, RO00075 and its ortholog OPAG_07226 form a subgroup within the medium PAPs (Fig. 3). Interestingly, we observed that the occurrence of different kinds of PAP enzymes in the studied strains coincided with their genome sizes and their abilities to accumulate TAG. Rhodococci with smaller genomes such as *R. fascians* (5.3 Mb) and *R. equi* (5.0 Mb) contain only PAP2-like proteins with short amino acid length (177–185 aa), whereas rhodococcal strains with larger genomes, such as *R. erythropolis* (6.9 Mb), *R. opacus* B4 (8.83 Mb), *R. opacus* PD630 (9.15 Mb), and *R. jostii* RHA1 (9.7 Mb) contain, additionally, larger PAP2-like proteins. Further, the oleaginous rhodococcal strains possess more than one PAP2 enzyme distributed in the two groups described above (small PAPs and medium/large PAPs), while strains described as non-oleaginous bacteria only contain enzymes in the group of small PAP2 proteins. Besides, the medium PAPs were present only in *Rhodococcus* strains able to accumulate the highest TAG levels, such as *R. jostii* RHA1 and *R. opacus* PD630; in fact, this last strain possesses only a medium PAP2 protein within the group of medium/large PAPs (Fig. 3). The transmembrane domain analysis using all PAPs of Fig. 3 revealed that small, medium, and large PAPs contain a five-, six-, and seven-transmembrane topology, respectively (data not shown).

Finally, when we extended alignment analyses to other actinobacteria such as *Mycobacterium*, *Streptomyces*, and *Nocardia*, RO00075 always grouped with proteins containing a PAP2_like_2 single-domain (cd03392) in those bacteria but with identities less than 40 %. Further, most of those proteins were represented by large or small PAPs (data not shown).

## Heterologous expression of ***ro00075*** in ***E. coli***

In order to characterize and to assign a functional role of RO00075 as a PAP2 enzyme, we cloned the *ro00075* gene into the pET23a expression vector and transferred it into *E. coli* C41 (DE3), an optimized strain for overproducing membrane proteins (Wagner et al. 2008). The strain producing a recombinant version of the membrane-associated lipid phosphatase (RO00075-his$_6$) was analyzed for DAG production. Recombinant cells were grown to mid-log phase and then cultivated for 16 h at 23 °C after induction with IPTG and

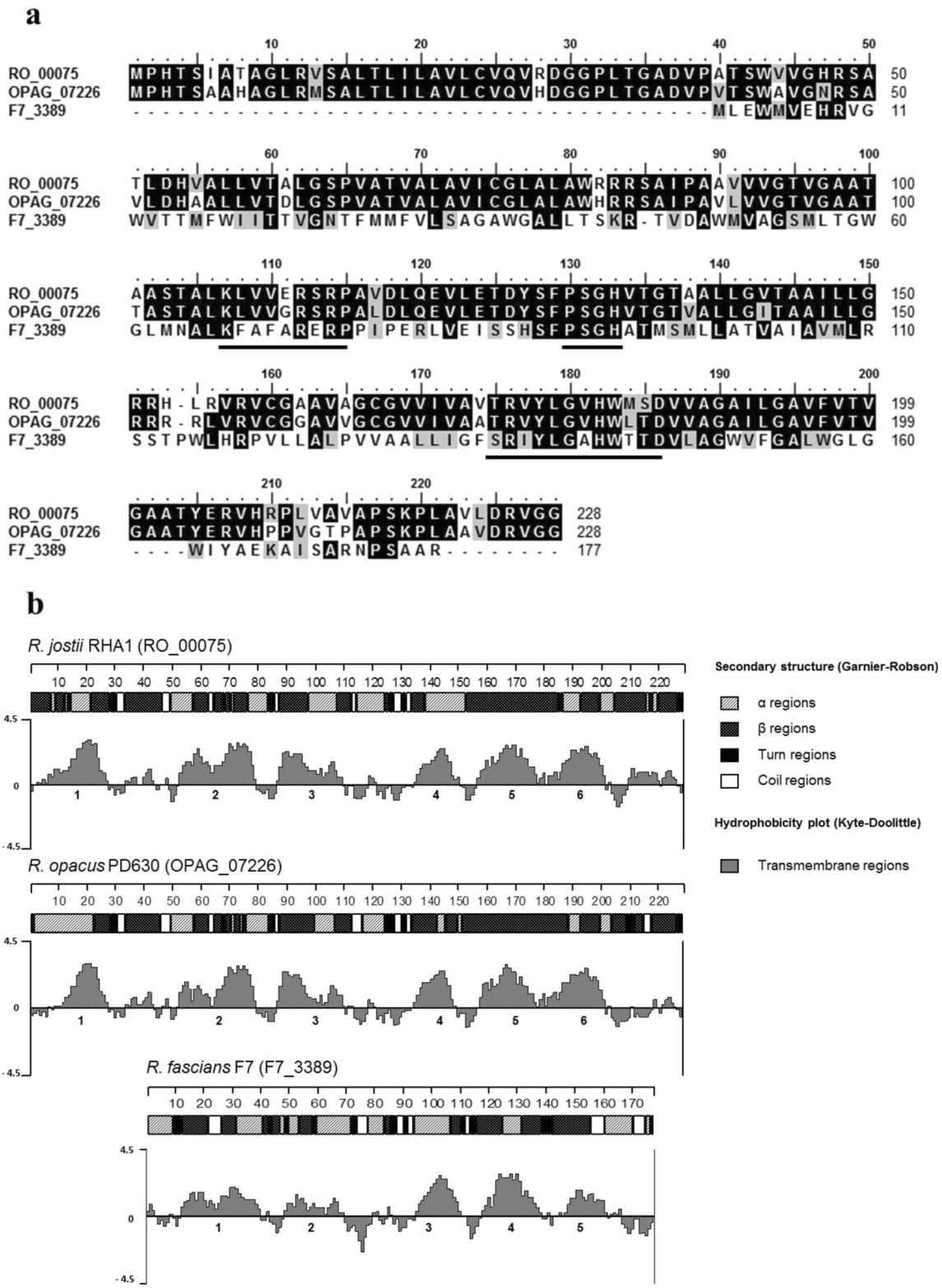


**Fig. 2** Analysis of RO00075 sequence. **a** Sequence alignment between RO00075 of *R. jostii* RHA1 and its homologs OPAG_ 07226 of *R. opacus* PD630 and F7_3389 of *R. fascians* F7. Identical amino acids (*black*), homologous residues (*gray*), putative signal peptide, and putative conserved PAP2-like domains (*underlined*) are shown. **b** Predicted secondary structure and hydrophobicity profiles of RO00075, OPAG_ 07226, and F7_3389. To obtain the hydrophobicity plots, a window size of 19 was used

the addition of [$^{14}$C]acetate. Then, we analyzed the total lipid profile of this recombinant strain by metabolic labeling of [$^{14}$C]acetate into the different lipid fractions. As shown in Fig. 4, heterologous expression of *ro00075* in *E. coli* C41

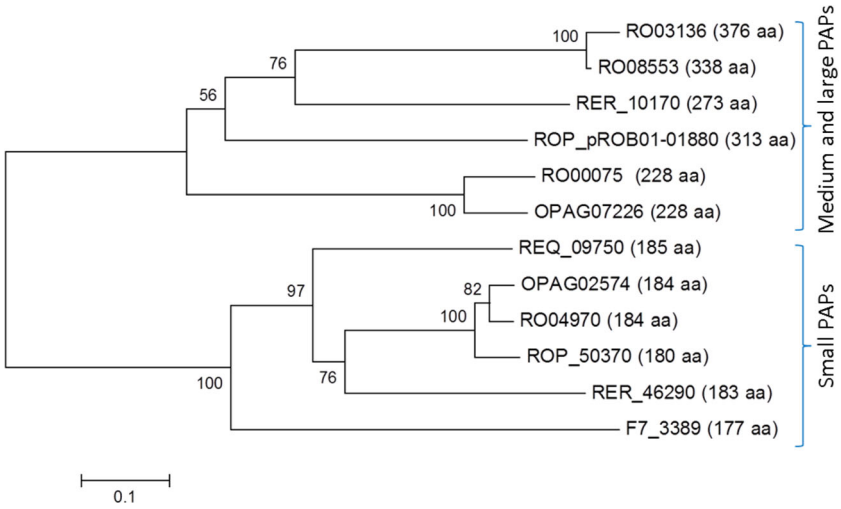


**Fig. 3** Phylogenetic tree of putative PAP2 enzymes from different *Rhodococcus* species. A neighbor-joining algorithm was used (software MEGA 5.1). Bootstrap values are shown along the branches

(DE3) resulted in a twofold increase in DAG levels in comparison with control cells, whereas the free fatty acid and phospholipids fractions showed no changes. On the other hand, the specific $Mg^{2+}$-independent PAP activity from purified *E. coli* membrane homogenate of C41 (DE) strain expressing *ro00075* was measured. As is shown in Table 4, the membrane fraction isolated from the strain expressing *ro00075* exhibited a fourfold increase in PAP activity.

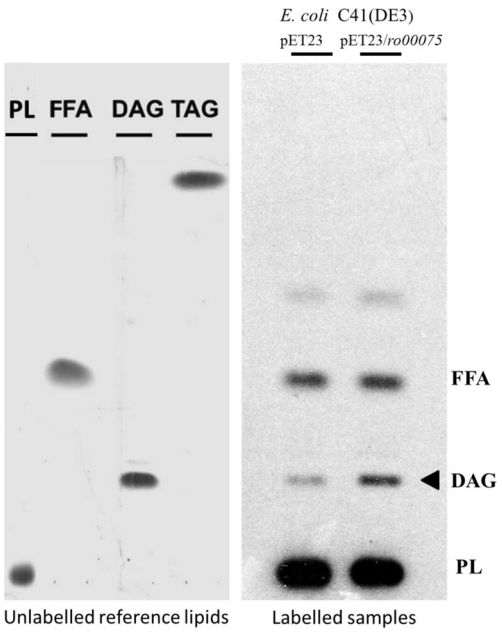


**Fig. 4** Heterologous expression of *ro00075* in *E. coli* and analysis of its lipid profile. Total lipid extract from [$^{14}$C]acetic acid-labeled culture of *E. coli* C41 (DE3) pET23/*ro00075* was analyzed on a silica gel TLC plate and developed in hexane/diethyl ether/acetic acid (70:30:1, *v*/*v*/*v*). *E. coli* C41 (DE3) containing pET23 empty was used as a control strain. Both control and sample were induced with IPTG. Unlabeled diacylglycerol (*DAG*), triacylglycerol (*TAG*), phospholipid (*PL*), and free fatty acid (*FFA*) were used as reference substances

## In vivo role of RO00075 in oleaginous ***R. jostii*** RHA1 strain

The in vivo role of RO00075 in TAG metabolism was studied by means of a mutagenesis strategy. To delete *ro00075*, an unmarked single-gene deletion strategy was performed in *R. jostii* RHA1 using the *sacB* counterselection system (see "Materials and methods"). A large number of transconjugant colonies were observed on LB plates after the first homologous recombination event of the suicide plasmid pK18*mobSacB*/Δ*ro00075*$_{RHA1}$. However, several attempts to delete the *ro00075* gene failed after the second recombination event on LBS plates, with the wild type genotype being restored in all cases. Only after transferring of the suicide plasmid pK18*mobSacB*-Δ*ro00075*$_{RHA1}$ in a RHA1 strain containing an extrachromosomal copy of *ro00075* in the pTip-QC2 vector, a mutant version was obtained after the second recombination event on LBS plates supplemented with the inducer (thiostrepton). The genotype of this strain was confirmed by PCR analysis from chromosomal DNA by means of several primers pairs (Table 2, Fig. S1).

Interestingly, the mutant strain was able to grow in liquid culture media with high nitrogen levels such as LB or MSM1 ($NH_4^+$, 1 g $L^{-1}$) without the inducer and only a slight delay on cell growth was observed (data not shown). In contrast, a significant decrease in cell growth was observed after cultivation in nitrogen-limiting MSM0.1 medium ($NH_4^+$, 0.1 g $L^{-1}$) in the absence of the inducer (Fig. S2). This growth was

**Table 4** PAP activity in membrane of *E. coli* expressing *ro00075*

| Strain | Specific PAP activity[a] |
|---|---|
| C41(DE3) pET23 | 1.07±0.08 |
| C41(DE3) pET23/*ro00075* | 4.1±0.2 |

[a] Values represent the mean ± S.D. of triplicate determinations

partially recovered after the addition of the inducer to the culture medium (Fig. S2).

On the other hand, in order to analyze the effect of chromosomal deletion of *ro00075* on TAG accumulation, both mutant and control strain were grown in LB medium in the presence of the inducer to generate cell biomass, and then cells were resuspended in a mineral medium free of nitrogen (MSM0) with and without the addition of the inducer. As is shown in Fig. 5a, a decrease in TAG and DAG contents was observed in the mutant strain without the inducer. When the inducer was added to the cell culture, TAG as well as DAG contents were restored at similar levels to that of the control strain (Fig. 5a). These results were in agreement with quantitative analyses; whereas the mutant strain showed lower amounts of total fatty acids (up to 20 % less by cellular dry weight) in the absence of the inducer, a similar lipid content was observed in both control and mutant strain when the inducer was added to this last (Fig. 5b).

## Overexpression of the *ro00075* gene in oleaginous *Rhodococcus* strains and its effect on TAG accumulation

To further evaluate the in vivo role of RO00075 in TAG metabolism of oleaginous *Rhodococcus* cells, *ro00075* was overexpressed in *R. jostii* RHA1 as well as in the taxonomically related strain *R. opacus* PD630 using both pJAM2 and pTip-QC2 vectors as expression systems. pJAM2 and pTipQC2 and its derivatives containing the *ro00075* gene were transferred into *Rhodococcus* cells by electroporation, and the resultant recombinant strains (Table 1) were evaluated for TAG production by TLC analysis and total fatty acid quantification from dried cells. Expression of *ro00075* using the pJAM2 expression vector promoted an increase of total fatty acid content in both *R. jostii* RHA1 and *R. opacus* PD630 cells during cultivation in MSM0.1 or MSM0 with glucose as the sole carbon source (Fig. 6a). Similar results were obtained when *ro00075* was expressed using the pTipQC2 expression vector under the P*tipA* promoter, although the absolute values of total fatty acid content were slightly higher than those obtained using pJAM2 vector with the *ace* promoter (Fig. 6b). However, the relative differences in total fatty acid content between controls cells and *ro00075*-overexpressing cells were similar after 48 and 120 h for both expression systems with an average increase of 10–15 % by cellular dry weight (Fig. 6a, b). Since the expression level of *ro00075* was comparable in both systems (pJAM2 and pTipQC2) as indicated by western blot analysis (data not shown), the slightly lower increase of total fatty acid content in pJAM2-harboring cells is attributed to the increase of the nitrogen level by the addition of acetamide into the culture media with the concomitant reduction of the total lipid content. In addition, TLC analysis revealed that the increase in the total fatty acid content in recombinant strains expressing *ro00075* was exclusively associated with the increase of the TAG fraction in both RHA1 and PD630 strains (Fig. 7a, b).

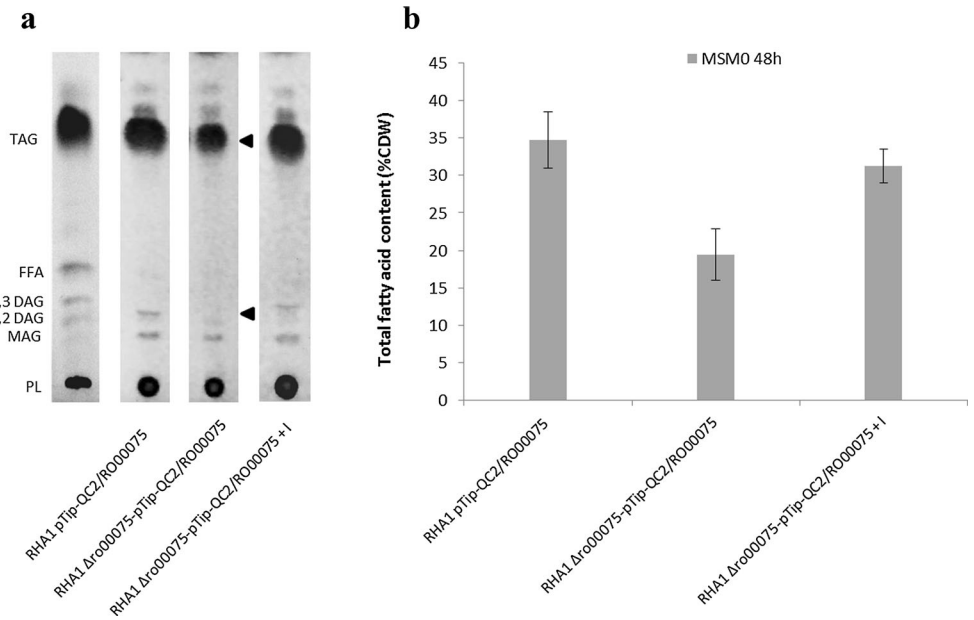


**Fig. 5** Lipid analysis of *R. jostii* Δro00075 mutant strain. Cells were grown overnight in LB medium with thiostrepton as the inducer, harvested, washed twice, and then incubated for 48 h in nitrogen-free MSM with glucose as the carbon source. **a** TLC analysis and **b** total fatty acid content of *R. jostii* RHA1 pTip-QC2/RO00075, RHA1 Δ*ro00075*-pTip-QC2/RO00075 without inducer, and RHA1 Δ*ro00075*-pTip-QC2/RO00075 with inducer (*I*). In the TLC analysis, a mixture of reference lipids was used as control (*TAG* triacylglycerol, *FFA* free fatty acid, *DAG* diacylglycerol, *MAG* monoacylglycerol, *PL* phospholipid). *Bars* represent the mean ± S.D. of triplicate determinations

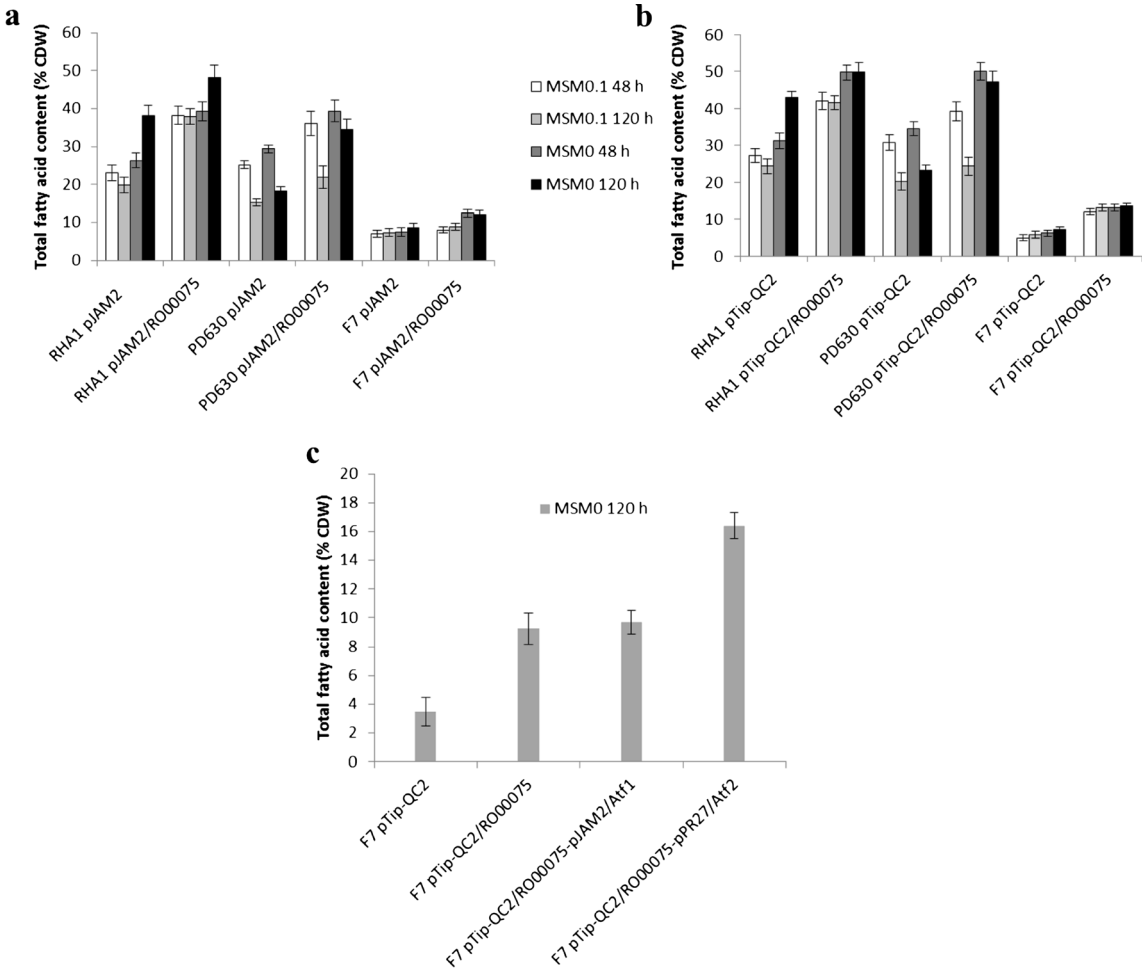


**Fig. 6** Total fatty acid content in overexpressing *ro00075*-recombinant strains. Cells were grown in LB medium for 24 h, harvested, washed, and then incubated for 48 and 120 h under storage lipid conditions (0.1 g $L^{-1}$ $NH_4^+$ MSM and nitrogen-free MSM) with glucose (RHA1 and PD630 strains) or fructose (F7 strain) as the carbon source. **a** pJAM2-derivative strains, **b** pTipQC2-derivative strains, and **c** F7-derivative strains co-expressing both *ro00075* and *atf* genes

## Effect of ***ro00075*** expression on TAG accumulation in non-oleaginous ***R. fascians*** F7

*R. fascians* F7 as well as other strains of this species is able to accumulate only low amounts of TAG after cultivation under nitrogen-limiting conditions (Alvarez et al. 1997, 2013; Alvarez 2003). For this reason, in this study, we consider the strain F7 as a non-oleaginous microorganism. Recombinant plasmid derivatives of pJAM2 and pTip-QC2 containing *ro00075* were introduced into *R. fascians* F7 cells in order to analyze the expression of this gene on TAG biosynthesis in a non-oleaginous bacterium. While F7 pJAM2/RO00075 strain did not present a significant difference in comparison with control strain (Fig. 6a), the strain F7 pTip-QC2/RO00075 showed an increase up to 7 % by cellular dry weight in total fatty acid content (Fig. 6b) under lipid accumulation conditions and fructose (cells exhibited low growth with glucose, but an optimum growth with fructose) as the sole carbon source. Interestingly, unlike what was observed in RHA1 and PD630 strains, the increase of total fatty acid content in F7 pTip-QC2/*ro00075* strain (Fig. 7c) occurred at the expense of several lipid fractions (FFA, DAG, and TAG). We also analyzed the effect of the co-expression of the coupled genes *ro00075*/*atf*1 and *ro00075*/*atf*2 on the biosynthesis and accumulation of TAG in this non-oleaginous strain. Both *atf1* and *atf2* genes actively involved in TAG accumulation in the oleaginous strain PD630 (Alvarez et al. 2008; Hernández et al. 2013) were expressed at similar levels in F7 pTip-QC2/*ro00075*-pJAM2/*atf*1 and F7 pTip-QC2/*ro00075*-pPR27/*atf*2 strains as indicated by western blot analysis (data not shown). While co-expression of *ro00075*/*atf*1 genes produced a similar effect on lipid accumulation than the expression of the single *ro00075* gene (Fig. 6c), the co-expression of *ro00075*/*atf*2 genes resulted approximately in a fourfold increase in total fatty acid content (Fig. 6c). In agreement

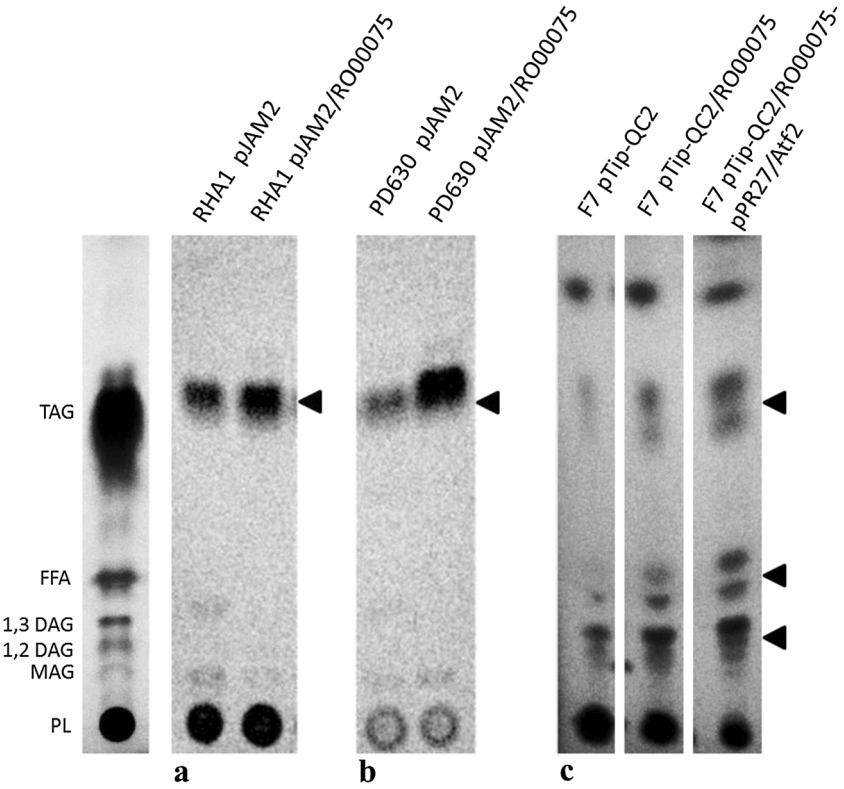


**Fig. 7** TLC-lipid analysis of whole-cell extracts of overexpressing *ro00075*-recombinant strains. Cells were grown in LB medium for 24 h, harvested, washed, and then incubated for 120 h under storage lipid conditions in MSM0 with glucose (RHA1 and PD630) or fructose (F7) as the carbon source. **a** Overexpression of *ro00075* in strain RHA1, **b** expression of *ro00075* in PD630, **c** expression of *ro00075*, and co-expression of *ro00075*/*atf*2 in F7 strain. The *arrows* indicate the increased lipid fractions in samples. *TAG* triacylglycerol, *FFA* free fatty acid, *DAG* diacylglycerol, *MAG* monoacylglycerol, *PL* phospholipid

with the quantitative analyses, TLC analysis revealed that the increase in the total fatty acids was produced mainly by a further increase of the FFA and TAG fractions in the strain F7 pTip-QC2/*ro00075*-pPR27/*atf*2 (Fig. 7c). Finally, when an analysis by BLAST using the foreign DGATs as query was performed, no ortholog of the *atf*1 product (OPAG_07257) was found in F7 strain, whereas the protein (F7_3568) of F7 strain exhibited a good identity (*I*, 53 %) with the *atf*2 product (OPAG_00138).

## Discussion

In this study, we report the occurrence of a diverse number and types of putative PAP2-like proteins coded in the genomes of different rhodococcal species. All investigated species possess small PAP2-like proteins (177–185 aa), whereas *R. jostii*, *R. opacus*, and *R. erythropolis* additionally possess a set of larger PAP proteins (228–376 aa). Interestingly, these last species are able to accumulate significant amounts of TAG, in contrast to *R. equi* or *R. fascians* which usually produce lower amounts of TAG with different carbon sources, such as gluconate, glucose, fructose, or hexadecane (Alvarez et al. 1997; Alvarez 2003). In addition, medium-PAPs (228 aa) were only present in TAG-accumulating specialists as *R. opacus* PD630 and *R. jostii* RHA1, but absent in the analyzed non-oleaginous rhodococcal strains. These results suggest that medium PAPs may be specific proteins of those specialized TAG-accumulating rhodococci. In this context, RO00075 and OPAG_07226, with similar primary and predicted secondary structures, may constitute key enzymes with identical function in specialized TAG-accumulating *R. jostii* RHA1 and *R. opacus* PD630, respectively.

The high diversity of PAP2-like enzymes suggests that different proteins may have appeared during evolution of rhodococci with differing or specialized functions in lipid metabolism of cells, probably to adapt glycerolipid biosynthesis to the fluctuating physiological and environmental conditions. The appearance of additional rhodococcal PAP proteins through the evolution might permit cells to form different cellular pools of DAG for the selective production of phospholipids or TAG or potentially may also allow independent regulation of isoenzymes at the gene expression level or enzymatic activity. Interestingly, all active PAP2 enzymes described to date, including the non-related eukaryotic type LPPs, have shown a predicted six-transmembrane topology (Dillon et al. 1996; Carman 1997; Toke et al. 1998a, b; Sigal et al. 2005; Carman and Han 2006; Zhang et al. 2008; Touze et al. 2008; Comba et al. 2013). Further, the crystal structure and the predicted six-transmembrane topology of the lipid phosphatase PgpB of *E. coli* have been confirmed experimentally in a recent work (Fan et al. 2014). In this context, a five-transmembrane topology observed in rhodococcal small PAPs might lead to a loss of their functionality during evolution.

This result fits to our finding that non-oleaginous rhodococcal strains have only small PAPs. However, additional experiments with the different types of PAPs enzymes analyzing their enzymatic activity and their participation in TAG metabolism are certainly needed.

Since RO00075 of RHA1 strain contains all the conserved domains of PAP2 proteins, and considering that it belongs to a phylogenetic subgroup only represented in TAG-accumulating specialist rhodococci, we considered this protein as the best candidate for being the PAP involved in the TAG biosynthesis pathway of *R. jostii*. In this context, we investigated the possible role of RO00075 in the accumulation of TAG in the strain RHA1 and its effect on other rhodococcal strains.

The functional role of RO00075 as a PAP enzyme was first analyzed by heterologous expression of the *ro00075* gene in *E. coli* C41 (DE3). The increase of DAG levels in *ro00075*-expressing strain suggests that RO00075 is a membrane-associated lipid phosphatase enzyme able to catalyze the in vivo formation of DAG from intracellular phosphatidic acid or other analogous lipids from the host cell. In addition, the significant increase in the PAP activity observed in the membrane fraction of the strain expressing the *ro00075* gene indicates that RO00075 is able to catalyze the in vitro formation of DAG from phosphatidic acid, confirming its role as a membrane-associated PAP enzyme type 2. Similar results were reported for two PAPs (Lppα and Lppβ) of the actinobacterium *S. coelicolor* (Comba et al. 2013).

In order to analyze the in vivo role of RO00075 in its native host *R. jostii* RHA1, we performed a mutagenesis strategy to delete the *ro00075* gene. Several attempts to delete *ro00075* failed, suggesting that this gene may be important for growth of parental RHA1 cells at least under the used conditions. However, when extrachromosomal copies of *ro00075* were present in RHA1 cells, the chromosomal deletion of *ro00075* occurred under similar conditions. This result suggested that a minimum expression level of the *ro00075* gene may be necessary for growing on solid media. A similar result was reported by Nakamura et al. (2007), in which a knockout mutant for LPPγ (the primary plastidic PAP2 in *Arabidopsis*) could not be obtained and a *lppγ* homozygous mutant was only isolated under ectopic overexpression of LPPγ, suggesting that loss of LPPγ may cause lethal effect on viability of that plant.

Interestingly, no inducer was necessary for growth of RHA1 mutant strain in liquid nitrogen-rich media, discarding the essentiality of *ro00075* under these conditions. However, some genes have been efficiently expressed in pTip-QC series vectors even in the absence of the inducer (Hirofumi et al. 2010). Due to this non-stringent response of the pTip-QC2 vector used in this study, a basal expression of *ro00075* supplied in *trans* might be sufficient to ensure the cellular growth under nitrogen-rich conditions. In contrast, a basal expression of *ro00075* in mutant strain seemed to be insufficient to ensure a normal cell growth under nitrogen-limiting conditions and being resumed only after inducer addition. Altogether, these results suggested that *ro00075* may play a possible role in cellular growth, especially under lipid accumulation conditions. Further studies are necessary to verify the potential importance of *ro00075* for initial cell growth in *R. jostii* RHA1.

Further, RO00075 seems to play a relevant role in the TAG metabolism of RHA1 strain. The decrease in total fatty acid, TAG, and DAG contents in mutant strain during cultivation of cells in the absence of the inducer demonstrates that this enzyme contributes actively to the DAG pool for TAG biosynthesis in RHA1 strain. Since the mutant without inducer was still able to accumulate TAG to some extent, the remaining PAP enzymes might be involved in providing DAG for TAG biosynthesis.

Overexpression of *ro00075* in RHA1 and PD630 resulted in an increase in TAG content under lipid accumulation conditions (MSM0.1 and MSM0). These results also confirm that RO00075 is involved in the biosynthesis and accumulation of TAG in the native strain *R. jostii* RHA1 as well as in the taxonomically related strain *R. opacus* PD630, which possesses an orthologous protein (OPAG_07226, also named PD630_LPD04081) very similar to RO00075 (*I*, 86 %). Interestingly, according to recent studies by Chen et al. (2013) in *R. opacus* PD630, expression of the gene encoding for the protein PD630_LPD04081 was higher in the low-nitrogen MSM than in the nitrogen-rich media. Since TAG accumulates to high levels under nitrogen-limiting conditions, the increased expression of this gene suggests that it may be involved in TAG synthesis. The results reported in this work are consistent with those studies.

On the other hand, expression of *ro00075* in the non-oleaginous strain *R. fascians* F7 resulted in a slight increase in total fatty acids at the expense of FFA, DAG, and TAG. This result also supports the involvement of RO00075, which possesses no ortholog in F7 strain, in DAG levels, and in concomitant TAG production. Interestingly, several works have been reported that both lipid fractions, TAG and FFA, which increased in F7 cells can be used for biofuel production (Kosa and Ragauskas 2011; Liang and Jiang 2013; Wu et al. 2014; Janßen and Steinbüchel 2014). However, single expression of *ro00075* from an oleaginous bacterium seems not to be sufficient to significantly increase TAG biosynthesis in the non-oleaginous strain *R. fascians* F7, at least under the conditions used in this study. A possible reason for this result might be due to the flux of precursors in central and lipid metabolism being not sufficient to support a significant increase in TAG accumulation in a non-oleaginous strain. Alternatively, since the predicted structure of RO00075 was different compared to the native PAP found in F7 cells, RO00075 may exhibit a limited activity in this non-

oleaginous strain (Fig. 2b). In this context, the different structure of the RO00075 protein might be not well recognized by the anchoring machinery of F7 cells or, alternatively, the activity of this protein may also be modulated by a posttranslational allosteric process in native cells, which may not occur in F7 cells. On the other hand, the two native WS/DGAT enzymes found in F7 strain, which were required to convert DAG to TAG, might have low activity. In order to analyze this last possibility, we analyzed the effect of the co-expression of the coupled genes *ro00075*/*atf*1 and *ro00075*/*atf*2 in TAG biosynthesis in this non-oleaginous strain. Since the effect on lipid accumulation by co-expressing of *ro00075*/*atf*1 genes was similar to that observed by expressing of the single *ro00075* gene, this result suggests that the *atf*1 product from PD630 is not active enough to support a significant TAG accumulation by F7 cells, at least under the conditions used in this study. However, co-expression of *ro00075*/*atf*2 genes in the strain F7 resulted in a higher total fatty acid content, at the expense of TAG and FFA fractions, mainly demonstrating that both RO00075 and Atf2 were sufficient to improve the de novo TAG biosynthesis using precursors from the glycerophospholipid metabolism of non-oleaginous rhodococci. Interestingly, these results are in agreement with the fact that only an homolog for the *atf*2 product could be found in F7 cells. On the other hand, co-expression of both PAP and WS/DGAT enzymes has been previously reported in *E. coli* as an attempt to establish TAG formation in this bacterium. For example, Rucker et al. (2013) co-expressed the native PgpB enzyme (with PAP activity) of *E. coli* and the AtfA enzyme of *A. baylyi* to produce TAG in *E. coli*. Although *E. coli* cells were able to produce TAG to some extent, a loss of its cellular viability after 8 h following induction was also observed. A similar study was performed by Comba et al. (2013) using the same biosynthetic route, but expressing the genes *sco0958* (DGAT) and *lppα* or *lppβ* (PAPs) from *S. coelicolor* in *E. coli*. Interestingly, the growth of F7 cells was no affected after co-expression of two genes from oleaginous rhodococcal strains ($ro00075_{RHA1}$/$atf2_{PD630}$), thus suggesting that this genetic procedure may be useful as an alternative form to significantly increase TAG production in a non-oleaginous strain. Further, the results observed in this study and those previously reported are in agreement with the hypothesis that both kinds of enzymes, DGAT and PAP, might catalyze the rate-limiting steps in TAG formation.

Understanding rhodococcal lipid metabolism is of great interest regarding the development of new technologies through the design and refinement of these oleaginous cell factories. In this work, we report the functional elucidation of an individual gene coding for a PAP2 enzyme in *R. jostii* RHA1 involved in the production of DAG as a precursor for TAG biosynthesis. The manipulation of this gene alone or in combination with other identified rhodococcal genes also involved in TAG accumulation may contribute to establish diverse strategies to increase lipid production for industrial applications. Thus, our results provide new elements and tools for further cell engineering to achieve sustainable and cost-effective single-cell oil production in oleaginous bacteria.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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