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Rewiring neutral lipids production for the *de novo* synthesis of wax esters in *Rhodococcus opacus* PD630

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ABSTRACT

Rhodococcus opacus PD630 accumulates significant amounts of triacylglycerols (TAG), but is not able to *de novo* synthesize wax esters (WE) from structural unrelated carbon sources, such as gluconate. In this study, strain PD630 was engineered to produce WE by heterologous expression of *maqu_2220* gene, which encodes a fatty acyl-CoA reductase for the production of fatty alcohols in *Marinobacter hydrocarbonoclasticus*. Recombinant cells produced ca. 46% of WE and 54% of TAG (of total WE + TAG) from gluconate compared with the wild type, which produced 100% of TAG. Cell growth was not affected by the heterologous expression of MAQU_2220. Several saturated and monounsaturated WE species were produced by cells, with C18:C16, C16:C16 and C16:C18 as main species. The fatty acid composition of WE fraction in PD630*maqu_2220* was enriched with $C_{16:0}$, $C_{18:0}$, whereas $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ predominated in the TAG fraction. Significant amounts of WE and TAG were accumulated by PD630*maqu_2220* from whey, an inexpensive waste material from dairy industries, without affecting cell biomass production. This is the first report on WE synthesis by *R. opacus* from gluconate, which demonstrates that lipid metabolism of this bacterium is flexible enough to assimilate heterologous components for the production of new lipid derivatives with industrial interest.

1. Introduction

Neutral lipids compounds stored by prokaryotes include triacylglycerols (TAG) and wax esters (WE). Nowadays, microbial WE production has gained increasing attention due to their multiple industrial applications, as additives for biolubricants, cosmetic and pharmaceutical products, surface coating, among others (Santala et al., 2014; Wenning et al., 2016; Iven et al., 2016). Gram positive actinobacteria mostly accumulate variable amounts of TAG, whereas Gram negative proteobacteria store predominantly WE, usually at low levels (reviewed in Alvarez, 2016). Synthesis of both compounds shares common characteristics, such as: (i) their production is stimulated by a high C:N ratio in the culture medium; (ii) their synthesis demands an available pool of acyl-CoA or acyl-ACP in the cell; and (iii) the final enzymatic step for their synthesis is usually performed by bifunctional acyltransferases known as wax ester synthase/diacylglycerol acyltransferases (WS/DGAT) that catalyze the esterification between fatty acids and diacylglycerol to form TAG or between fatty acids and fatty alcohols to produce WE (Alvarez et al., 2017). The paradigm of TAG biosynthesis and accumulation among Gram positive bacteria is *Rhodococcus opacus* PD630. This strain as well as *R. jostii* RHA1, are able to synthesize WE at very low levels when cells are cultivated on specific pre-formed substrates as carbon sources, such as phenyldecane or hexadecane (Alvarez and Steinbüchel, 2002; Hernández et al.,2008). Nevertheless, strains PD630, RHA1 or related strains did not produce detectable amounts of WE when grown on gluconate as sole carbon source, the best simple substrate for TAG accumulation (Hernández et al., 2008; Silva et al., 2010). Presumably, the inability to accumulate WE was due to the absence of a significant pool of fatty alcohols when cells are cultivated on a structural unrelated carbon source, such as gluconate or glucose. In this context, we reasoned that engineering the cells with an efficient acyl-CoA reductase might provide such a pool and thus result in the production of WE.

Marinobacter hydrocarbonoclasticus VT8 (formerly *Marinobacter aquaeolei* VT8) is one of the most studied Gram negative WE-producer, which possesses two fatty acyl-CoA reductases, MAQU_2220 and MAQU_2507 for producing fatty alcohol residues from acyl-CoA as substrate (Lenneman et al., 2013). Fatty alcohols are produced via a two-step reduction of either acyl-CoA or acyl-ACP to the respective fatty alcohol via an intermediate fatty aldehyde (Hofvander et al., 2011). Both fatty acyl-CoA reductases of *M. hydrocarbonoclasticus* VT8, MAQU_2220 and MAQU_2507, are able to reduce not only fatty

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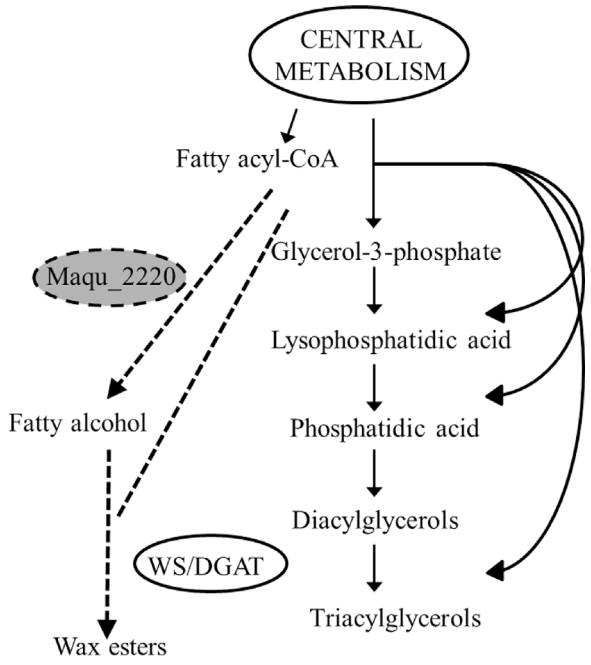

Fig. 1. Schematic flow of neutral lipid pathways in engineered *Rhodococcus opacus* PD630 for wax ester production. The foreign pathway is indicated by dotted lines.

aldehydes, but also acyl-CoAs or acyl-ACPs to fatty alcohols in an NADPH-dependent manner (Hofvander et al., 2011; Willis et al., 2011). The highest production of fatty alcohols in *Escherichia coli* and *Saccharomyces cerevisiae* was obtained by the heterologous expression of *maqu_2220* (Liu et al., 2014; Wenning et al., 2016). Based on these previous studies, we analyzed the feasibility to produce WE with the oleaginous *R. opacus* PD630 after heterologous expression of the gene encoding a fatty acyl-CoA reductases from *M. hydrocarbonoclasticus* DSM8798 which is 100% identical to *maqu_2220*. The ability of *R. opacus* PD630 to maintain a high carbon flux for massive fatty acid synthesis during cultivation under nitrogen-limiting conditions might facilitate the coupling of the fatty alcohol production reaction necessary for WE synthesis. Thus, the genetic endowment that makes *R. opacus* a specialist in TAG accumulation might be utilized as support to redirect lipid metabolism to WE synthesis after the expression of a heterologous fatty acyl-CoA reductase (Fig. 1). We expected that a simple genetic modification, such as the expression of a single heterologous gene, would be enough for redirecting the metabolism toward WE synthesis, since strain PD630 possesses several native WS/DGAT enzymes potentially involved in WE synthesis (Hernández et al., 2013).

The aim of this study was to explore the flexibility of the metabolic network of *R. opacus* PD630 for the *de novo* synthesis of WE from structural unrelated substrates, such as gluconate and whey, through the heterologous expression of a fatty acyl-CoA enzyme as a proof of concept.

2. Material and methods

2.1. Bacterial strains, plasmids and culture conditions

The strains and plasmids used in this study 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 was used in MSM media as the sole carbon source at a final concentration of 1% (w/v). Cells were also cultivated in whey as indicated by Herrero and Alvarez, 2015. In some experiments, hexadecane 0.1% (v/v) was used. Nitrogen-limiting conditions consisted of MSM with no addition of nitrogen to allow lipid accumulation (storage conditions) (MSM0). To cultivate cells in MSM0, cells were grown in LB overnight, harvested, washed in a NaCl solution (0.85%, w/v) twice and then resuspended in MSM0 medium. To cultivate PD630 in MSM0.1 or MSM1 pre-inoculum consisted of cells grown over-night in LB washed twice in a NaCl solution (0.85%, w/v). Cells were harvested at specific time-points, washed with a NaCl solution (0.85%, w/v) and dried at 37 °C to a constant weight for chemical analyses. Antibiotics were used at the following final concentrations: 100 μg ml^{-1} ampicillin (Ap), 50 μg ml^{-1} kanamycin (Km), 34 μg ml^{-1} chloramphenicol (Cm) (Table 1). To characterize growth, LB or MSM medium containing a concentration of ammonium chloride 0.1 g L^{-1} or 1 g L^{-1} were used. Induction of the thiostrepton (PtipA) promoter of pTip-QC2 was achieved by addition of 3 μg ml^{-1} thiostrepton at the beginning of the experiment.

Table 1
Strains, plasmids and primers used in this study.

Strain	Reference
Marinobacter hydrocarbonoclasticus	DSM 8798
Escherichia coli DH5α	Hanahan (1983)
Rhodococcus opacus PD630	DSM 44193
PD630 pTip, Cm^R	This study
PD630 pTip/*maqu_2220*, Cm^R	This study
PD630 *atf1* ΩKm, Km^R	Alvarez et al. (2008)
PD630 *atf1* ΩKm pTip/*maqu_2220*, Km^R, Cm^R	This study

Plasmids	Reference
pGEM-T-easy vector Ap^R	Promega
pGEM-T-easy/*maqu_2220* Ap^R	This study
pTip-QC2 Cm^R	Nakashima and Tamura (2004)
pTip-QC2/*maqu_2220* Cm^R	This study

Primers	Reference
MAQU2220NdeI TCATATGGCAATACAGCAGGTACA	This study
MAQU2220BamHI GGATCCTCAGGCAGCTTTTTTGCGC	This study
FpTip GCAGCGTGGACGGCGTCTAGAAAT	This study
RpTip TTTGTGCAGGTTTCGCGTGTTGCAGT	This study

2.2. Cloning of MAQU_2220

Genomic DNA from *M. hydrocarbonoclasticus* was extracted with Fast DNA SPIN kit for Soil (MP Biomedicals) following manufacturer instructions. To obtain the whole sequence of the gene, MAQU2220NdeI and MAQU2220BamHI primers were designed to include the beginning and end of the gene of interest, respectively (Table 1). PCR amplification was carried out under these conditions: 94 °C for 120 s followed by 30 cycles of 94 °C for 60 s, 57 °C for 30 s, 72 °C for 180 s and a final cycle at 72 °C for 5 min. The reaction was maintained at 4 °C until analysis. The PCR product was ligated into pGEM-T Easy vector and cloned in *E. coli*.

2.3. Analysis of gene sequence

To verify the correct gene sequence, the products were purified using Wizard SV Gel and PCR Clean-Up System (Promega). Sequences were obtained using the BigDye terminator cycle sequencing kit v3.1 (Applied Biosystems) according to the manufacturer's instructions, and electrophoretic separation in a 3130xl and 3500xl Genetic Analyzers (Applied Biosystems) operated by the Genomic Unit of Biotechnology Institute in INTA Castelar, Argentina. The generated sequences were edited with BioEdit Sequence Alignment Editor v 7.0.9 (Hall 1999).

2.4. Construction of plasmid for MAQU_2220 expression

The final construction was achieved by subcloning an NdeI/BamHI

fragment from pGEM-T Easy/MAQU_2220 into the NdeI/BamHI site of pTip-QC2. The correct fusion and building of pTip/MAQU_2220 was confirmed by PCR amplification using primers FpTip/MAQU2220NdeI.

2.5. Heterologous expression of MAQU_2220 in Rhodococcus opacus PD630

The construction obtained in the previous section was transferred to *R. opacus* PD630 by electroporation. Electroporation assays were carried out as described by Kalscheuer et al. (1999) using a Model 2510 electroporator (Eppendorf-Netheler-Hinz, Hamburg, Germany). As control, the empty vector pTip-QC2, was also transferred in an independent event. Also, an *atf1* mutant of *R. opacus* PD630 strain was electroporated with the pTip vector containing the *maqu_2220* gene. Those *R. opacus* PD630 colonies carrying the *maqu_2220* gene under pTip promoter were selected by antibiotic selection and confirmed by PCR using the primers FpTip/MAQU2220NdeI and RpTip/MAQU2220BamHI.

2.6. Lipid analysis

The qualitative and semiquantitative analyses of total intracellular lipids in *R. opacus* PD630 were performed by thin layer chromatography (TLC). For this, lipids were extracted from 5 mg of dried cells with 300 µL chloroform/methanol (2:1, v/v) for 90–120 min at 4 °C. For neutral lipids analysis, 15 µL (cells grown in whey) or 30 µL (cells grown in hexadecane or gluconate) of the extract was subjected to TLC on silica gel 60F254 plates (Merck) using hexane/diethyl ether/acetic acid (80:20:1, v/v/v) as the solvent. In some cases, solvent proportions were modified to (90:10:1, v/v/v) to achieve a better separation of TAG from WE fraction. Spot samples were compared with triolein, dipalmitin (DAG) and cetyl palmitate (Merck) as standards. Lipid fractions were visualized by iodine vapours staining. TAG or WE fractions were obtained by preparative TLC from 40 mg of dried cells extracted with 1 mL chloroform/methanol (2:1, v/v) for 90–120 min at 4 °C.

To determine the total fatty acid content of *R. opacus* PD630 carrying *maqu_2220* as well as from its control strain containing the empty pTip vector, 5–10 mg dried whole cells were subjected to methanolysis in the presence of 15% (v/v) sulfuric acid as described by Brandl et al. (1988). An HP 5890A gas chromatograph equipped with a TR-FAME capillary column (30 m; 0.25 mm; 0.25 µm) and a flame ionization detector was used to detect the resulting acyl-methylesters. The injection volume was 0.5 µL of chloroformic phase and H_2 at 95 mm/min was used as carrier gas. For the efficient separation of the methyl esters, a temperature program of 100 °C min^{-1} kept for 1 min and raised with an initial ramp of 8 °C min^{-1} up to 180 °C, then an increase of 2 °C min^{-1} up to 200 °C and a final ramp of 30 °C min^{-1} up to 240 °C maintained for 5 min to allow column cleaning. For quantitative analysis, tridecanoic acid was used as an internal standard. The same analysis was also carried out from WE and TAG fractions. For this, total lipid extracts were fractionated by preparative TLC.

Gas chromatography-mass spectrometry (GC–MS) analyses were carried out with an HP 6890 gas chromatograph connected to a mass-selective detector (Agilent model 5975C VL). The samples were injected splitless at 360 °C. The operating conditions were as follow: a capillary column 30 m × 0.25 mm inner diameter, 0.25 µm film thickness (Zebron ZB-5HT Inferno; Phenomenex). The oven temperature was programmed from 50 (hold time 1 min) to 200 °C at 50 °C min^{-1} and then to 380 °C at 7 °C min^{-1} with a holding time of 20 min. The carrier gas (He) flow was 2.0 mL/min. The injector was operated in splitless mode at 360 °C, the electron energy was 70 eV and the ion source temperature was 250 °C. Identification of WE was based on spectra interpretation (Urbanová et al., 2012) and comparison with data from NIST05 library. Peak areas were calculated and expressed as a percentage of total peak area.

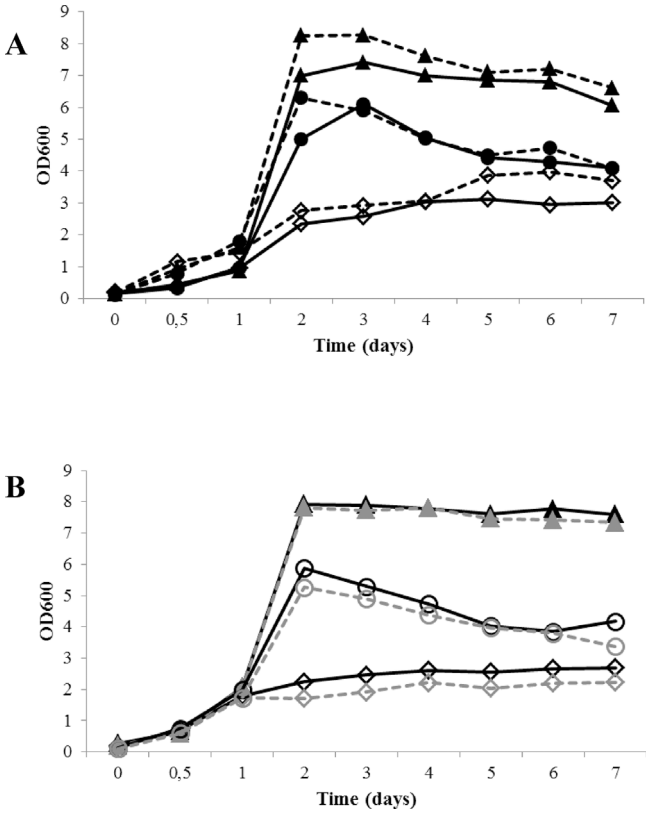

Fig. 2. Growth curves of *R. opacus* PD630 in different carbon sources. The strain harboring pTip/*maqu_2220* (continuous line) or an empty pTip (dotted line) (A) and *R. opacus* PD630 harboring pTip/*maqu_2220* (continuous line) or *R. opacus* PD630 ΔAtf1 harboring pTip/*maqu_2220* (dotted line), (B) in MSM1 with gluconate 1% (w/v) (triangle), LB medium (circle), and MSM0.1 with gluconate 1% (w/v) (diamond). In all cases thiostrepton was added as inducer.

3. Results

3.1. Effect of the fatty acyl-CoA reductase (MAQU_2220) expression on cell growth

In order to analyze the impact of MAQU_2220 expression on cell growth, PD630 was cultivated in three different growth conditions in relation to the nitrogen source: (i) in MSM0.1 medium (nitrogen-limiting condition), (ii) in MSM1 medium (nitrogen-rich condition) with gluconate (1%, w/v) as sole carbon source, and (iii) in LB medium (complex nutrient-rich medium), with the addition of the inducer thiostrepton at the beginning of the experiment. The growth kinetics of MAQU_2220-expressing PD630 (PD630*maqu_2220*) was comparable to that of the wild type strain (WT) carrying the empty pTip vector under all culture conditions used in this study, as is shown in Fig. 2A. After approximately 2 days of incubation, the proliferation of cells was restricted, growth ceased and both strains began to enter the stationary growth phase. In sum, results demonstrated a similar growth dynamics between PD630*maqu_2220* and the WT strain under the conditions used in this study, indicating that the production of fatty alcohols in the presence of the acyl-CoA reductase does not have a negative impact in cell growth.

3.2. Synthesis of neutral lipids by MAQU_2220-expressing R. opacus PD630

To analyze the ability of *R. opacus* carrying pTip/MAQU_2220 to synthesize and accumulate WE, cells were cultivated in nitrogen-free medium (MSM0) with gluconate as sole carbon source to promote lipid accumulation and with thiostrepton as inducer. After 24 h of

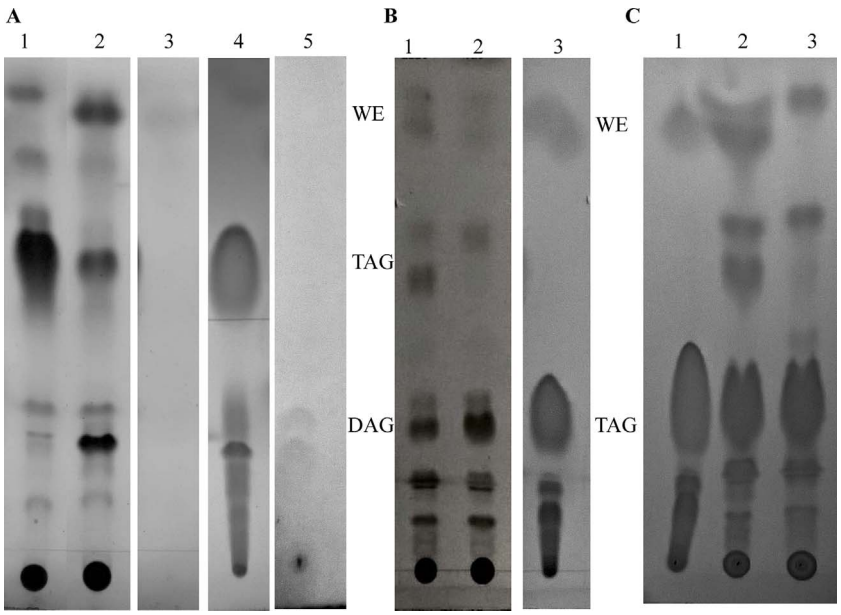

Fig. 3. TLC of neutral lipids extracted from *R. opacus* PD630 cultivated in different carbon sources. (A) Cells in MSM0 plus gluconate 1% (w/v). Lane 1 *R. opacus* PD630 carrying empty pTip. Lane 2 *R. opacus* PD630 harboring pTip/*maqu_2220*. Lane 3 Cetyl palmitate (WE). Lane 4 Triolein (TAG). Lane 5 dipalmitin (DAG). In this case, the solvent mixture used for TLC was hexane/diethyl eter/acetic acid (80:20:1 v/v/v). (B) Cells grown in hexadecane. Lane 1 *R. opacus* PD630 harboring pTip/*maqu_2220*. Lane 2 *R. opacus* PD630 carrying empty pTip. Lane 3 reference neutral lipids cetyl palmitate (WE) and triolein (TAG). (C) Cells grown in whey. Lane 1 cetyl palmitate (WE) and triolein (TAG) as standards. Lane 2 *R. opacus* PD630 harboring pTip/*maqu_2220*. Lane 3 *R. opacus* PD630 carrying empty pTip. In this case, the solvent mixture used for TLC was hexane/diethyl eter/acetic acid (90:10:1 v/v/v).

cultivation, cells were harvested, dried and analyzed for their lipid composition. The induced expression of MAQU_2220 in gluconate-grown PD630 resulted in a decrease of TAG accumulation, an increase of the DAG fraction, and in the appearance of a spot with a R_f compatible to that of the cetyl palmitate (C16:0-C16:0) used as standard compared to the control strain, as revealed by semiquantitative TLC analysis (Fig. 3A).

In a previous study, we demonstrated that strain PD630 was able to produce WE as minor lipid components during cultivation of cells on hexadecane as sole carbon source (Alvarez et al., 1996). When cells were cultivated in MSM0 with hexadecane as sole carbon source in this study, a decrease of the TAG fraction and the appearance of a WE spot were observed in PD630*maqu_2220* as compared with PD630 strain (Fig. 3B).

The expression of MAQU_2220 promoted a slight decrease of the total fatty acid content in gluconate-grown PD630 (1.19-fold) and a higher reduction in cells grown on hexadecane as sole carbon source (1.6-fold) (Fig. 4).

In other experiment, we compared the neutral lipid profile of PD630*maqu_2220* grown on gluconate in the presence and the absence of the inducer (thiostrepton) after TLC analysis. The WE spot appeared only in cells cultivated in the presence of the inducer in the medium (SFig. 1). Altogether, these results confirmed that the WE-like spot in TLC is produced when MAQU_2220 is expressed in PD630.

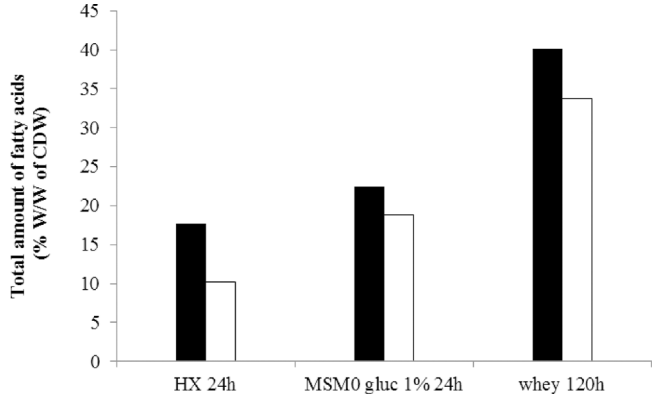

Fig. 4. Total fatty acid content in *Rhodococcus opacus* under different carbon sources. Cells carrying pTip:*maqu_2220* (■) or the wild type carrying an empty pTip vector (□) were cultivated in MSM0 with hexadecane for 24 h, MSM0 with gluconate 1% (w/v) for 24 h, and whey for 120 h.

3.3. Identification of WE produced by MAQU_2220-expressing R. opacus PD630

To confirm the identity of the WE fraction from gluconate-grown cells, the chemical composition of that fraction obtained from preparative TLC was analyzed by GC–MS. The so-called "WE fraction" of gluconate-grown cells represented c. 46% of the total neutral lipid (estimated as WE + TAG, by dry weight), whereas the TAG fraction accounted for c. 54% (Fig. 3A). The analysis of that fraction revealed the presence of a large mixture of saturated and unsaturated WE mainly formed by condensation of fatty alcohols with chain lengths from C_{16} to C_{18} with odd- and even-fatty acids from C_{14} to C_{18} chain lengths (Table 2 and SFig. 2A). The most abundant WE accumulated by PD630*maqu_2220* included octadecyl hexadecanoate (C34:0) and hexadecyl hexadecanoate (C32:0) among other saturated WE (Table 2). The structural identification of WE was based on the interpretation of their mass spectra and the analysis of mass spectrometric fragmentations. The EI mass spectra of the two most abundant WE produced by PD630*maqu_2220* are shown in SFig. 2B and C.

Table 2
Most abundant wax esters formed in *R. opacus* PD630 heterologously expressing MAQU_2220 reductase.

Compound*	MW$^{\perp}$	Alcohol part	Acid part	WE^{+}	Relative amounts^ (%)
4	452	hexadecanol	tetradecanoic	C30:0	0.78
5	466	hexadecanol	pentadecanoic	C31:0	0.85
8	480	hexadecanol	hexadecanoic	C32:0	6.36
11	494	heptadecanol	hexadecanoic	C33:0	3.23
14	508	octadecanol	hexadecanoic	C34:0	8.17
21	536	octadecanol	octadecanoic	C36:0	4.71
13	506	hexadecanol	octadecenoic	C34:1	25.6
16	520	octadecanol	heptadecenoic	C35:1	16.9
20	534	octadecanol	octadecenoic	C36:1	19.5

*Numbers refer to peaks shown in Fig. 1 (TIC).
$^{\perp}$Molecular weight (MW) in g/mol.
$^{+}$Wax ester (WE) refers to the most abundant isomer found.
^For saturated WE, amount of each compound is relative to the total. For unsaturated WE, percentages are relative to the total of its type.

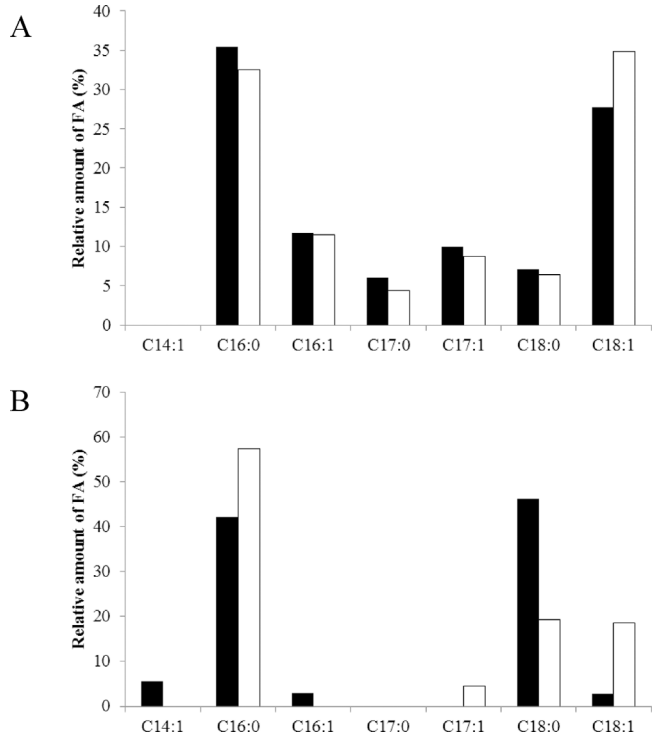

Fig. 5. Fatty acid composition of total and each lipid fraction. Total lipids produced by *R. opacus* PD630 harboring pTip/*maqu_2220* (white bars) or the empty pTip vector (black bars) (A) and TAG (white bars) and WE (black bars) fractions obtained from preparative TLC of lipid extracts of *R. opacus* PD630 harboring pTip/*maqu_2220* cells (B).

3.4. Fatty acid analysis from TLC preparative samples

To analyze the fatty acid composition of neutral lipids produced by PD630*maqu_2220* in comparison to PD630 carrying the empty pTip vector, cells were cultivated in MSM0 with gluconate as sole carbon source and the presence of thiostrepton from the beginning of the experiment. After 24 h of cultivation, cells were harvested and lipids were analyzed by preparative TLC and GC. Both strains exhibited similar fatty acid composition in their total lipid extracts, with a slight increase (approx. 7.1%, w/w) of the $C_{18:1}$ fatty acid in PD630*maqu_2220* (Fig. 5A). On the other hand, the fatty acid composition of TAG and WE fractions extracted from preparative TLC was also analyzed by GC (Fig. 5B). Whereas the TAG fraction was enriched in unsaturated $C_{18:1}$ (representing approx. 20% of the total fatty acids), WE fraction contained predominantly saturated fatty acids with $C_{16:0}$, $C_{18:0}$ chain lengths (both representing approx. 90% of the total fatty acids). The dominant occurrence of saturated fatty acids with $C_{16:0}$- and $C_{18:0}$-chain lengths in WE produced by PD630*maqu_2220* was in concordance with GC–MS results (Table 2).

3.5. WE production by PD630maqu_2220 from industrial dairy wastes

In a previous study we demonstrated that *R. opacus* was the most robust rhodococcal species for supporting high yields of cellular biomass and lipid production from whey (Herrero et al., 2016). For this reason, in this study we analyzed the ability of PD630*maqu_2220* to produce WE during cultivation of cells on whey. After 120 h of incubation MAQU_2220-expressing cells were able to accumulate WE in addition to TAG, as is shown in Fig. 3C. The heterologous expression of MAQU_2220 in PD630 and the synthesis of WE had no effect on cell growth, since both strains (PD630*maqu_2220* and PD630 with the empty vector) produced similar cell biomass after growth on whey (5.92–5.95 g/L), whereas the total fatty acid content was slightly reduced (1.19-fold) in cells (Fig. 4).

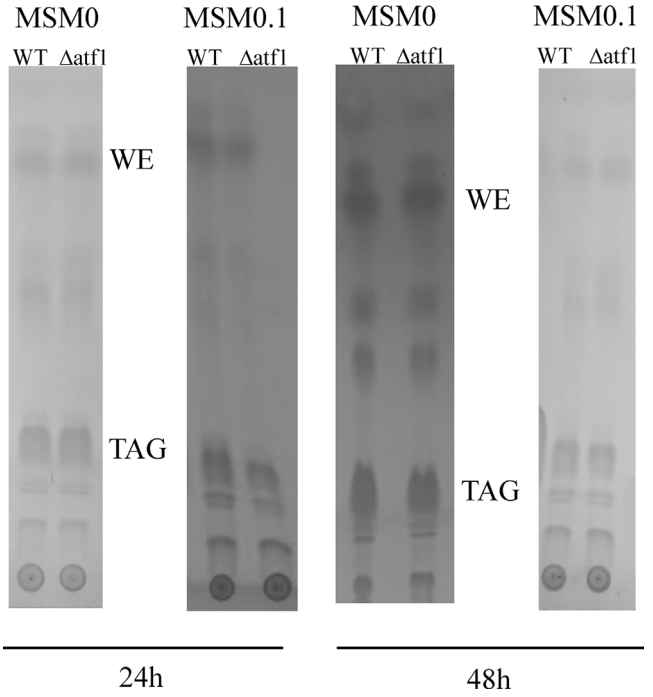

Fig. 6. TLC of neutral lipids extracted from *R. opacus* PD630 (WT) and its *atf1*-mutant (ΔAtf1), both harboring pTip/*maqu_2220* cultivated in MSM0 (left panel) or MSM0.1 (right panel) with gluconate (1%, w/w) during 24 h and 48 h. Triolein (TAG) and cetyl palmitate (WE) were used as standards.

These results demonstrated that engineered *R. opacus* PD630 is able to achieve an efficient bioconversion of whey to WE.

3.6. WE synthesis in the mutant R. opacus atf1 ΩKm carrying maqu_2220 gene

In a previous study, we demonstrated that *atf1* gene, which encodes a wax ester/diacylglycerol acyltransferase (WS/DGAT) enzyme, was involved in TAG synthesis and accumulation in *R. opacus* PD630 (Alvarez et al., 2008). The disruption of the *atf1* gene (*R. opacus atf1* ΩKm) resulted in a significant decrease of TAG content (up to 50% less total fatty acids) in comparison to the WT strain, and also in a substantial reduction of DGAT activity in crude extracts (Alvarez et al., 2008). However, when heterologously expressed in *E. coli*, Atf1 exhibited significant WE synthase activity and almost no DGAT activity (Alvarez et al., 2008). To analyze the putative role of Atf1 in WE synthesis in PD630, we cloned and expressed *maqu_2220* gene in *R. opacus atf1* ΩKm and compared its ability of produce TAG and WE to that of PD630*maqu_2220* as control. Both recombinant strains exhibited similar growth profiles when cultivated in LB medium or MSM0.1 and MSM1 with gluconate as sole carbon source as is shown in Fig. 2B. On the other hand, PD630 and the mutant *R. opacus atf1* ΩKm, both expressing *maqu_2220* gene, produced similar amounts of WE as revealed semi-quantitative TLC analysis after 24 and 48 h of incubation (Fig. 6). However, a slight decrease in the TAG fraction was observed in the *atf1*mutant in comparison to the PD630*maqu_2220*, principally after 24 h of incubation.

4. Discussion

In this study, we have successfully expressed MAQU_2220, a fatty acyl-CoA reductase from the marine bacterium *Marinobacter hydrocarbonoclasticus* in the oleaginous *R. opacus* PD630, the paradigm in TAG synthesis and accumulation. Although oleaginous rhodococci possess a protein annotated as fatty acyl-CoA reductase (**LPD02872** in *R. opacus* and **RS30405** in *R. jostii* RHA1), they do not seem to be

involved in WE synthesis from carbon sources (glucose or gluconate) entering at the glycolytic pathways for degradation, since WE are usually not detected during cultivation of cells on those substrates (Alvarez et al., 1996; Hernández et al., 2008; Wältermann et al., 2000). We propose that the heterologous expression of MAQU_2220 in PD630 provides a pool of fatty alcohol moieties available for WE synthesis by a native bifunctional WS/DGAT enzyme. Accordingly, synthesis and accumulation of WE from gluconate as sole carbon source only occurred when cells contained *maqu_2220* gene cloned into the pTip vector in the presence of the inducer (thiostrepton). This result indicated that the expression of a single gene coding for an enzyme responsible for the generation of a fatty alcohol pool by reduction of acyl-CoA residues was enough for expanding lipid synthesis to WE in addition to TAG by the oleaginous *R. opacus* PD630 from structural unrelated carbon sources. It was evident that the function of this heterologous enzyme has been well integrated in the anabolic network of strain PD630, deviating at least part of the carbon flux to the production of fatty alcohols, which were efficiently used by native WS/DGAT enzymes for WE synthesis (Fig. 1). The MAQU_2220 expression in PD630 had no effect on cell growth probably because free fatty acyl and fatty alcohol residues were not accumulated, since they were mostly used for WE and TAG synthesis by cells. Interestingly, synthesis and accumulation of WE promoted a decrease in TAG content in PD630*maqu_2220* indicating that the cloned fatty acyl reductase efficiently competes with acyltransferases of the Kennedy pathway (TAG synthesis pathway) for acyl-CoA residues (Fig. 1). Moreover, we detected a surplus of DAG precursors in PD630*maqu_2220* probably generated by a decreased flux of carbon into TAG synthesis.

The expression of *maqu_2220* gene in cells cultivated on gluconate as sole carbon source did not cause significant changes in the total fatty acid content or in fatty acid profiles compared to PD630 carrying the empty pTip vector. In both cases, fatty acid composition of cells was similar to those previously reported for PD630 (Alvarez et al., 1996; Wältermann et al., 2000). However, when we separately analyzed WE and TAG fractions after preparative TLC, some qualitative differences in fatty acid compositions were detected (Fig. 5B). $C_{16:0}$ and $C_{18:0}$ were the main fatty acids contained in WE, whereas $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ predominated in the TAG fraction. These differences might be the result of the different substrate-specificities of the acyltransferases involved in WE and TAG synthesis in recombinant cells. GC–MS analysis revealed the presence of a complex mixture of saturated and unsaturated WE mainly formed by condensation of fatty alcohols with chain lengths from C_{16} to C_{18} with odd- and even-fatty acids from C_{14} to C_{18} chain lengths, including C34:1, C35:1 and C36:1 as the main WE species (Table 2). This WE composition exhibited by gluconate-grown PD630*maqu_2220* cells probably was determined at least in part by the specificities of the cloned fatty acyl-CoA reductase and the native WS/DGAT's involved in WE synthesis. Similar WE compositions, with the dominance of C32:0 and C34:0 species, were reported for natural and/or recombinant WE-synthesizing bacteria (Ishige et al., 2003; Kalscheuer et al., 2007; Teerawanichpan et al., 2010; Barney et al., 2012). In other study, Wenning et al. (2016) reported the production of similar saturated or monounsaturated C_{32} to C_{40} WE by *S. cerevisiae* expressing *maqu_2220* and a wax synthase after cultivation on glucose. In the case of *R. opacus* PD630, the expression of a heterologous wax synthase gene was not necessary, since some of the native WS/DGAT isoenzymes can assume this role for WE synthesis when fatty alcohol residues are available. We expected that the WS/DGAT called Atf1 was one of the main enzymes involved in the WE synthesis in the recombinant PD630 strain, since this protein exhibited significant WE synthase activity and almost no DGAT activity, when heterologously expressed in *E. coli* (Alvarez et al., 2008). However, the disruption of *atf1* in PD630 did not affect growth nor WE accumulation when expressing *maqu_2220* in comparison with PD630*maqu_2220* (Figs. 2 B and 6), indicating that Atf1 is dispensable for the accumulation of WE by PD630 expressing MAQU_2220. Thus, some other WS/DGAT might be involved in WE synthesis when a fatty alcohol pool is available in cells.

It is interesting to consider that the synthesis and accumulation of WE by recombinant *R. opacus* PD630 harboring *maqu_2220* was fueled directly by gluconate as sole carbon source, and the feeding of exogenous fatty acid or fatty alcohol residues was not necessary. In this context, Santala et al. (2014), who engineered WE production in *Acinetobacter baylyi* ADP1, considered that selection of the correct substrate should be sufficient to obtain customized WE with biotechnological applications. Based on this criterion, we explored the possibility to use PD630*maqu_2220* as platform for producing WE from an industrial waste. In a previous study, we demonstrated that *R. opacus* PD630 is able to produce significant amounts of TAG during cultivation of cells on whey (Herrero and Alvarez, 2015). The heterologous expression of *maqu_2220* in PD630 promoted the conversion of whey into WE in addition to TAG, without affecting cell growth. Thus, the cultivation of engineered *R. opacus* on residual whey could be optimized for the biotechnological production of WE with commercial interest. Moreover, the use of this recombinant strain can be extrapolated for the utilization of other industrial wastes for the production of precursor WE for biolubricants, cosmetic additives, among other bioproducts.

Finally, results of this study suggested that the metabolism of *R. opacus* is robust enough to successfully incorporate and integrate heterologous reactions and pathways for the production of broad range of lipids with commercial interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2017.09.009.

References

Alvarez, H.M., Steinbüchel, A., 2002. Triacylglycerols in prokaryotic microorganisms. Appl. Microbiol. Biotechnol. 60, 367–376.
Alvarez, H.M., Mayer, F., Fabritius, D., Steinbüchel, A., 1996. Formation of intracytoplasmic lipid inclusion by *Rhodococcus opacus* PD630. Arch. Microbiol. 165, 377–386.
Alvarez, A.F., Alvarez, H.M., Kalscheuer, R., Wältermann, M., Steinbüchel, A., 2008. Cloning and characterization of a gene involved in triacylglycerol biosynthesis and identification of additional homologous genes in the oleaginous bacterium *Rhodococcus opacus* PD630. Microbiology 154, 2327–2335.
Alvarez, H.M., Hernández, M.A., Herrero, O.M., Lanfranconi, M.P., Silva, R.A., Villalba, M.S., 2017. Wax ester and triacylglycerol biosynthesis in bacteria. In: Geiger, O. (Ed.), Biogenesis of Fatty Acids, Lipids and Membranes, Handbook of Hydrocarbon and Lipid Microbiology. Springer International Publishing AG, Switzerland, pp. 1–12.
Alvarez, H.M., 2016. Triacylglycerol and wax ester-accumulating machinery in prokaryotes. Biochimie 120, 28–39.
Barney, B.M., Wahlen, B.D., Garner, E., Wei, J., Seefeldt, L.C., 2012. Differences in substrate specificities of five bacterial wax ester synthases. Appl. Environ. Microbiol. 78, 5734–5745.
Brandl, H., Gross, R.A., Lenz, R.W., Fuller, R.C., 1988. *Pseudomonas oleovorans* as a source of poly(hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl. Environ. Microbiol. 54, 1977–1982.
Hanahan, D., 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol Biol. 166, 557–580.
Hernández, M.A., Mohn, W.W., Martínez, E., Rost, E., Alvarez, A.F., Alvarez, H.M., 2008. Biosynthesis of storage compounds by Rhodococcus jostii RHA1 and global identification of genes involved in their metabolism. BMC Genom. 12, 600.
Hernández, M.A., Arabolaza, A., Rodríguez, E., Gramajo, H., Alvarez, H.M., 2013. The

atf2 gene is involved in triacylglycerol biosynthesis and accumulation in the oleaginous Rhodococcus opacus PD630. Appl. Microbiol. Biotechnol. 97, 2119–2130.
Herrero, O.M., Alvarez, H.M., 2015. Whey as a renewable source for lipid production by Rhodococcus strains: physiology and genomics of lactose and galactose utilization. Eur. J. Lipid Sci. Technol. 118, 262–272.
Hofvander, P., Doan, T.T., Hamberg, M., 2011. A prokaryotic acyl-CoA reductase performing reduction of fatty acyl-CoA to fatty alcohol. FEBS Lett. 585, 3538–3543.
Ishige, T., Tani, A., Sakai, Y.R., Kato, N., 2003. Wax ester production by bacteria. Curr. Opin. Microbiol. 6, 244–250.
Iven, T., Hornung, E., Heilmann, M., Feussner, I., 2016. Synthesis of oleyl oleate wax esters in Arabidopsis thaliana and Camelina sativa seed oil. Plant Biotechnol. J. 14, 252–259.
Kalscheuer, R., Arenskötter, M., Steinbüchel, A., 1999. Establishment of a gene transfer system for Rhodococcus opacus PD630 based on electroporation and its application for recombinant biosynthesis of poly(3-hydroxyalkanoic acids). Appl. Microbiol. Biotechnol. 52, 508–515.
Kalscheuer, R., Stoveken, T., Malkus, U., Reichelt, R., Golyshin, P.N., Sabirova, J.S., Ferrer, M., Timmis, K.N., Steinbüchel, A., 2007. Analysis of storage lipid accumulation in Alcanivorax borkumensis: evidence for alternative triacylglycerol biosynthesis routes in bacteria. J. Bacteriol. 189, 918–928.
Lenneman, E.M., Ohlert, J.M., Palani, N.P., Barney, B.M., 2013. Fatty alcohols for wax esters in Marinobacter aquaeolei VT8: two optional routes in the wax biosynthesis pathway. Appl. Environ. Microbiol. 79, 7055–7062.
Liu, R., Zhu, F., Lu, L., Fu, A., Lu, J., Deng, Z., Liu, T., 2014. Metabolic engineering of fatty acyl-ACP reductase-dependent pathway to improve fatty alcohol production in Escherichia coli. Metab. Eng. 22, 10–21.
Nakashima, N., Tamura, T., 2004. Isolation and characterization of a rolling-circle-type plasmid from *Rhodococcus erythropolis* and application of the plasmid to multiple-recombinant-protein expression. Appl. Environ. Microbiol. 70, 5557–5568.
Santala, S., Efimova, E., Koskinen, P., Karp, M.T., Santala, V., 2014. Rewiring the wax ester production pathway of *Acinetobacter baylyi* ADP1. ACS Synth. Biol. 3, 145–151.
Schlegel, H.G., Kaltwasser, H., Gottschalk, G., 1961. Ein submersverfahren zur kultur wasserstoffoxydierender bakterien: wachstumsphysiologische untersuchungen. Arch. Mikrobiol. 38, 209–222.
Silva, R.A., Grossi, V., Olivera, N., Alvarez, H.M., 2010. Characterization of the indigenous *Rhodococcus* sp. 602: a strain able to accumulate triacylglycerides from naphthyl- compounds under nitrogen-starved conditions. Res. Microbiol. 161, 198–207.
Teerawanichpan, P., Robertson, A.J., Qiu, X., 2010. A fatty acyl-CoA reductase highly expressed in the head of honey bee (*Apis mellifera*) involves biosynthesis of a wide range of aliphatic fatty alcohols. Insect Biochem. Mol. Biol. 40, 641–649.
Urbanová, K., Vrkoslav, V., Valterová, I., Háková, M., Cvacka, J., 2012. Structural characterization of wax esters by electron ionization mass spectrometry. J. Lipid. Res. 53, 204–213.
Wältermann, M., Luftmann, H., Baumeister, D., Kalscheuer, R., Steinbüchel, A., 2000. Rhodococcus opacus PD630 as a source of high-value single cell oil? Isolation and characterization of triacylgycerols and other storage lipids. Microbiology 146, 1143–1149.
Wenning, L., Yu, T., David, F., Nielsen, J., Siewers, V., 2016. Establishing very long-chain fatty alcohol and wax ester biosynthesis in Saccharomyces cerevisiae. Biotechnol. Bioeng. http://dx.doi.org/10.1002/bit.26220.
Willis, R.M., Wahlen, B.D., Seefeldt, L.C., Barney, B.M., 2011. Characterization of a fatty acyl-CoA reductase from *Marinobacter aquaeolei* VT8: a bacterial enzyme catalyzing the reduction of fatty acyl-CoA to fatty alcohol. Biochemistry 50, 10550–10558.