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Physiological and genetic differences amongst *Rhodococcus* species for using glycerol as a source for growth and triacylglycerol production

O. Marisa Herrero,[1,2] Gabriel Moncalián[3] and Héctor M. Alvarez[1]

Correspondence
Héctor M. Alvarez
halvarez@unpata.edu.ar

[1]Centro Regional de Investigación y Desarrollo Científico Tecnológico, Facultad de Ciencias Naturales, Universidad Nacional de la Patagonia San Juan Bosco y CIT-CHUBUT CONICET, Km 4-Ciudad Universitaria, 9000 Comodoro Rivadavia (Chubut), Argentina

[2]Oil m&s, Avenida Hipólito Yrigoyen 4250, 9000 Comodoro Rivadavia (Chubut), Argentina

[3]Departamento de Biología Molecular e Instituto de Biomedicina y Biotecnología de Cantabria, Universidad de Cantabria-Consejo Superior de Investigaciones Científicas-SODERCAN, Calle Albert Einstein 22, 39011 Santander, Spain

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We analysed the ability of five different rhodococcal species to grow and produce triacylglycerols (TAGs) from glycerol, the main byproduct of biodiesel production. *Rhodococcus fascians* and *Rhodococcus erythropolis* grew fast on glycerol, whereas *Rhodococcus opacus* and *Rhodococcus jostii* exhibited a prolonged lag phase of several days before growing. *Rhodococcus equi* only exhibited poor growth on glycerol. *R. erythropolis* DSMZ 43060 and *R. fascians* F7 produced 3.9–4.3 g cell biomass l^{-1} and 28.4–44.6 % cellular dry weight (CDW) of TAGs after 6 days of incubation; whereas *R. opacus* PD630 and *R. jostii* RHA1 produced 2.5–3.8 g cell biomass l^{-1} and 28.3–38.4 % CDW of TAGs after 17 days of growth on glycerol. Genomic analyses revealed two different sets of genes for glycerol uptake and degradation (here named clusters 1 and 2) amongst rhodococci. Those species that possessed cluster 1 (*glpFK1D1*) (*R. fascians* and *R. erythropolis*) exhibited fast growth and lipid accumulation, whereas those that possessed cluster 2 (*glpK2D2*) (*R. opacus*, *R. jostii* and *R. equi*) exhibited delayed growth and lipid accumulation during cultivation on glycerol. Three glycerol-negative strains were complemented for their ability to grow and produce TAGs by heterologous expression of *glpK2* from *R. opacus* PD630. In addition, we significantly reduced the extension of the lag phase and improved glycerol assimilation and oil production of *R. opacus* PD630 when expressing *glpK1D1* from *R. fascians*. The results demonstrated that rhodococci are a flexible and amenable biological system for further biotechnological applications based on the reutilization of glycerol.

INTRODUCTION

Biofuels, such as biodiesel and bioethanol, are renewable and environmentally friendly alternative products used for substituting fossil fuels. Biodiesel is produced by trans-esterification of vegetable oils, animal fats or microbial oils (single-cell oils) with alcohol, in which glycerol is the main byproduct, representing 10 % by weight (Papanikolaou & Aggelis, 2002; da Silva *et al.*, 2009; Easterling *et al.*, 2009). Although glycerol is used for different applications, such as in emulsifiers, softening agents, stabilizers and wetting agents for the food industry, and in the pharmaceutical and cosmetic industries, the increasing biodiesel production in the world is causing an excess of this waste material which exceeds their market (da Silva *et al.*, 2009; Galan *et al.*, 2009). For this reason, glycerol can be considered an abundant and cheap raw material that could be utilized as a substrate for single-cell oil production by oleaginous micro-organisms. The production of triacyl-glycerols (TAGs) from glycerol has been extensively investigated in oleaginous yeasts and fungi (Yen *et al.*, 2012; Chatzifragkou *et al.*, 2011; Xu *et al.*, 2012), but not yet in oil-accumulating bacteria. Amongst these, some bacteria belonging to the genus *Rhodococcus* are specialists in the accumulation of TAGs, producing significant

Abbreviations: CDW, cellular dry weight; DHAP, dihydroxyacetone phosphate; G3P, glycerol 3-phosphate; NCBI, National Center for Biotechnology Information; TAG, triacylglycerol.

The GenBank/EMBL/DDBJ accession numbers of the amplified 16S rRNA and *glpK* sequences are KT253457, KT253455, KT253456, KT253462, KT253461 and KT253460.

Five supplementary tables and one supplementary figure are available with the online Supplementary Material.

amounts of those lipids under nitrogen-limiting conditions (Alvarez *et al.*, 1996; Alvarez & Steinbüchel, 2010). In particular, *Rhodococcus opacus* PD630 and *Rhodococcus jostii* RHA1 are well-known oleaginous bacteria with the ability to produce large amounts of TAGs [> 20 % cellular dry weight (CDW)] from different carbon sources, including single substrates, and agro-industrial wastes, such as sugar beet molasses, orange wastes and whey (Alvarez *et al.*, 1996; Voss & Steinbüchel, 2001; Alvarez & Steinbüchel, 2002; Gouda *et al.*, 2008; Alvarez *et al.*, 2013; Herrero & Alvarez, 2015). Other rhodococcal species, such as *Rhodococcus fascians*, *Rhodococcus erythropolis* and *Rhodococcus equi*, generally produced lower amounts of TAGs compared with *R. opacus* and *R. jostii* during cultivation on sugars, organic acids or hydrocarbons (Alvarez *et al.*, 1997; Alvarez, 2003). In spite of the importance of glycerol as a potential renewable source for lipid production by rhodococci, the genetic and physiological ability of these micro-organisms to utilize this substrate has been poorly explored. Recently, Kurosawa *et al.* (2015) studied the capability of *R. opacus* PD630 for growing on glycerol to evaluate the use of this substrate for the production of lipid-derived materials. The authors reported that the cell growth of strain PD630 was extremely poor in glycerol; thus, they employed an adaptive evolution approach to improve growth. The evolved *R. opacus* strain (MITGM-173) showed improved glycerol utilization in comparison with the parental strain (Kurosawa *et al.*, 2015). In another study, Ciapina *et al.* (2006) reported the biosurfactant production by a *R. erythropolis* strain grown on glycerol as the sole carbon source. Understanding fundamental aspects of glycerol-assimilating mechanisms in rhodococci will enable the design of efficient bacterial biofactories for high yields of oil production from this substrate. Glycerol metabolism has not yet been investigated thoroughly within the genus *Rhodococcus*, although this process has been extensively studied in other bacteria. Glycerol can cross the cytoplasmic membrane through passive diffusion or by a specific glycerol transporter protein (GlpF), facilitating the uptake especially at low substrate concentration (Richey & Lin, 1972; Voegele *et al.*, 1993). The respiratory pathway of glycerol dissimilation involves a glycerol kinase (GlpK) enzyme, which phosphorylates glycerol to glycerol 3-phosphate (G3P), and a G3P dehydrogenase aerobic (GlpD) or anaerobic (GlpABC) enzyme oxidizes G3P to dihydroxyacetone phosphate (DHAP) as an intermediate in the glycolysis. This pathway for glycerol catabolism has been reported in *Escherichia coli* (Lin, 1976), *Pseudomonas aeruginosa* (Schweizer & Po, 1996), *Pseudomonas putida* (Nikel *et al.*, 2014), *Klebsiella pneumoniae* (Forage & Lin, 1982), *Bacillus subtilis* (Mindich, 1968; Holmberg *et al.* 1990), *Streptomyces clavuligerus* (Baños *et al.*, 2009) and *Mycobacterium smegmatis* (Titgemeyer *et al.*, 2007), amongst others. In several bacteria, such as *E. coli* (Dharmadi *et al.* 2006) or *K. pneumoniae* (Németh *et al.*, 2003), glycerol can be further metabolized by a fermentative pathway, in which glycerol is oxidized by a glycerol dehydrogenase (GldA) to dihydroxyacetone, which is further converted to DHAP by a dihydoxyacetone kinase (DhaK) enzyme. Our previous proteomic study showed a significant increase in the abundance of glycerol-degrading enzymes, GlpK and GlpD, during cultivation of *R. jostii* RHA1 cells with gluconate under TAG-accumulating conditions (Dávila Costa *et al.*, 2015). These enzymes seem to be part of a TAG recycling mechanism that occurs in oleaginous rhodococcal cells during lipid accumulation, in order to control NADPH and G3P availability in cells during the production process.

With the aim to improve our understanding of glycerol metabolism and the ability of rhodococci to utilize this substrate for lipid production, we investigated the genetic and physiological capability of different strains from five different rhodococcal species for using glycerol as a substrate for growth and TAG production. In addition, we restored the ability of some glycerol-negative rhodococcal strains to grow on this substrate by heterologous expression of key genes, and improved glycerol assimilation and oil production yields of *R. opacus* PD630 when transformed with glycerol-related genes from *R. fascians*.

METHODS

Bacterial strains, culture conditions and plasmids. Strains and plasmids used in this work are listed in Table S1 (available in the online Supplementary Material). For the analysis of growth and lipid production of *Rhodococcus* strains, a liquid Luria–Bertani (LB) preculture was inoculated from a fresh LB plate and cultivated overnight. Then, cells were collected, washed with sterile NaCl solution (0.85 %, w/v) and inoculated in minimal salt medium (MSM; Schlegel *et al.*, 1961) to OD_{600} 0.2. MSM1 medium (containing 1 g $NH_4Cl\ l^{-1}$) was used to analyse cell growth and MSM0.1 (containing 0.1 g $NH_4Cl\ l^{-1}$) to promote accumulation of lipids (nitrogen-limiting conditions). Growth and TAG production by rhodococcal cells were tested at different glycerol concentrations (Fig. S1). According to the results, we used a glycerol concentration of 0.3 % (v/v) for cell cultures. *Rhodococcus* cultivations were performed in 250 ml Erlenmeyer flasks with 50 ml medium at 28 °C and 150 r.p.m.

E. coli DH5α was used as host for cloning and cultivated in LB medium or LB agar plates at 37 °C and 150 r.p.m. When appropriate, ampicillin, kanamycin and chloramphenicol were used at concentrations of 100, 30 and 34 μg ml^{-1}, respectively, for both *E. coli* and *Rhodococcus* strains. For analysis of gene overexpression under the acetamidase promoter (P_{ace}) of pJAM2 and the thiostrepton promoter of pTip-QC2, 0.5 % (w/v) acetamide and 1–3 μg thiostrepton ml^{-1} were, respectively, added to cell cultures at time zero.

Electron microscopy analysis. Cells were washed, suspended in 0.1 M potassium phosphate buffer (pH 7.5) and fixed with glutaraldehyde for 24 h. Then, cells were washed with a solution of 0.32 M sucrose in phosphate buffer and embedded in low-viscosity resin (Spurr, 1969). Thin sections were contrasted with uranyl acetate and ruthenium red (Vogt *et al.*, 1995). Images were obtained utilizing a Zeiss 109T electron microscope with a Gatan ES camera.

DNA analysis, amplification, cloning and sequencing. Chromosomal DNA, plasmids and DNA fragments were isolated and analysed by standard methods (Marmur, 1961; Sambrook *et al.*, 1989). To identify three native strains (006, 346 and G212) exhibiting a glycerol-negative phenotype, DNA was extracted from isolated

colonies, and the 16S rRNA gene was amplified by PCR with universal primers for eubacteria and the conditions shown in the Table S2. PCR products were sequenced by INTA Genomic Services. Sequences were screened against the National Center for Biotechnology Information (NCBI) database using the BLAST search program.

glpK2 genes were amplified using genomic DNA from *R. jostii* strains 006, 346 and G212 and *R. opacus* strain PD630, whereas *glpF* and *glpK1D1* genes were obtained from *R. erythropolis* DSMZ 43060 and *R. fascians* F7 genomic DNA, respectively. The primer pairs used for amplification were *glpK2*-F/*glpK2*-R, *glpF*-F/*glpF*-R and *glpK1*-F/*glpD2*-R. Primer sequences and cycling conditions used in this work are listed in Table S2.

Nucleotide sequence accession numbers. The GenBank accession numbers for the amplified nucleotide sequence of the 16S rRNA and *glpK* genes from the three glycerol-negative rhodococcal strains are: *R. jostii* G212 (KT253457), *R. jostii* 346 (KT253455) and *R. jostii* 006 (KT253456), and $glpK_{G212}$ (KT253462), $glpK_{346}$ (KT253461) and $glpK_{006}$ (KT253460).

Subcloning, electroporation and genotyping. Inserts were excised from pGEM-T Easy vector using the restriction sites introduced by primers and subcloned into shuttle *E. coli–Mycobacterium–Rhodococcus* vector pJAM2, yielding pJAM2/$glpK2_{PD630}$, pJAM2/$glpK2_{006}$ and pJAM2/*glpF*. This vector contains an inducible acetamidase promoter (P_{ace}) and six His codons downstream of the multiple cloning site. To obtain pTip-QC2/*glpK1D1*, the fragment of 3.2 kb containing both *glpK1* and *glpD1* from the *R. fascians* F7 *glpFK1D1* cluster was taken from pGEM-T Easy/*glpK1D1* using *Bam*HI/*Hin*dIII enzymes and subcloned in the thiostrepton inducer vector pTip-QC2, carrying six His codons downstream of the *Hin*dIII restriction site.

All replicative plasmids were transferred to *R. jostii* (strains 006, 346 and G212) and *R. opacus* PD630 by electroporation. Electroporation was carried out as described by Kalscheuer *et al.* (1999) using a model 2510 electroporator (Eppendorf-Netheler-Hinz). The electrotransformants carrying $glpK2_{PD630}$, $glpK2_{006}$ and *glpF* genes under the P_{ace} promoter were checked by colony PCR using primer Pace-F and the reverse primers listed in Table S2. Transformant strains of *R. opacus* PD630 carrying pTip-QC2/*glpK1D1* were checked using the primers pTip-F/*glpK1*-R and *glpD1*-F/pTip-R (Table S2) to detect the presence of both *glpK1* and *glpD1* genes.

Bioinformatic and phylogenetic analyses. Genomes analysed in this study are listed in the supplementary Material and methods. Genes involved in glycerol metabolism were searched by gene and protein name in the NCBI database, UniProt website and RAST server (Aziz *et al.*, 2008), and by homology using the BLASTP program. The search was run using the default parameters set by the program, considering as homologous proteins those with significant alignments (*E*-value $\leq 10^{-50}$). We also used well-characterized protein sequences (GlpF, GlpK and GlpD) of *E. coli* (Voegele *et al.*, 1993; Feese *et al.*, 1998), *B. subtilis* (Holmberg *et al.*, 1990; Beijer *et al.*, 1993), *Mycobacterium smegmatis* (Titgemeyer *et al.*, 2007) and *Enterococcus casseliflavus* (Yeh *et al.*, 2004) to find their homologues in different species of the genus *Rhodococcus* analysed. The criteria considered for predicting the possible function of proteins are listed in the supplementary Material and methods.

For phylogenetic analyses, sequences of the 16S rDNA gene and glycerol kinase (GlpK) and G3P dehydrogenase (GlpD) proteins from five *Rhodococcus* species used in this study were aligned using CLUSTAL W (Thompson *et al.*, 1994). Evolutionary relationships were inferred using the neighbour-joining method (Saitou & Nei, 1987). The resultant tree topologies were evaluated by bootstrap analyses based on 1000 resamplings. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

Structural prediction and modelling of GlpK1 from *R. fascians* F7 and GlpK2 from *R. opacus* PD630 were performed using the Phyre (protein homology/analogy recognition engine) web server (Kelley & Sternberg, 2009) in the intensive modelling mode. Modelling was based on glycerol kinase from *Cellulomonas* sp. NT3060 (Protein Data Bank ID: 2D4W), with 74 % and 70 % amino acid identity with *R. fascians* GlpK1 and *R. opacus* GlpK2, respectively.

Analytical methods: determination of cell biomass, lipids and glycerol concentration. Growth was evaluated by measuring OD_{600} in a PG-Instrument 700 spectrophotometer. For cell biomass dry weight determination, 10 ml culture was centrifuged at 6000 ***g*** for 15 min, washed twice with sterile saline solution and dried at 60 °C until constant weight.

Details of the qualitative and quantitative analyses of lipids are included in the supplementary Material and methods.

To determine residual glycerol, 3 ml culture media was collected at different times and centrifuged at 8500 ***g*** for 30 min. The concentration was analysed by an enzymic-colorimetric assay using a Wiener lab triglyceride kit (TG Color GK/GPO/PAP).

RESULTS

Growth and lipid accumulation by rhodococci from glycerol

We analysed the ability of *R. opacus*, *R. jostii*, *R. erythropolis*, *R. fascians* and *R. equi* strains to grow and produce TAGs from glycerol as the sole carbon and energy source. According to the capability of the investigated rhodococcal strains for growing from glycerol, they could be divided into three groups. (1) Strains that exhibited fast growth, which included *R. erythropolis* strains (DSMZ 43060, 17 and DM1-21) and *R. fascians* strains (F7, S1.17b, 123 and D188-5). Cells reached the stationary phase after ~3 days of incubation (Fig. 1, Table 1). (2) Strains exhibiting growth on glycerol after a prolonged lag phase of ~5 days of incubation, including *R. opacus* strains (PD630 and MR22), *R. jostii* strains (RHA1 and 602) and *R. equi* ATCC 6939. Cells of these microorganisms reached the stationary phase after ~13 days of incubation (Fig. 1a, Table 1). (3) Strains belonging to *R. jostii* strains (006, 346 and G212), which were unable to grow with glycerol as the sole carbon and energy source, after 30 days of incubation (Fig. 1a).

Rhodococcal strains from groups 1 and 2 produced significant amounts of lipids during cultivation on glycerol, but with differences in the extension of time used by cells for cell biomass and TAG production (Fig. 1b, Table 1). *R. erythropolis* and *R. fascians* produced 3.9–4.3 g l^{-1} cell biomass l^{-1} and 28.4–44.6 % CDW of TAGs (with a maximum lipid yield of 1.2–1.8 g l^{-1}) after 6 days of incubation, whereas *R. opacus* and *R. jostii* produced 2.5–3.8 g cell biomass l^{-1} and 28.3–38.4 % CDW of TAGs (with a maximum lipid yield of 0.8–1.4 g l^{-1}) after 17 days of growth on glycerol (Fig. 1a, Table 1). *R. equi* produced lower amounts of cell biomass (1.5 g l^{-1}) and TAGs

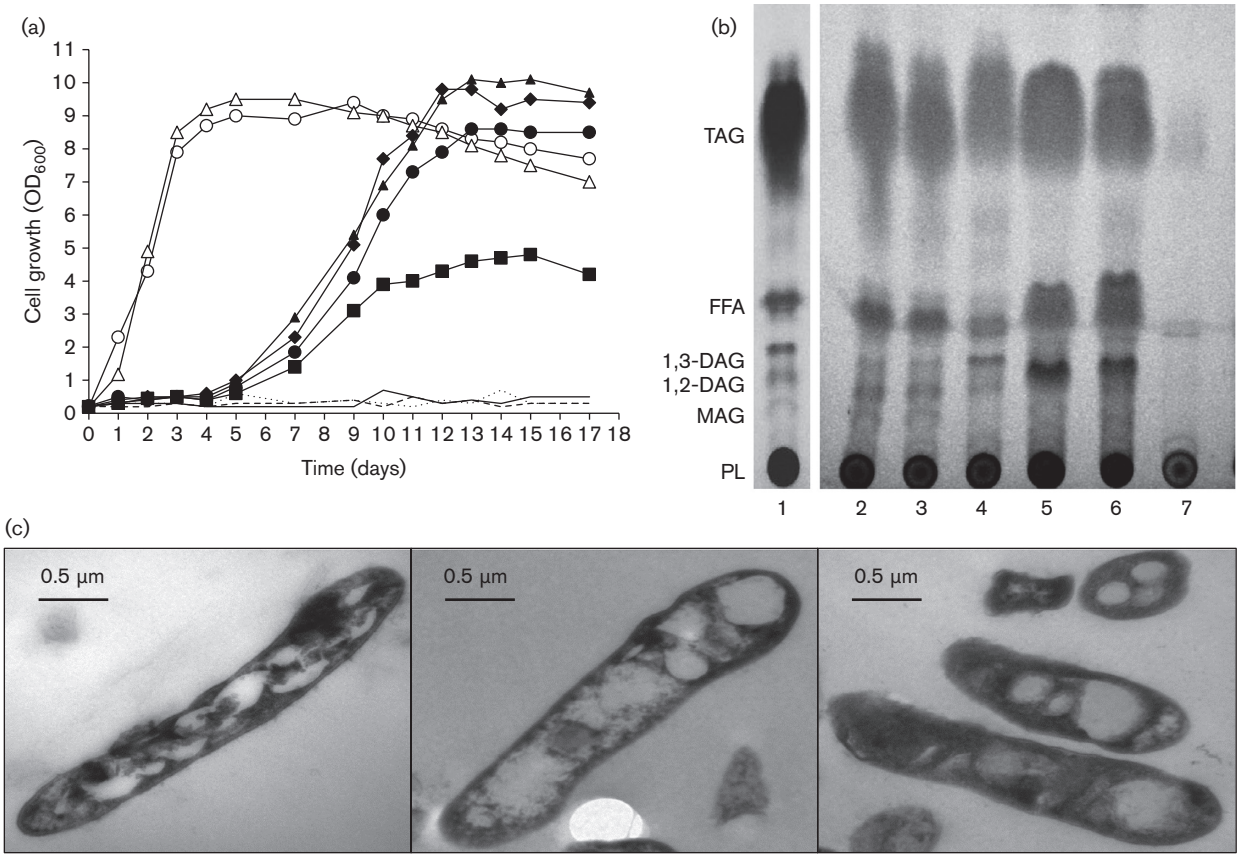

Fig. 1. Growth and lipid accumulation of different species of the genus *Rhodococcus* using glycerol as the sole carbon source. (a) Growth kinetics on glycerol of *R. fascians* F7 (open triangles), *R. erythropolis* DSMZ 43060 (open circles), *R. opacus* PD630 (filled diamonds), *R. opacus* MR22 (filled triangles), *R. jostii* RHA1 (filled circles) and *R. equi* ATCC 6939 (filled squares). Three strains belonging to *R. jostii* unable to grow on glycerol are represented by lines without symbols: *R. jostii* 006 (continuous line), *R. jostii* 346 (thin dotted line) and *R. jostii* G212 (thick dotted line). (b) TLC analysis of neutral lipids extracted from glycerol-grown rhodococcal strains. Cells were collected at the stationary phase. Lanes: 1, mixture of reference lipids used as control (FFA, free fatty acid; DAG, diacylglycerol; MAG, monoacylglycerol; PL, phospholipids); 2, *R. opacus* PD630; 3, *R. opacus* MR22; 4, *R. jostii* RHA1; 5, *R. fascians* F7; 6, *R. erythropolis* DSMZ 43060; 7, *R. equi* ATCC 6939. (c) Lipid inclusions produced by *R. fascians* F7 after growth in minimal media with glycerol as the sole carbon source as revealed by transmission electron microscopy.

(5.2 % CDW) from glycerol in comparison with the other investigated rhodococcal species (Fig. 1a, b, Table 1).

Table 2 shows the fatty acid composition of lipids from the representative rhodococcal species used in this study analysed by GC-flame ionization detection as described in the supplementary Material and methods. In general, palmitic acid ($C_{16:0}$) and oleic acid ($C_{18:1}$) were the major fatty acids synthesized by rhodococcal cells during cultivation on glycerol. However, significant amounts of stearic acid ($C_{18:0}$) were produced by *R. erythropolis* and *R. fascians* strains, approximately twofold higher than in *R. opacus* and *R. jostii* lipids (Table 2).

Interestingly, *R. fascians* and *R. erythropolis*, which were considered in previous studies as non-oleaginous bacteria when grown on glucose or gluconate (Alvarez *et al.*, 1997; Alvarez 2003), exhibited an oleaginous phenotype after cultivation on glycerol as the sole carbon and energy source (Fig. 1b, Table 1). In this context, Fig. 1(c) shows lipid inclusion bodies produced by *R. fascians* F7 cells during growth on glycerol.

Survey of key genes for glycerol catabolism in the available genomic databases

The available genomic information for the rhodococcal species used in this study provides the possibility for comparative genome analysis on the occurrence and distribution of key genes involved in glycerol uptake and degradation. For this, genomes of *R. opacus* (PD630 and B4), *R. jostii* RHA1, *R. erythropolis* PR4, *R. fascians* F7 and *R. equi* 103S were examined in detail.

Table 1. Maximum cell biomass and lipid production of different *Rhodococcus* strains grown in minimal medium with glycerol as the sole carbon source

Strain	Biomass ($g\ l^{-1}$)	Fatty acids (% CDW)	Fatty acids ($g\ l^{-1}$)	Time (days)
R. opacus PD630	3.8	38.4	1.4	17
R. jostii RHA1	2.5	30.5	0.8	17
R. jostii 602	2.7	28.3	0.8	17
R. erythropolis DSMZ 43060	4.1	38.3	1.6	6
R. erythropolis 17	3.9	40.4	1.6	6
R. erythropolis DM1-21	4.0	42.7	1.7	6
R. fascians F7	4.3	44.6	1.8	6
R. fascians S1.17b	3.9	37.6	1.1	6
R. fascians D188-5	4.1	28.4	1.2	6
R. fascians 123	3.9	30.6	1.2	6
R. equi ATCC 6939	1.5	5.2	1.1^{-1}	17

Our bioinformatic analysis of rhodococcal genomes showed no evidence for the occurrence of fermentative pathways for glycerol degradation, as genes coding for glycerol dehydrogenase (*dhaD*) and dihydroxyacetone kinase (*dhaK*) enzymes were not found. In contrast, we found genes coding for the putative glycerol kinase (*glpK*) and G3P dehydrogenase (*glpD*) enzymes in all genomes analysed. According to this, we propose the catabolic glycerol pathway shown in Fig. 2(a) as the starting framework for our analysis of DNA and protein sequence databases. In the proposed pathway, the entrance of glycerol into the cell occurs by simple diffusion or is mediated by a glycerol facilitator protein called GlpF; then glycerol is activated to G3P by a glycerol kinase enzyme (GlpK) and finally G3P is oxidized by the membrane-bound G3P dehydrogenase (GlpD) enzyme to DHAP, which enters into the central metabolic pathways (Fig. 2a).

Genes encoding a putative glycerol uptake facilitator protein (*glpF*) were found in genomes of *R. erythropolis* PR4 and *R. fascians* F7, within a cluster including genes that encode putative GlpK and GlpD enzymes (RER_46060–RER_46040 for strain PR4 and ACG96_05165–ACG96_05175 for strain F7) (Fig. 2b). Protein sequences of these gene clusters from *R. erythropolis* PR4 and *R. fascians* F7 (here named *glpF*, *glpK1* and *glpD1*) show high identity with homologous proteins involved in glycerol metabolism of *M. smegmatis* (63–77 %), *B. subtilis* (30–50 %) and *E. coli* (33–52 %) (Holmberg *et al.*, 1990; Beijer *et al.*, 1993; Voegele *et al.*, 1993; Feese *et al.*, 1998; Titgemeyer *et al.*, 2007). This gene cluster (*glpFK1D1*) was absent in the genomes of *R. opacus*, *R. jostii* and *R. equi*. However, these micro-organisms possess an alternative gene cluster (here named cluster 2) containing *glpK2* and *glpD2* with opposite transcription directions, which in general exhibited high identity with GlpK1 (73–75 %) and GlpD1 (57–59 %) from *R. erythropolis* PR4 and *R. fascians* F7 (Fig. 2b). Interestingly, *R. erythropolis* PR4 was the unique species amongst the rhodococcal strains investigated in this study, simultaneously containing both gene clusters (clusters 1 and 2) in its genome (Fig. 2b). In addition, an additional gene coding for a putative G3P dehydrogenase enzyme (GlpD3) was present in a different locus of the investigated rhodococcal genomes, sharing 30–33 % identity with GlpD1 and 31–32 % identity with GlpD2.

The predicted GlpK enzymes (from gene clusters 1 and 2) from rhodococci possessed the two conserved signatures reported for well-characterized GlpK enzymes from *E. classeliflavus* (Yeh *et al.*, 2004), *B. subtilis* (Wehtje *et al.*, 1995) and *E. coli* (Ormö *et al.*, 1998): signature 1 (FGGY_KINASES_1, PS00933), which corresponds to the central region, and signature 2 (FGGY_KINASES_2, PS00445) located in the C-terminal region (Table S3). Moreover, they contained the conserved residues T, Y and D, which are required for the binding of glycerol, as well as the N-terminal conserved domain 'DQGTTSSR' and several amino acids involved in ATP-binding (Flaherty *et al.*, 1991; Feese *et al.*, 1998; Alvarez *et al.*, 2004) (Table S3).

We performed structural prediction and modelling of GlpK1 from *R. fascians* F7 and GlpK2 from *R. opacus*

Table 2. Fatty acid composition of lipids accumulated by different *Rhodococcus* strains

Strain	Relative proportion of fatty acids (%, w/w)								Proportion SFA/UFA*
	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{17:1}$	$C_{18:0}$	$C_{18:1}$	
R. opacus PD630	3.4	6.9	28.1	8.3	10.1	7.2	12.0	24.0	1.5
R. jostii RHA1	3.3	6.3	25.5	9.0	12.0	9.4	11.3	23.2	1.5
R. fascians F7	3.7	1.1	33.4	1.6	1.0	0.8	27.2	31.2	2.0
R. erythropolis DSMZ 43060	5.0	1.7	33.2	4.0	1.9	1.0	24.2	29.2	2.0
R. equi ATCC 6939	3.0	6.3	27.1	9.1	9.3	8.0	11.1	21.5	1.3
R. opacus PD630-pTip-QC2/*glpK1D1*$_{F7}$	3.5	6.7	28.9	6.9	8.7	7.2	12.0	25.6	1.5
R. opacus PD630-pTip-QC2	3.2	6.2	29.5	6.4	8.1	7.5	12.4	25.5	1.5

*SFA, Saturated fatty acid; UFA, unsaturated fatty acid.

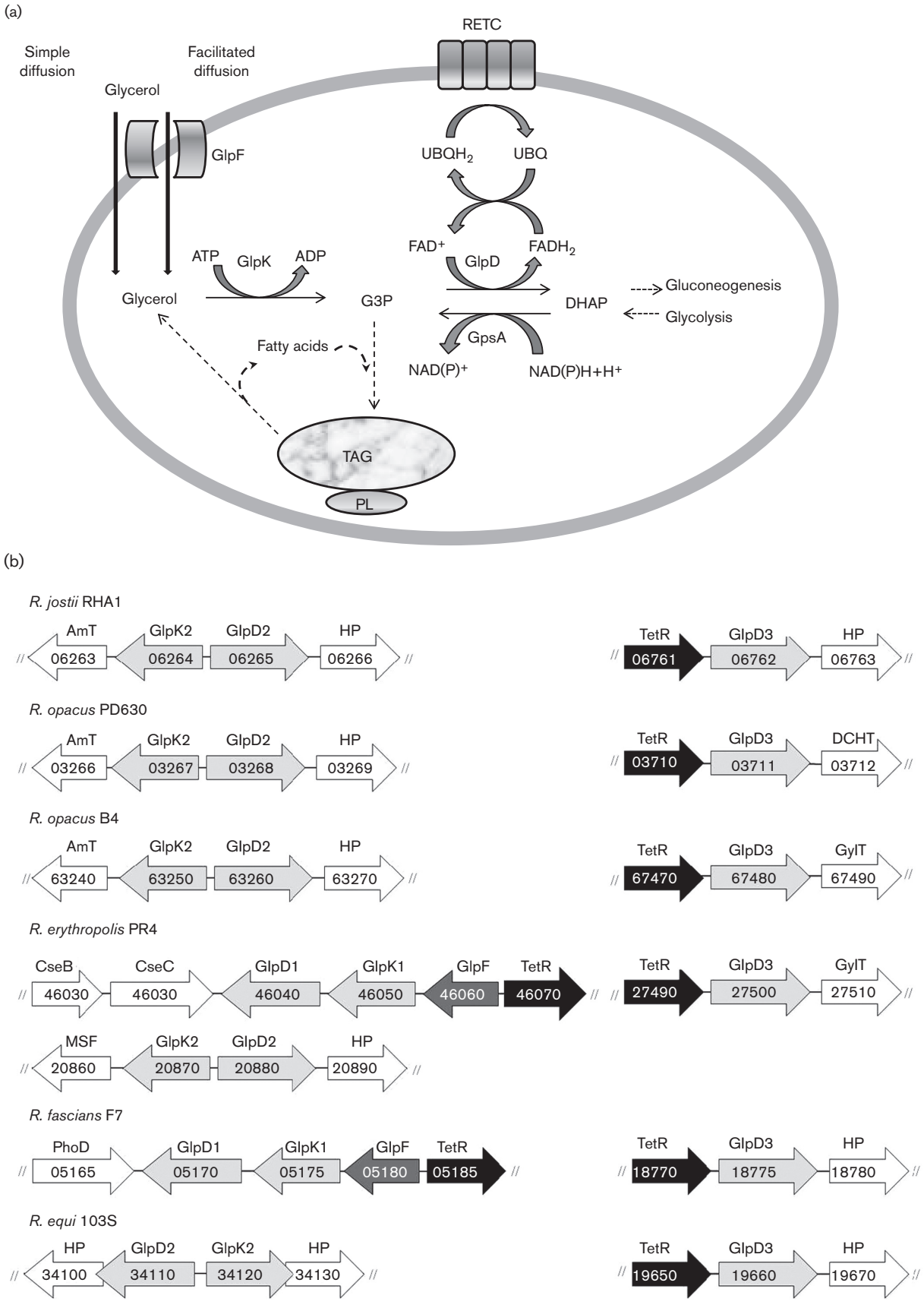

Fig. 2. Glycerol catabolism in *Rhodococcus* species. (a) Schematic representation of the possible glycerol catabolism pathway occurring in *Rhodococcus* species. (b) Organization of glycerol-related genes in *Rhodococcus* species. The arrows indicate the length and transcriptional orientation of genes. Genes encoding glycerol transport systems are depicted in dark grey arrows, glycerol catabolic genes in light grey arrows and regulatory genes probably related to glycerol metabolism in black arrows. Other genes are in white arrows. Genes are shown by their number without their corresponding prefixes: 'RHA1_ro', *R. jostii* RHA1; 'OPAG_', *R. opacus* PD630; 'ROP_', *R. opacus* B4; 'RER_', *R. erythropolis* PR4; 'ACG96_', *R. fascians* F7; 'REQ_', *R. equi* 103S. The names on the arrows represent the putative proteins encoded by each gene. GlpF, Glycerol uptake facilitator protein; GlpK, glycerol kinase; GlpD, G3P dehydrogenase; GpsA, NAD(P)H-dependent G3P dehydrogenase; PL, phospholipid; UBQ, ubiquinone; RETC, respiratory electron transport chain. CesB, Two-component response regulator; CesC, two-component histidine kinase; AmT, aminotransferase; GylT, glycosyltransferase; DCHT, alanine-rich transferase; HP, hypothetical protein.

PD630 using the Phyre2 web server (Kelley & Sternberg, 2009). Modelled structures of both putative enzymes exhibited the characteristic glycerol kinase fold consisting of two large domains, one on either side of a deep and narrow cleft. At the interface of the two domains The catalytic site, responsible for the binding of ATP and glycerol (Fig. 3), is localized at the interface of the two domains. In the Gram-positive bacteria *E. classeliflavus* and *B. subtilis*, GlpK enzymes are activated by phosphorylation at an activation loop containing conserved histidine residues (His232 and His230), which is located ~25 Å from the catalytic site of the enzyme (Wehtje *et al.*, 1995; Yeh *et al.*, 2009). Interestingly, a major conformational difference between rhodococcal GlpK1 and GlpK2 was observed in the putative activation loop of enzymes (Fig. 3). The putative activation loop of GlpK1 from *R. fascians* is more prominent than that of GlpK2 from *R. opacus* PD630, exhibiting a major exposed surface in the structure, which may determine a higher accessibility for its activation by phosphorylation. In addition, the alignment of rhodococcal GlpK1 and GlpK2 with glycerol kinase enzymes from *E. casseliflavus* and *B. subtilis* showed the occurrence of the conserved histidine for phosphorylation in GlpK2 from *R. opacus* PD630, whereas this amino acid was replaced at this position by an arginine in GlpK1 from *R. fascians* F7 (Table S3). Interestingly, it has been reported that the replacement of histidine for arginine at this position in *E. casseliflavus* (His232Arg) and *B. subtilis* (His230Arg) promotes a conformational change in the activation loop, which significantly increases the enzyme activity between seven- and 19-fold (Yeh *et al.*, 2009; Wehtje *et al.*, 1995). Whether the observed differences in the predicted protein structures of rhodococcal GlpK1 and GlpK2 affect the enzyme activity or efficiency remains to be investigated in the future.

Table S4 shows the different putative G3P dehydrogenase FAD-dependent (*glpD*) enzymes occurring in the investigated rhodococci, including GlpD1, GlpD2 and GlpD3, which exhibit the conserved domains of short-chain dehydrogenases/reductases (SDR, Cl21454) and characteristic signatures FAD_G3PD_1 (PS00977) and FAD_G3PD_2 (PS00978) (Table S4), responsible for the oxidation of G3P to DHAP.

Finally, the glycerol facilitator proteins (GlpF) detected in *R. erythropolis* and *R. fascians* genomes (Fig. 2b) showed the characteristic MIP (major intrinsic protein) family signature [(HNQA)-D-N-P-(STA)-(LIVMF)-(ST)-(LIVMF)-(GSTAFY)] (PS00221), and NPA1 and NPA2 domains located in the loops connecting transmembrane segments 2/3 and 5/6, respectively (Lagrée *et al.* 1999; Unger, 2000) (Table S5).

Phylogenetic analysis using proteins from the investigated rhodococci allowed us to separate the different GlpK and GlpD enzymes into diverse evolutionary groups (Fig. 4a, b).

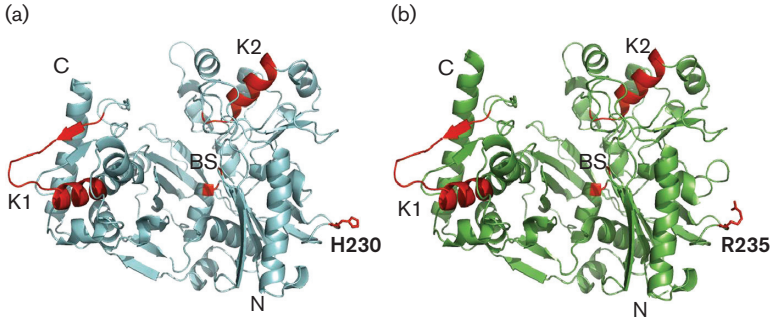

Fig. 3. Modelled structures of (a) *R. opacus* PD630 GlpK2 and (b) *R. fascians* F7 GlpK1. The locations of conserved signature motifs FGGY_KINASES_1 (PS00933, K1), FGGY_KINASES_2 (PS00445, K2) and glycerol binding site (BS) are shown. The position of the putative activation site (H230 or R235) is also shown.

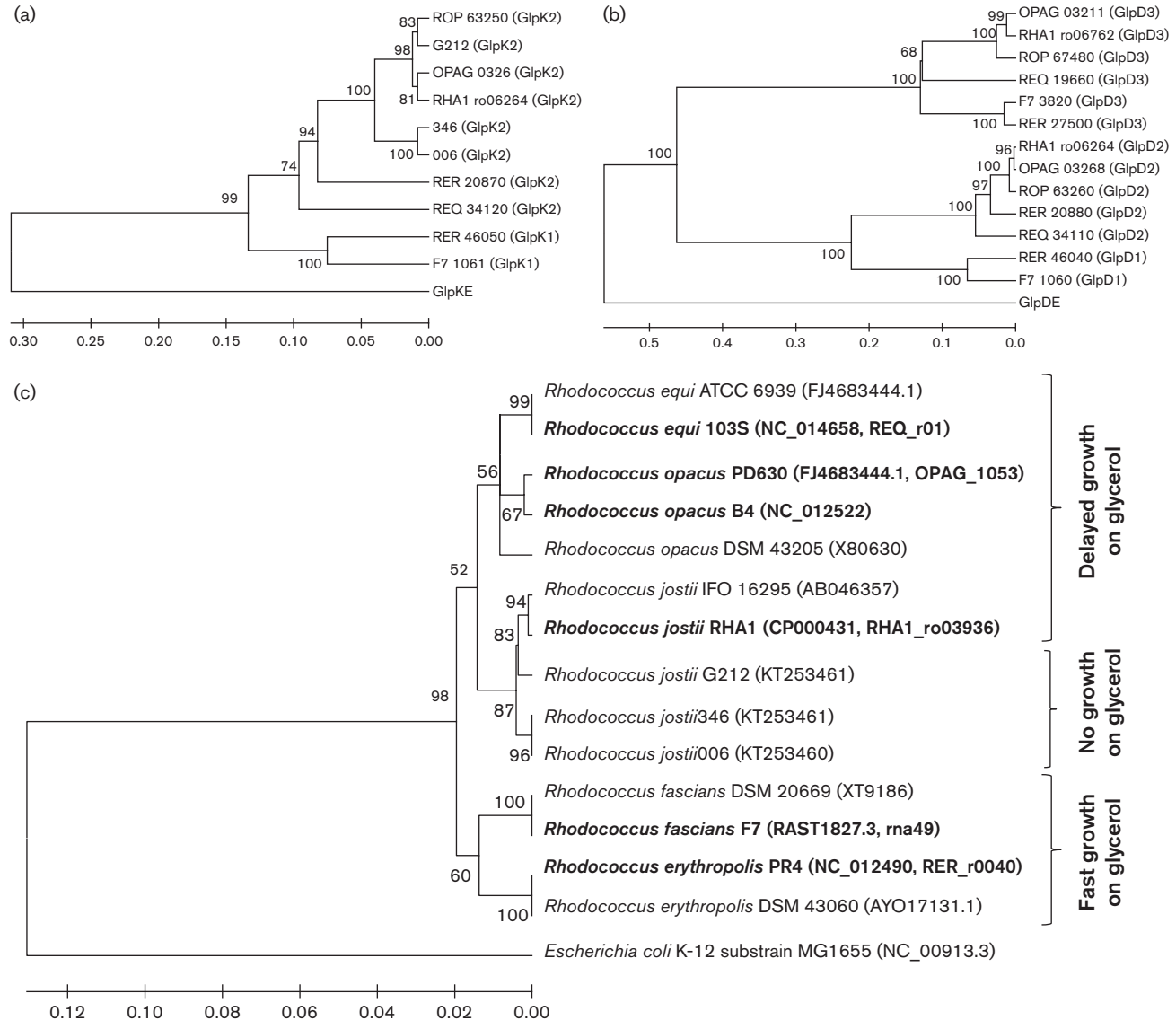

Fig. 4. Evolutionary relationship of glycerol-related genes between different *Rhodococcus* species. (a) Phylogenetic tree of glycerol kinase enzymes (GlpK) present in different *Rhodococcus* species. (b) Phylogenetic tree of G3P dehydrogenase FAD-dependent (GlpD). (c) Unrooted neighbour-joining tree based on 16S rRNA sequences showing the relationships between species of the genus *Rhodococcus*. Those *Rhodococcus* strains used in this study for genome analyses are in bold. 16S rDNA, GlpK (GlpKE) and GlpD (GlpDE) from *E. coli* K-12 substrain MG1655 were used as outgroups in the respective trees. GenBank nucleotide accession numbers and UniProt accession numbers are shown. Access numbers of the different GlpK and GlpD proteins are listed in Table S1 and S2, respectively.

Analysis of GlpK rhodococcal sequences showed the separation of two defined phylogenetic groups, including GlpK2 enzymes of *R. opacus*, *R. jostii*, *R. erythropolis* and *R. equi* in one group, and GkpK1 enzymes of *R. fascians* and *R. erythropolis* in the second group (Fig. 4a). This result suggested some evolutionary differences between GlpK1 and GlpK2 in rhodococci. Similar results were obtained after analysing GlpD sequences, as shown in Fig. 4(b). GlpD3 from all studied rhodococci formed a subgroup in the phylogenetic tree separated from the other subgroups formed by GlpD1 and GlpD2. In addition, GlpD1 from *R. fascians* and *R. erythropolis* formed a subgroup separated from the other subgroup, which includes GlpD2 from *R. opacus*, *R. jostii*, *R. erythropolis* and *R. equi* (Fig. 4b). Fig. 4(c) shows an unrooted neighbour-joining tree based on 16S rRNA sequences of diverse species of rhodococci. Interestingly, in the three phylogenetic trees there was a relation between the groups formed and the physiological ability of cells to grow on glycerol (Fig. 4c). The rhodococcal species that grow fast on glycerol (*R. fascians* and

R. erythropolis) possessed the gene cluster *glpFK1D1*, whereas those species that grow on glycerol after a long lag phase possessed the *glpK2D2* cluster (Figs 1, 2 and 4). Taken together, these results suggested that the ability to utilize and metabolize glycerol was modelled in different ways during the evolution within the species of the *Rhodococcus* genus.

Complementation of *R. jostii* strains unable to grow on glycerol

Three *R. jostii* strains (006, 346 and G212) closely related to *R. jostii* RHA1 (Fig. 4c) were unable to grow on glycerol as the sole carbon and energy source after 30 days of incubation (Fig. 1a). However, we demonstrated in this study that *R. jostii* strains RHA1 and 602 were able to grow on glycerol after a lag phase of ~5 days of incubation (Fig. 1a). The differential ability to grow on this substrate has also been reported for diverse strains of mycobacteria in which alterations in the *glpK* sequence occur, including SNPs promoting a shift in the reading frame, the generation of truncated proteins or changes in enzyme activity (Keating *et al.*, 2005). Taking this into account, we amplified and sequenced *glpK* genes from strains 006, 346 and G212 using specific primers designed with *glpK2* sequences from *R. jostii* RHA1 and *R. opacus* PD630. Sequence analysis revealed high amino acid identities (94–98 %) with GlpK putative enzymes from *R. jostii* RHA1 and *R. opacus* (PD630 and B4), and the absence of evident alterations in their sequences. Conserved domains of GlpK enzymes were present in the amplified sequences of the glycerol-negative rhodococcal strains (Table S3). Thus, to test whether the inability to use glycerol as the sole carbon source was related to the GlpK enzyme, as the reaction catalysed by this enzyme is the rate-limiting step of the pathway, we cloned the *glpK2* gene of *R. opacus* PD630 into the pJAM2 expression vector containing an acetamide-inducible promoter and transferred it into *R. jostii* strains 006, 346 and G212. The recombinant strains together with WT strains carrying the empty vectors were analysed for growth and TAG production from glycerol. As shown in Fig. 5(a), the three glycerol-negative rhodococcal strains expressing $glpK2_{PD630}$ were now able to grow on glycerol as the sole carbon and energy source. The delayed growth profiles of recombinant strains *R. jostii* G212-pJAM2/$glpK2_{PD630}$, 346-pJAM2/$glpK2_{PD630}$ and 006-pJAM2/$glpK2_{PD630}$ were similar to those of *R. opacus* PD630 and *R. jostii* RHA1 (Figs 1a and 5a) during cultivation with glycerol. In addition, cells of the three recombinant strains were able to produce and accumulate significant amounts of TAGs (22–24 % CDW) from glycerol after 17 days of incubation under nitrogen-limiting conditions (Fig. 5b). These results indicated that the inability of *R. jostii* strains 006, 346 and G212 to grow on glycerol was related with their GlpK enzymes/genes.

To test the *in vivo* functionality of the *glpK2* gene from *R. jostii* 006, we cloned the native $glpK2_{006}$ into the pJAM2 expression vector under the control of an acetamide-inducible promoter (pJAM2/$glpK2_{006}$) and transferred it into *R. jostii* 006. As shown in Fig. 5(a), the expression of $glpK2_{006}$ under the control of an external promoter promoted the growth of *R. jostii* 006 on glycerol.

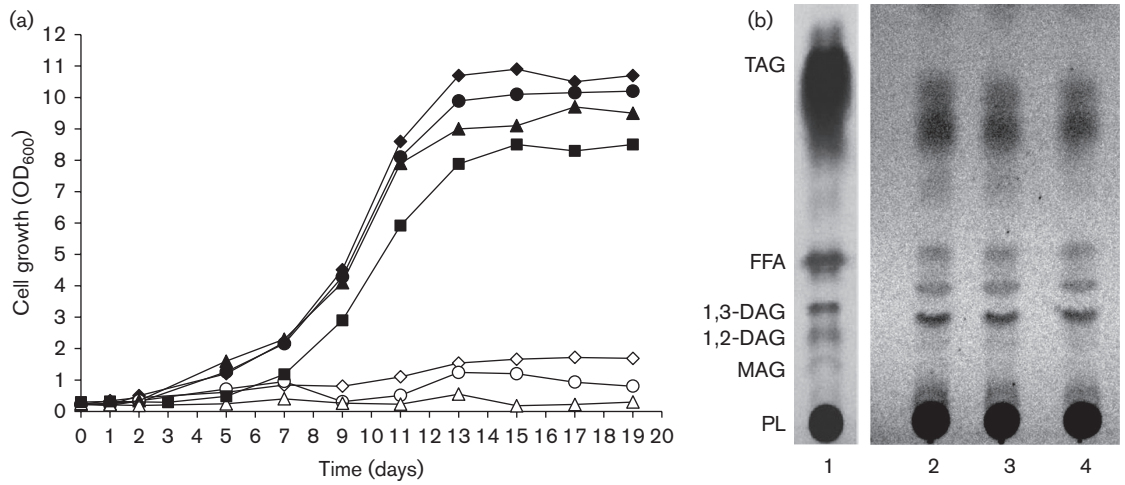

Fig. 5. Growth and lipid accumulation by glycerol-negative *R. jostii* strains 006, 346 and G212 after expressing *glpK* genes from *R. opacus* PD630 and *R. jostii* 006 during cultivation of cells in MSM medium containing 0.3 % (v/v) glycerol as the sole carbon source: (a) *R. jostii* 006-pJAM2 (open diamonds) and pJAM2/$glpK2_{PD630}$ (filled diamonds), *R. jostii* G212-pJAM2 (open circles) and pJAM2/$glpK2_{PD630}$ (filled circles), *R. jostii* 346-pJAM2 (open triangles) and pJAM2/$glpK2_{PD630}$ (filled triangles), and *R. jostii* 006-pJAM2/$glpK2_{006}$ (filled squares). (b) TLC analysis of neutral lipids extracted from glycerol-grown cells. Lanes: 1, mixture of reference lipids used as control (FFA, free fatty acid; DAG, diacylglycerol; MAG, monoacylglycerol; PL, phospholipid); 2, *R. jostii* 006-pJAM2/$glpK2_{PD630}$; 3, *R. jostii* G212-pJAM2/$glpK2_{PD630}$; 4, *R. jostii* 346-pJAM2/$glpK2_{PD630}$.

These results demonstrated that, at least in strain 006, the *glpK2* structural gene was functional and that the inability of cells to grow on glycerol could be due to a regulatory effect or to an alteration of native promoter sequences.

Engineering *R. opacus* PD630 for improving glycerol utilization

R. opacus PD630 is able to grow and produce TAGs from glycerol, but after a long lag phase of incubation (Fig. 1a). With the aim to improve the utilization of glycerol by strain PD630 for cell biomass and lipid production, we analysed the effect of the heterologous expression of genes involved in glycerol uptake and catabolism from *R. fascians* and *R. erythropolis*, which contain the *glpFK1D1* cluster (Fig. 2b).

First, we hypothesized that the expression of *glpF*, which is lacking in cluster 2 (*glpK2D2*) in the *R. opacus* PD630 genome, could improve the entrance of glycerol into the cell, and enhance growth and lipid accumulation. Thus, the *glpF* gene of *R. erythropolis* DM1-21 was amplified, cloned into the pJAM2 expression vector and transferred into strain PD630. As shown in Fig. 6(a), no changes in growth kinetics and glycerol uptake were observed in recombinant PD630 cells expressing *glpF* in comparison with those of $PD630_{WT}$. In addition, expression of *glpF* in *R. opacus* PD630 produced no significant changes in TAG production, as shown in Fig. 6(b, c).

To test the effect of the glycerol catabolic genes (*glpK1D1*) on grow and TAG accumulation in the oleaginous PD630, we transferred *glpK1D1* genes from *R. fascians* F7 into this strain, cloned in the pTip-QC2 vector as an expression system, which possessed a thiostrepton-inducible promoter. The expression of $glpK1D1_{F7}$ in *R. opacus* PD630 improved growth and consumption of glycerol, reducing the extension of the lag phase in the culture, as shown in Fig. 6(d). In this context, *glpK1D1*-expressing cells of strain PD630 consumed 67 % of glycerol and grew to a cell biomass concentration of 4.4 g l^{-1} after 6 days of incubation, whereas the WT strain (PD630-pTip-QC2) showed only incipient growth and glycerol consumption at this time point (Fig. 6d). In addition, the genetic modification significantly accelerated the time ($\sim$10 days of incubation) needed to reach the maximal content of TAGs in recombinant cells (41 % of TAGs by CDW and 1.8 g l^{-1}) in comparison with PD630-pTip-QC2 (30 % of TAGs by CDW and 1.1 g l^{-1}) (Fig. 6e, f). The expression of $glpK1D1_{F7}$ improved cell biomass and TAG production $\sim$1.2- and $\sim$1.4-fold, respectively, as shown in Fig. 6(e). Analysis of lipids accumulated by *R. opacus* PD630-pTip-QC2/$glpK1D1_{F7}$ showed no differences in the fatty acid composition of lipids compared with those of *R. opacus* PD630-pTip-QC2 (Table 2).

Taken together, these results demonstrated that it was possible to improve oil yields from glycerol in rhodococci by moving gene modules encoding part of the glycerol metabolic pathways from one rhodococcal species to another.

DISCUSSION

In this study, we analysed the genetic potential and physiological ability of diverse rhodococcal species to grow and accumulate lipids from glycerol as the sole carbon and energy source. Our results suggested that the capability of using glycerol as the carbon source may be widespread amongst several rhodococcal species. However, we found important differences in the time needed by cells for starting growth from glycerol, which seems to be a species-dependent feature amongst rhodococci. In several previous studies, *R. opacus* and *R. jostii* species were considered the canonical oleaginous micro-organisms because they usually exhibit robust growth and high capability for accumulating TAGs from diverse substrates, such as gluconate, glucose and fructose, amongst others (Alvarez *et al.*, 1996, 1997; Alvarez, 2003). In contrast, members of *R. fascians* and *R. erythropolis* usually showed a lower capability for producing cell biomass and TAGs from these carbon sources; thus, they were considered as non-oleaginous species amongst rhodococci (Alvarez *et al.*, 1997; Alvarez, 2003). Interestingly, in this study we demonstrated that *R. fascians* and *R. erythropolis* are able to develop an oleaginous and more robust phenotype during growth on glycerol in comparison with the canonical *R. opacus* and *R. jostii* species. The latter species needed a prolonged lag phase before developing good growth and TAG accumulation. Once all these rhodococcal species started growth, they were able to produce significant amounts of cell biomass (0.8–1.8 g l^{-1}) and TAGs (30–44 % CDW) during cultivation of cells on glycerol. *R. equi* exhibited a lower capability for assimilating glycerol for biomass and TAG production compared with *R. opacus*, *R. jostii*, *R. fascians* and *R. erythropolis* species. These results suggested that the different *Rhodococcus* species have the potential to develop an oleaginous phenotype when the selected carbon source fits into the metabolic and regulatory network needed for activating the metabolic reactions that provide precursors, energy and NADPH for cell growth and lipid synthesis. The differences in the time-dependent growth regimes exhibited by rhodococci in this study may be based in the different genetic endowment, fluxes of carbon/precursors through metabolism and specific regulatory circuits in each rhodococcal species/strains. In this context, we found two different sets of genes for glycerol uptake and degradation (here named clusters 1 and 2). Phylogenetic analyses suggested that these gene modules possess different evolutionary origins within rhodococci (Fig. 4c). Results of this study indicated that the species/strains that possess cluster 1 (*glpFK1D1*) (*R. fascians* and *R. erythropolis*) exhibit fast growth and lipid accumulation, whereas those rhodococcal species/strains that possess cluster 2 (*glpK2D2*) (*R. opacus*, *R. jostii* and *R. equi*) exhibit delayed growth and lipid accumulation during cultivation on glycerol (Fig. 2a). The putative enzymes coded by these different sets of genes found in the investigated rhodococci exhibited phylogenetic and structural differences as revealed by

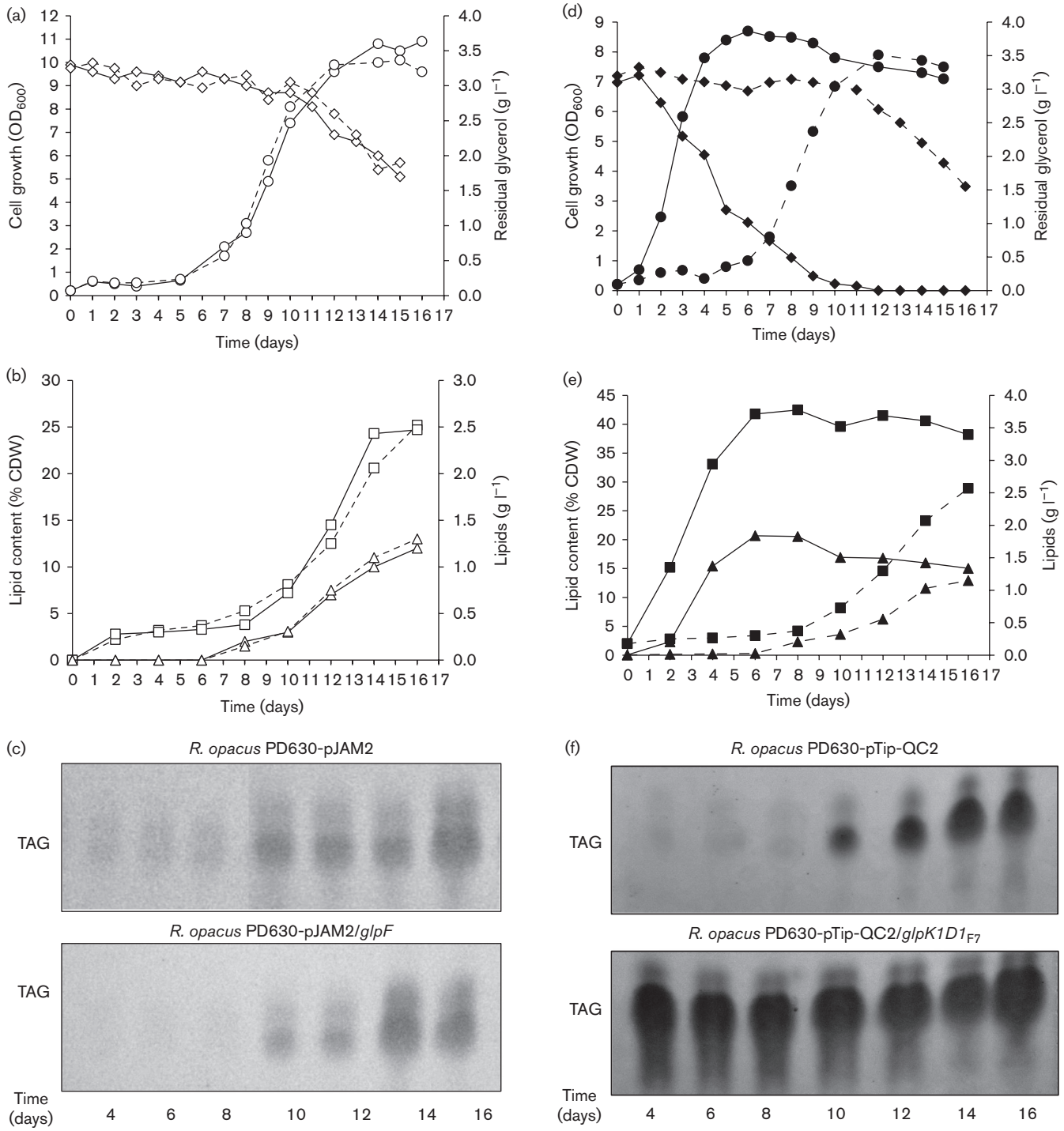

Fig. 6. Effect of heterologous expression of (a–c) pJAM2/*glpF* and (d–f) pTip-QC2/*glpK1D1* in *R. opacus* PD630 on growth, glycerol consumption (a, d) and lipid production (b, c, e, f). Recombinant strains are represented by continuous lines and control strains by dotted lines. The recombinant strains *R. opacus* PD630-pJAM2/*glpF* and *R. opacus* PD630-pTip-QC2/*glpK1D1*, and their respective controls *R. opacus* PD630-pJAM2 and *R. opacus* PD630-pTip-QC2, were grown in MSM1 with 0.3 % (v/v) glycerol as the sole carbon source. Growth (circles) was analysed by measuring the OD_{600} and glycerol remaining in the culture medium (diamonds) by colorimetric methods as described in Methods. (b, e) Recombinant strains and their respective controls were grown in MSM0.1 with 0.3 % (v/v) glycerol as the sole carbon source. Samples were collected at different times and lipids were quantified by GC. TAG values are expressed as % CDW (squares) and g l^{-1} (triangles). (c, f) TLC analysis of lipids produced by strains.

alignment and structural predictive analyses. Whether these differences can determine differential *in vivo* enzyme activities and regulatory events must be investigated in the future. We can speculate that rhodococci could have developed through the evolution of a differential arrangement of metabolic pathways and regulatory circuits as intrinsic characteristics of each micro-organism, which define the potential of bacterial cells to synthesize and accumulate TAGs from glycerol. Clusters 1 and 2 for glycerol degradation may work in a different metabolic context in rhodococcal cells. Cluster 1, which is more similar in gene composition and arrangement to those found in other glycerol-degrading bacteria (Schweizer & Po, 1996; Titgemeyer *et al.*, 2007; Baños *et al.*, 2009; Nikel *et al.*, 2014), may be designed for utilization of external glycerol taken up from the environment. The occurrence of the *glpF* gene only in cluster 1, but not in cluster 2, may be related to this feature. In this context, glycerol is not an abundant carbon source in the environment (e.g. in bulk soil), but can be found frequently in plant exudates (Nikel *et al.*, 2014); thus, some rhodococci that adapted to new nutritional niches may have selected specific metabolic arrangements to cope with environmental challenges. In this context, *R. fascians* is the only reported phytopathogen amongst rhodococci (Cornelis *et al.*, 2001); thus, they must have evolved diverse traits for interacting with plants. Interestingly, *R. fascians* possesses a robust metabolic configuration for producing cell biomass and TAGs from glycerol, as shown in this study. In a previous proteomic study performed in *R. jostii* RHA1, we demonstrated that lipolytic and glycerol-degrading enzymes (GlpK2 and GlpD2) increased in abundance during TAG-accumulating conditions, together with the typical G3P-synthesizing enzyme GpsA (Dávila Costa *et al.*, 2015). We proposed the occurrence of a dynamic cycling of cellular TAGs, resulting in the activation of glycerol catabolic enzymes, which works together with enzymes of glyceroneogenesis in order to regulate the availability of G3P in oleaginous cells. Thus, cluster 2 may play a key role in *R. opacus*, *R. jostii*, *R. erythropolis* and *R. equi* for the control of the availability of G3P in the metabolic network of cells during TAG accumulation, with a secondary role in the catabolism of external glycerol.

In this study, we found three *R. jostii* strains (006, 346 and G212) which were unable to grow on glycerol as the sole carbon source. As the driving force for the uptake of glycerol is the substrate phosphorylation to G3P by GlpK (Nikel *et al.*, 2014), we expressed $glpK2_{PD630}$ under an inducible promoter in order to complement the phenotype of the three strains regarding the utilization of glycerol. After heterologous expression of $glpK2_{PD630}$, the three glycerol-negative *R. jostii* strains used in this study were able to grow and produce TAGs from glycerol. However, the expression of its native $glpK2_{006}$ gene cloned into the pJAM2 expression vector with the P_{ace} promoter in *R. jostii* strain 006 allowed cells to grow on glycerol as the sole carbon source, suggesting a defect at its expression level in the native organism. Taken together, these results suggested that the alteration of the *glpK* gene at the structural or expression level is the major defect of glycerol-negative rhodococcal strains, similar to that reported for mycobacteria. Keating *et al.* (2005) demonstrated that some particular strains of *Mycobacterium bovis* and *Mycobacterium microti* were unable to use glycerol as a carbon source due to SNPs in *glpK* genes which affected enzyme activity or generated truncated proteins.

R. opacus PD630 is an important oleaginous model for its ability to produce significant amounts of lipids from diverse organic wastes, such as whey (Herrero & Alvarez, 2015), beet molasses (Voss & Steinbüchel, 2001), lignocellulosic wastes (Kurosawa *et al.*, 2013) and orange waste (Gouda *et al.*, 2008). Despite the ability of strain PD630 to grow and produce high amounts of TAGs from glycerol as the sole carbon source, cells need a prolonged time of adaptation (of several days) before growing. This is a negative feature of *R. opacus* PD630, if we consider its biotechnological potential for producing valuable oils for industry. For this reason, and in order to improve the process, we separately expressed *glpF* and *glpK1D1* genes from glycerol-fast-growing rhodococci in *R. opacus* PD630. The heterologous expression of *glpF* in strain PD630 did not produce any significant effect on glycerol utilization and TAG accumulation. This was in agreement with previous studies in *Streptomyces clavuligerus* (Baños *et al.*, 2009) and *Corynebacterium glutamicum* (Rittmann *et al.*, 2008) reporting that *glpF* expression by itself does not generate changes in growth and glycerol capture, which requires the simultaneous expression of genes coding for GlpK and GlpD enzymes. However, the heterologous expression of *glpK1D1* from *R. fascians* F7 in *R. opacus* PD630 was sufficient for improving the use of glycerol for growth and lipid production. *R. opacus* PD630-pTip-QC2/$glpK1D1_{F7}$ exhibited a similar growth profile to *R. erythropolis* and *R. fascians* strains, reducing the extension of time for accumulating TAGs to ~10 days of incubation (Figs 1a and 6c). Cells of recombinant PD630 accumulated up to 40 % (CDW) of lipids after 6 days of incubation. These results demonstrated that *R. opacus* PD630 can be used for the design and construction of an efficient cell factory that can ensure robust conversion of waste materials derived from the biodiesel industry to valuable oils.

In this study, we also demonstrated that alternative rhodococci could be considered for technological conversion of glycerol into commercial lipids, such as *R. erythropolis* and *R. fascians*, which exhibit an oleaginous phenotype during growth on this substrate. Interestingly, these rhodococcal species produced TAGs with a different fatty acid composition in comparison with those lipids produced by *R. opacus* and *R. jostii*, which contain an adequate composition for biodiesel production (Refaat, 2009; Schlagermann *et al.*, 2012). In contrast, TAGs produced by *R. erythropolis* and *R. fascians* contained ~66 % saturated fatty acids with a high content of stearic acid ($C_{18:0}$) (29–31 % CDW), similar to cocoa butter (23–30 % $C_{16:0}$, 32–37 %

$C_{18:0}$, 30–37 % $C_{18:1}$ and 2–4 % $C_{18:2}$), in which saturated fatty acids represent ~60–64 % (Papanikolaou *et al.*, 2003). These results highlight the metabolic versatility exhibited by rhodococci for producing interesting oils from industrial wastes.

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