Research article

Genome sequences of triacylglycerol metabolism in *Rhodococcus* as a platform for comparative genomics

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Received on January 5, 2013; Accepted on June 8, 2013; Published on June 22, 2013

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Abstract

Bacteria belonging to the *Rhodococcus* genus are usually able to synthesize and accumulate variable amounts of triacylglycerols (TAG) from diverse carbon sources. Although some significant advances in the basic knowledge on TAG metabolism in rhodococci have been made, the fundamental understanding of this process and its regulation remains to be clarified. The abundantly available genomic information for several rhodococcal species provides the possibility for comparative genome analysis on the occurrence and distribution of key genes and pathways involved in TAG metabolism. Our bioinformatic analyses of available databases from six rhodococcal strains demonstrated that genes/enzymes for reactions related to TAG biosynthesis and degradation, and fatty acid β-oxidation are surprisingly abundant in rhodococcal genomes. Several genes/enzymes of glycerolipids and fatty acid metabolism are highly represented in the analyzed genomes. A number of previously undescribed, new putative genes for glycerolipid metabolism in rhodococci have been identified and the size of each family has been estimated.

Introduction

Triacylglycerol (TAG) biosynthesis and accumulation is one of the most interesting features of actinobacteria belonging to the *Rhodococcus* genus. Some members of this genus can be considered as oleaginous microorganisms since they are able to accumulate more than 20% (of cellular dry weight) of TAG (Alvarez *et al.* 1996). The huge catabolic repertoire of these microorganisms, in addition to their capability to adapt their metabolism to a wide range of nutritional conditions, make such bacteria promising candidates for bioremediation of polluted environments (Larkin *et al.* 2005, Martínková *et al.* 2009, Warhurst & Fewson 1994). During nitrogen starvation and high C:N rates, as is the case during an oil spill in arid soil, cells are able to reduce their metabolic activity and their ability to mineralize the carbon source, but can significantly increase the biosynthesis and accumulation of TAG (Alvarez *et al.* 2000). This process permits a continuous degradation of environmental pollutants under unbalanced nutritional conditions, as found in the environment (Alvarez *et al.* 2002, Silva *et al.* 2010). In this context, the study of lipid metabolism in rhodococci can enhance our understanding of their physiology and their responses to diverse environmental conditions during bioremediation processes. On the other hand, basic knowledge on TAG metabolism in rhodococci may provide a new production platform for oils, in the biotechnology field. The applied potential of bacterial TAGs may be similar to that of vegetable sources, such as additives for feed, cosmetics, oleochemicals, lubricants and biofuels (Alvarez 2010).

Among rhodococci, *R. opacus* PD630 and *R. jostii* RHA1 have been used as research models for unraveling the physiology and molecular biology of TAG metabolism. Significant advances in the understanding of physiology, biochemistry and metabolic relationship of TAG accumulation to other pathways have occurred in the past few years (Alvarez *et al.* 1996, Alvarez *et al.* 1997, Alvarez *et al.* 2000, Hernández & Alvarez 2010). Moreover, new molecular studies have allowed the identification of certain genes/proteins involved in TAG biosynthesis and their accumulation in rhodococci (Alvarez *et al.* 2008, Hernández *et al.* 2008, Hernández *et al.* 2012, MacEachran *et al.* 2010). Recently, a large quantity of DNA and genomic sequences from rhodococci were produced. The comparative analysis of this available genomic information may contribute to new insights of the lipid field and extend the basic knowledge on TAG metabolism to lesser studied rhodococci. In this work,

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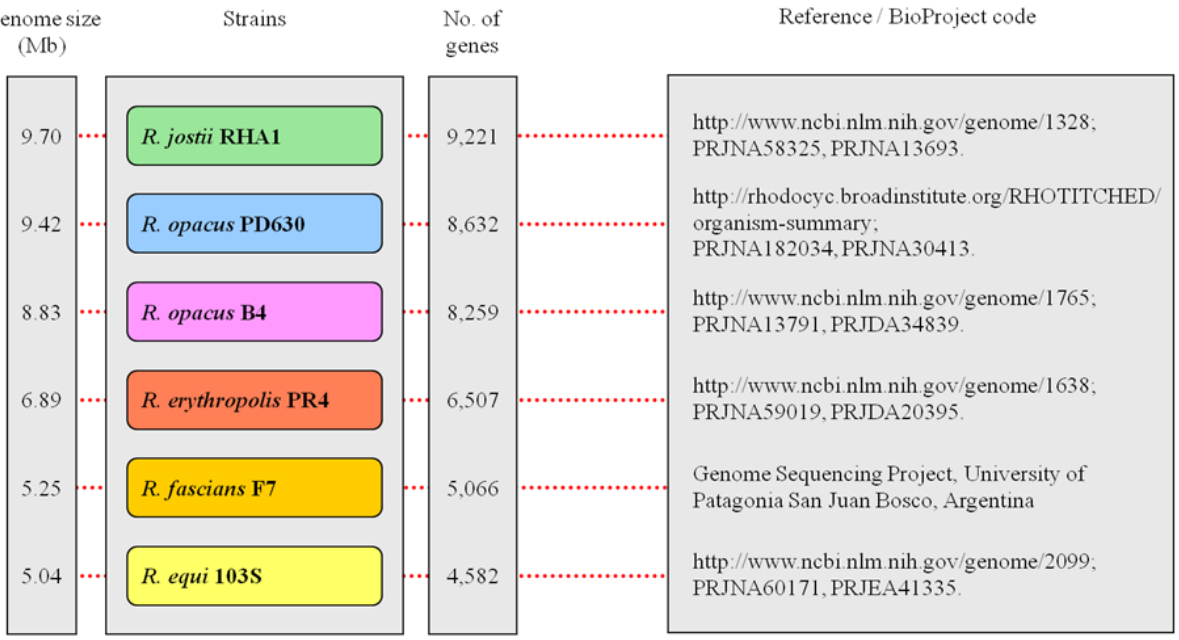

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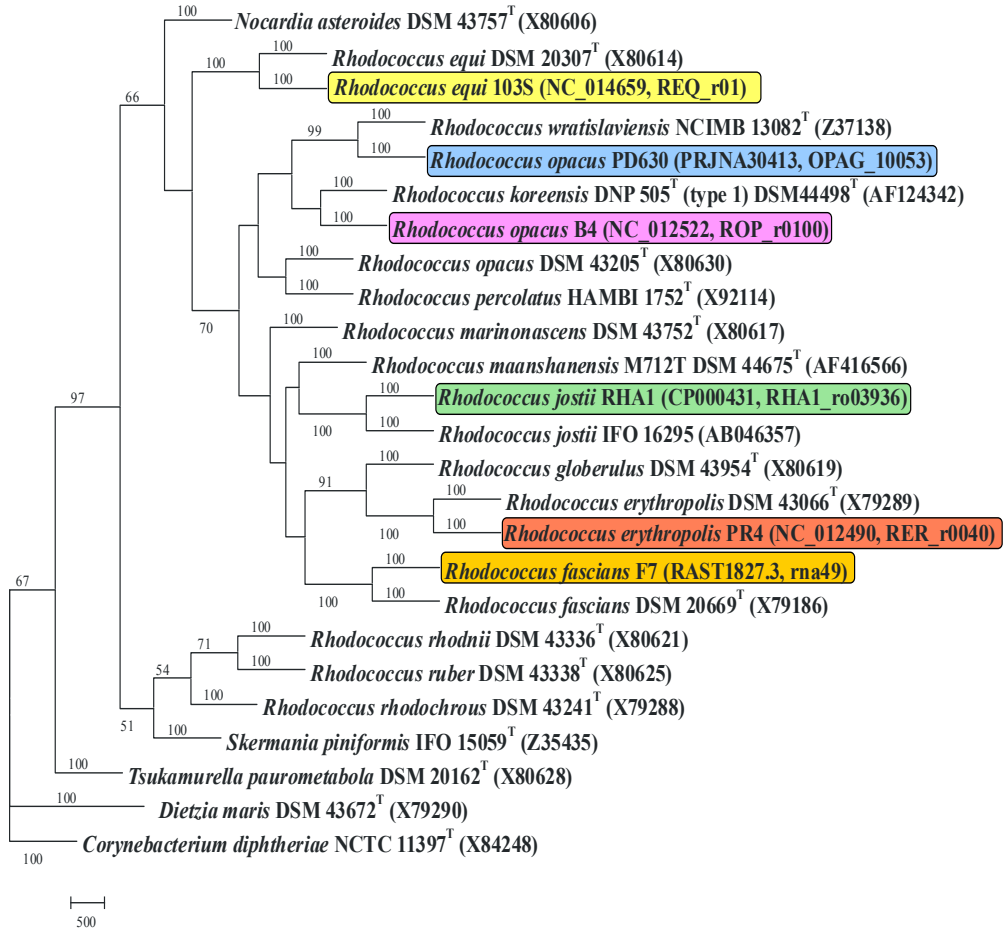

Figure 1. A) Comparative genomic information of the six *Rhodococcus* strains used in this study. B) Unrooted neighbour-joining tree based on 16S rRNA sequences (approximately 1,250 bp) showing the relationships between type strains of *Rhodococcus* genus and representatives of the *Corynebacterineae* suborder. Strains with an available genomic project used in this study are highlighted with different colours. Numbers at the nodes indicate percentages of bootstrap support based on neighbour-joining analyses of 1,000 resampled datasets. GenBank accession numbers are given in parentheses. The relationship of the species is shown by the horizontal lines that are proportional to the number of nt changes in the 16S rRNA (the scale is shown by the bar). *Corynebacterium diphtheriae* NCTC 11397^T 16S rRNA gene was used as outgroup.

we report a global and comparative analysis of genes/proteins involved in TAG metabolism identified from the genomes of *R. opacus* PD630, *R. opacus* B4, *R. jostii* RHA1, *R. erythropolis* PR4, *R. equi* 103S and *R. fascians* F7. Figure 1 shows the genome size, the number of genes and the database the data were retrieved from, as well as the phylogenetic relationship for each rhodococcal strain used in this study.

Materials and Methods

Phylogenetic analyses

For phylogenetic analyses, sequences were aligned using the Clustal W program (Thompson *et al.* 1994) and were processed by the Genedoc program (Nicholas *et al.* 1997). Evolutionary trees were inferred using maximum-likelihood (Felsenstein 1981), maximum-parsimony (Kluge & Farris 1969) and neighbor-joining (Saitou & Nei 1987) methods. *Corynebacterium diphtheriae* NCTC 11397^T 16S rRNA gene was used as outgroup. The resultant tree topologies were evaluated by bootstrap analyses (Felsenstein 1985), based on 1,000 resamplings, using the SEQBOOT, DNADIST and CONSENSE programs in the PHYLIP package (Felsenstein 1993).

Analyses of sequences

Genomes analyzed in this work correspond to the following strains: *R. jostii* RHA1 (McLeod *et al.* 2006), *R. opacus* PD630 (Holder *et al.* 2011), *R. opacus* B4 (Na *et al.* 2005), *R. erythropolis* PR4 (Sekine *et al.* 2006), *R. equi* 103S (Letek *et al.* 2010) and *R. fascians* F7 (unpublished results). In this study we retrieved the sequences of the genes/proteins involved in the rhodococcal TAG metabolism. The protein sequences were downloaded from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/), and in the case of *R. fascians* F7 from Rapid Annotation using the Subsystem Technology (RAST) server (Aziz *et al.* 2008).

To establish gene occurrence, genes involved in each metabolic reaction were identified using pathways presented in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa & Goto 2000) and were also searched by gene annotation or enzyme names using the NCBI and RAST Internet servers. For most genes and proteins, these sequences were used as queries for further searches using the BLASTP program in order to find homolog proteins. The search was run using the default parameters set by the program, considering as homologous proteins those with significant alignments (E value $\leq 10^{-50}$). Additionally, in these cases we performed specific analyses of gene/protein sequences searching for active sites, conserved domains and/or signal peptides using diverse bioinformatic tools: Conserved Domain Database (CDD), GenPept, Protein Family Database and Pfam. Such sequence analyses allowed us to tentatively identify several previously undescribed proteins in rhodococci. Further analyses using the BLASTP pro-

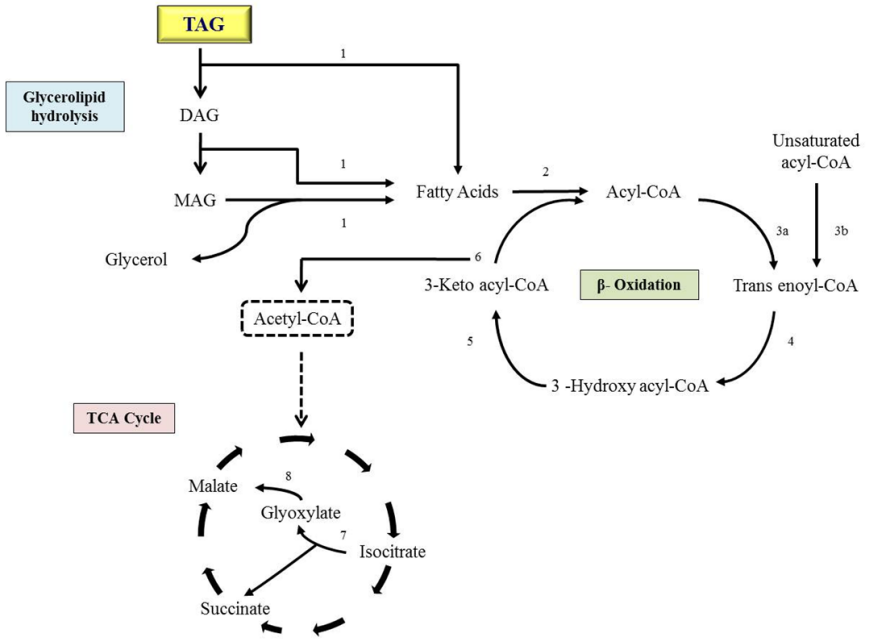

Figure 2. Putative pathways involved in TAG degradation in rhodococci. Each reaction in the figure has been numbered to provide a reference to Table 3.

Table 1. Genes coding for key enzymes of TAG biosynthesis in *Rhodococcus* species. Presence of the putative active site HHxxxDG or acyl_WS_DGAT region and the PAP2-like2 and "PlsC" domains were used as selection criteria for DGAT, PAP and AGPAT, respectively. Gene occurrence is represented in accordance to the locus name (RHA1_, OPAG_, ROP_, RER_, REQ_ and F7_) obtained from the NCBI database or the RAST server in the case of *R. fascians* F7 strain. Orthologs are shown in the same lines. EC, enzyme classification.

Key enzymes for TAG biosynthesis	*R. jostii* RHA1	*R. opacus* PD630	*R. opacus* B4	*R. erythropolis* PR4	*R. fascians* F7	*R. equi* 103S	EC
Multifunctional fatty acid synthase type 1a (FASI)	RHA1_ro01426	OPAG_09195	ROP_11350	RER_38730	F7_2364	REQ_30820	2.3.1.86
Glycerol-3-phosphate O-acyltransferase (GPAT)	RHA1_ro05648	OPAG_04306	ROP_57110	RER_16280	F7_1759	REQ_16780	2.3.1.15
1-acylglycerol-3-phosphate O-acyltransferase (AGPAT)	RHA1_ro04182	OPAG_06288	ROP_41110	RER_13990	F7_1968	-	2.3.1.51
	RHA1_ro01115	OPAG_05898	ROP_08430	RER_35800	F7_2673	REQ_28080	
	RHA1_ro04047	OPAG_06418	ROP_39250	RER_02010	F7_3258	REQ_01880	
	RHA1_ro04048	OPAG_06417	ROP_39260	RER_02020	-	-	
	RHA1_ro05647	OPAG_04305	ROP_57100	RER_16270	F7_1760	REQ_16770	
	RHA1_ro02025	OPAG_00578	ROP_16990	RER_16780	F7_1712	REQ_37300	
	RHA1_ro02057	OPAG_00610	ROP_17290	RER_16370	F7_0439	REQ_37680	
	RHA1_ro06465	OPAG_03466	ROP_65180	RER_23760	F7_1802	REQ_31700	
	-			pREL1_0275	-	-	
Phosphatidic acid phosphatase (PAP)	RHA1_ro00075	OPAG_07226	-	-	-	-	3.1.3.4
	RHA1_ro03136	-	ROP_pB01-01880	RER_10170	-	-	
	RHA1_ro08553	-			-	-	
	RHA1_ro04970	OPAG_02574	ROP_50370	-	-	-	
	-	-	-	RER_46290	-	-	
	-	-	-	-	F7_3389	-	
	-	-	-	-	-	REQ_09750	
Diacylglycerol acyltransferases (DGAT)	RHA1_ro00087	OPAG_03922	ROP_02100	-	-	-	2.3.1.20
	RHA1_ro08645	OPAG_03918	-	-	-	-	
	RHA1_ro06332	OPAG_03334	ROP_63930	RER_21760	F7_4458	REQ_33280	
	RHA1_ro06855	OPAG_03807	ROP_68400	RER_28450	-	REQ_20560	
	RHA1_ro05649	OPAG_04307	ROP_57120	RER_16290	-	REQ_16790	
	RHA1_ro05565	OPAG_07837	ROP_56340	-	-	-	
	RHA1_ro05356	OPAG_07648	ROP_54550	-	-	REQ_01690	
	RHA1_ro00583	OPAG_05286	ROP_04650	-	-		
	RHA1_ro08660	OPAG_05208	-	-	-	-	
	-	OPAG_06690	-	-	-	-	
	-	OPAG_07212	-	-	-	-	
	RHA1_ro00039	OPAG_07257	-	RER_33950	-	-	
	RHA1_ro00024	OPAG_07266	-	RER_34070	-	-	
	RHA1_ro00023	OPAG_07267	-	-	-	-	
	-	OPAG_06225	-	-	-	-	
	RHA1_ro01601	OPAG_00138	ROP_13050	RER_11860	F7_3568	REQ_40870	
	RHA1_ro02966	OPAG_01553	ROP_26950	-	-	-	
	-	-	-	RER_12460	-	-	
	-	-	-	RER_15300	-	REQ_12970	
	-	-	-	RER_15290	-	REQ_44660	
	-	-	-	-	-	REQ_29020	

gram were carried out to identify orthologs among the detected homologous proteins, considering the best hit with the highest percentage identity.

The presence of a signal peptide in lipase genes (for extracellular lipases) was analyzed by SignalP Server 4.0 for Gram positive bacteria using default parameters. This method provides a prediction of the cleavage sites of a probable signal peptide in the primary sequence of the protein (Petersen *et al.* 2011).

Results and Discussions

Triacylglycerol biosynthesis by rhodococci

The biosynthesis of fatty acids is performed in rhodococci by a multienzymatic complex known as fatty acid synthase type I (FASI). This complex consists of a unique large protein which catalyzes the successive reactions of condensation, reduction, dehydration and reduction. A unique FASI multienzyme gene is present in the genome of all rhodococci examined in this study (Table 1). FASI may provide fatty acids for phospholipids and TAG synthesis or for mycolic acid production after an elongation process mediated by FASII.

The main pathway for TAG biosynthesis in rhodococci seems to occur through three sequential acyl transfers from acyl-CoA to a glycerol backbone. The pathway involves the sequential acylation of the *sn*-1, 2 positions of glycerol-3-phosphate, resulting in the formation of phosphatidic acid. The removal of the phosphate group catalysed by the phosphatidic acid phosphatase enzyme occurs before the final acylation step. In the third acylation reaction, an acyl-residue is transferred to the vacant position of diacylglycerol, which is the final step of TAG biosynthesis. In general, a large number of genes seem to be involved in the rhodococcal TAG biosynthesis pathway as is shown in Table 1. Whereas the glycerol-3-phosphate O-acyltransferase enzyme is encoded by one gene in each *Rhodococcus* strain, the genes coding for 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT), phosphatidic acid phosphatase (PAP) and diacylglycerol acyltransferase (DGAT) are more abundant (Table 1). Interestingly, *R. fascians* F7 and *R. equi* 103S possess only one gene encoding a putative PAP enzyme, whereas at least four PAP genes occur in the *R. jostii* RHA1 genome (Table 1). The PAP enzyme catalyzes the removal of the phosphate group of phosphatidic acid to produce diacylglycerols. The two intermediates, phosphatidic acid and diacylglycerols, are also substrates for the synthesis of membrane phospholipids. Thus, the last step in the pathway, catalyzed by DGAT, is the only dedicated step in triacylglycerol synthesis. Only two rhodococcal DGATs have been cloned and characterized in detail, both from *R. opacus* PD630 (Alvarez *et al.* 2008, Hernández *et al.* 2012). Both genes, called *atf1* and *atf2*, were *in vivo* involved in TAG biosynthesis and accumulation in the PD630 strain. In this study, we used multiple sequence alignments to determine the potential number of genes encoding DGAT enzymes for TAG biosynthesis in the available rhodococcal genomes. In general, the latter contain several genes coding for putative DGATs, even though the DGAT gene number found in genomes seems to be a strain-dependent feature (Table 1). All deduced proteins showed the conserved putative active-site motif HHxxxDG described for bacterial DGAT enzymes. The sequence of the RHA1_ro06332 gene was the most conserved among the rhodococcal genomes included in this study (Table 1). Interestingly, *R. opacus* and *R. jostii* exhibited higher redundancy of DGAT genes in their genomes in comparison with *R. fascians*, *R. erythropolis* and *R. equi* (Table 1) (Alvarez *et al.* 2008, Holder *et al.* 2011, Hernández *et al.* 2012). The high content of DGAT genes in *R. opacus* and *R. jostii* is consistent with their ability to produce significant amounts of TAG from different substrates, such as gluconate or hexadecane (Alvarez *et al.* 1996, Hernández *et al.* 2008, Hernández *et al.* 2012). The redundancy and diversity of DGAT isoenzymes in *Rhodococcus* may permit cells to incorporate fatty acids into TAG with different specificity or to activate lipid accumulation under different conditions. Detailed studies on different rhodococcal DGATs and the other enzymes of the pathway may reveal the physiological role of each isoenzyme.

Degradation of triacylglycerols and fatty acids

Lipid metabolism, particularly the degradation pathways of fatty acids and TAG, have been only poorly studied in rhodococci in comparison to other bacteria, such as *E. coli* and *B. subtillis*. Based on the sequence homology and some shared biochemical characteristics of genes or enzymes from mycobacteria that are involved in lipolysis, it is generally assumed that the basic pathways of fatty acid and TAG degradation are analogous to those reported in other better-studied bacteria. Since the evidence obtained from lipolysis in rhodococci and related genera is still fragmentary, some broad generalizations are made in this section based on the putative identification of genes and limited experimental data. The genomes of rhodococci exhibit a very robust lipid catabolic network, with a large expansion in homologous genes involved in TAG and fatty acid degradation. This indicates that the carbon assimilation through lipid metabolism is a relevant feature for rhodococcal physiology. Figure 2 represents the starting framework for our analysis of the

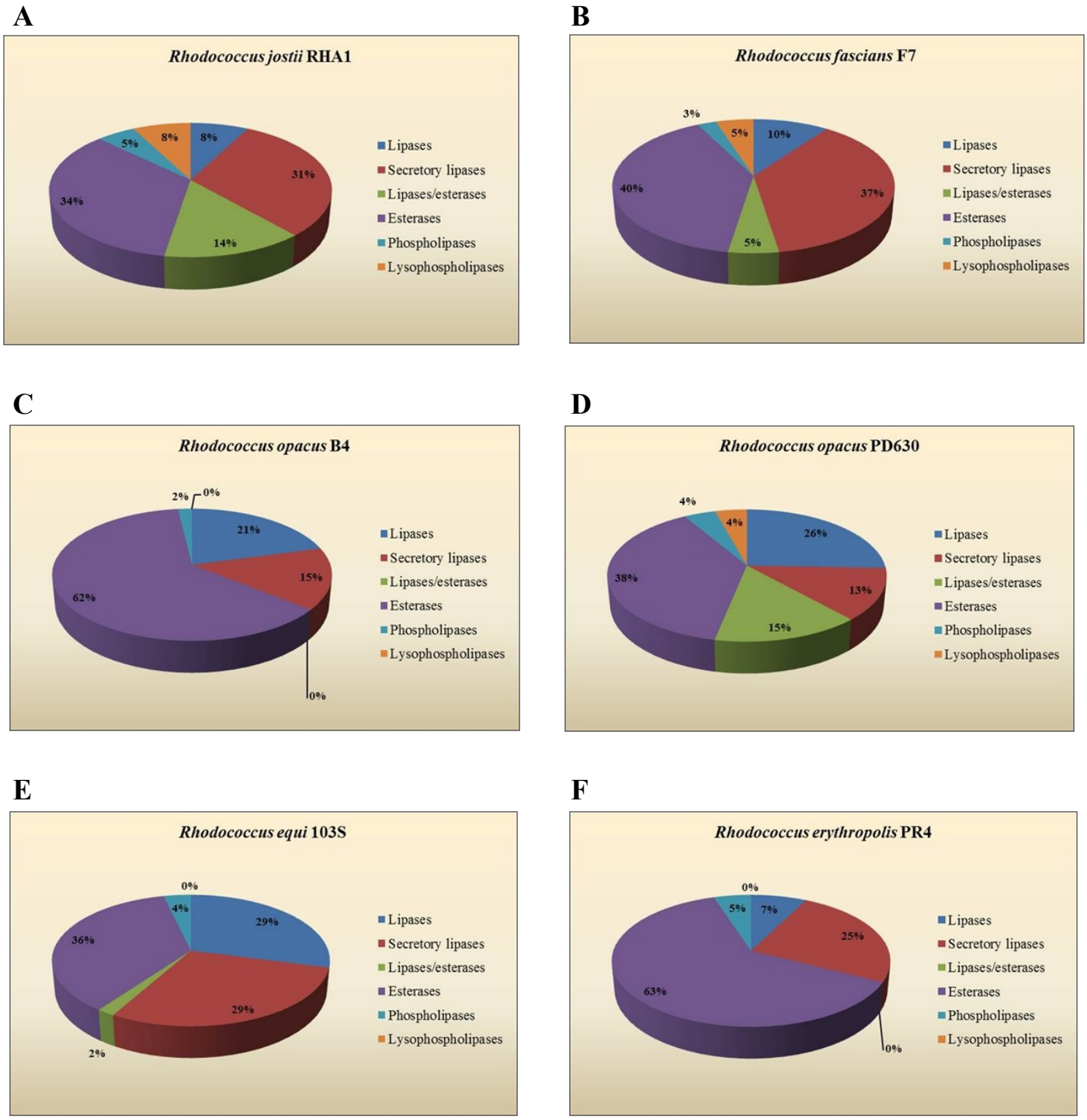

Figure 3. Relative proportions of diverse lipolytic enzymes occurring in different *Rhodococcus* species. Note that genes annotated as "lipases/esterases" in most rhodococcal genomes were annotated as "esterases" for *R. opacus* B4 and *R. erythropolis* PR4.

public DNA and protein sequence databases. Each reaction in Figure 2 has been numbered to provide a reference to Table 2. Our search of available databases demonstrated that genes/enzymes for reactions of TAG hydrolysis and fatty acid β-oxidation are surprisingly abundant in rhodococcal genomes. Several genes/enzymes of glycerolipid and fatty acid oxidation are highly represented in genomes (Table 2). The high expansion of enzymes involved in glycerolipids and other esterified compound hydrolysis indicates the importance of endogenous as well as exogenous lipid degradation for rhodococcal metabolism. In this context, it was proposed that lipids are a major growth carbon source for *R. equi* during infection *in vivo* (Letek *et al.* 2010). This microorganism is a unique animal pathogen within the *Rhodococcus* genus, with

Table 2. Gene occurrence related to triacylglycerol catabolism for species of the *Rhodococcus* genus.

Triacylglycerol catabolism	*R. jostii* RHA1	*R. opacus* PD630	*R. opacus* B4	*R. erythropolis* PR4	*R. fascians* F7	*R. equi* 103S	Reaction number	Enzyme classification	Genes/Proteins
Glycerolipids hydrolysis	6	12	11	3	4	16	1	3.1.1.3/ 3.1.1.23	Triacylglycerol lipases/ Monoacylglycerol lipases
	27	18	33	25	16	20	1	3.1.1.3	Esterases
	11	7			2	1	1	3.1.1.3	Lipasas/esterases
B-Oxidation	75	16	63	61	28	45	2	6.2.1.3	Long chain fatty acid CoA ligase
	1	1	1	1	1	1	3a	1.3.3.6	Acyl CoA oxidase
	92	30	101	58	29	47	3a	1.3.99.3	Acyl CoA dehydrogenase
	11	1			1		3a	1.3.99.13	Long chain acyl CoA dehydrogenase
	10	2			9		3a	1.3.8.1/1.3.99.2	Butyryl CoA deydrogenase
	4	4	3	1	2	1	3b	1.3.1.34	2,4-dienoyl CoA reductase
	60	5	68	41	15	24	4	4.2.1.17	Enoyl CoA hydratase
	11	3	9	4	5	2	5	1.1.1.35	3-Hydroxyacyl CoA dehydrogenase
	3	4	3	1	4	1	4/5	ND	Enoyl CoA hydratase/ 3-Hydroxyacyl CoA dehydrogenase complexes
	38	10	33	28	11	18	6	2.3.1.16/2.3.1.9	Acetyl CoA acyl transferase
Glyoxylate cycle	1	1	1	1	1	1	7	4.1.3.1	Isocitrate lyase
	2	3	2	1	1	1	8	2.3.3.9	Malate synthase
Extracellular glycerolipid hydrolysis	24	6	8	10	15	16		ND	Secreted lipases

Gene occurrence	
0	
1-10	
11-20	
21-30	
31-40	
>40	

the ability to inhabit within macrophages. There, cells find a lipid-rich environment with abundant availability of host cell lipids for fatty acid oxidation. The abundance of secreted lipases in the *R. equi* 103S genome (16 enzymes) reflects this situation (Table 2). Several secreted lipases as well as intracellular lipid-degrading enzymes also occur in the genome of saprophyte rhodococci, as shown in Table 2. Extracellular lipolytic enzymes may permit rhodococcal cells to degrade diverse lipids in the environment, which may occur in plants exudates or in the extracellular polymeric substances produced by cells during stress conditions, such as desiccation. In addition, intracellular lipids may account for 70% of dry weight of rhodococcal cells; thus, diverse lipases or esterases may contribute to the efficient lipid homeostasis during the life cycle of cells. Figure 3 shows the relative distribution of intracellular and extracellular lipolytic enzymes in rhodococcal genomes. In general, phospholipase enzymes responsible for phospholipid degradation and turnover are less abundant than neutral lipid-degrading enzymes in all analyzed genomes (Figure 3). Some additional generalizations could be made from Figure 3: (i) *R. jostii* RHA1 and *R. fascians* F7 seem to be enriched with secretory lipases genes (31 and 37% of total lipolytic enzymes, respectively), followed by *R. erythropolis* PR4 and *R. equi* 103S (25 and 29%, respectively). The genome of *R. opacus* (strains B4 and PD630) possesses a lesser proportion of secretory lipases in comparison to other rhodococci (Figure 3). In contrast, *R. opacus* exhibits the higher content of intracellular lipolytic enzymes, which is concordant with its ability to accumulate large amounts of intracellular TAG. Taken together, rhodococcal genomes possess a large set of lipolytic enzymes which may be involved in the mobilization of lipids in concert with other enzymes and proteins. Cells may require a complex regulatory network to finely control lipid metabolism under diverse environmental conditions. Although all studied rhodococci exhibit a high redundancy of lipolytic enzymes in their genomes, the relative proportion of each type of enzyme may depend on their particular physiology. There is only one report on the characterization of a rhodococcal lipase available. Bassegoda *et al.* (2012) cloned, purified and characterized the first lipase enzyme of this genus; in a strain of *R. erythropolis* (CR-53) isolated from a subtropical soil sample in Puerto Iguazú, Argentina (Ruiz *et al.* 2005). The enzyme, named LipR, was a secretory lipase exhibiting preference for medium-chain length acyl groups and showing high activity in a wide range of temperatures (4 to 60 ºC) (Bassegoda *et al.* 2012). Interestingly, this enzyme seems to belong to a new family of bacterial lipases which was included in family X, showing similar conserved motifs to the *Candida antartica* lipase clan. On the other hand, some lipases have been purified and characterized in related mycobacteria. Low *et al.* (2010) reported the occurrence of a lipolytic enzyme (BCG1721) associated to the lipid inclusion bodies, among other proteins involved in TAG metabolism. The authors assumed that BCG1721 was a bifunctional enzyme with lipase and potential long chain

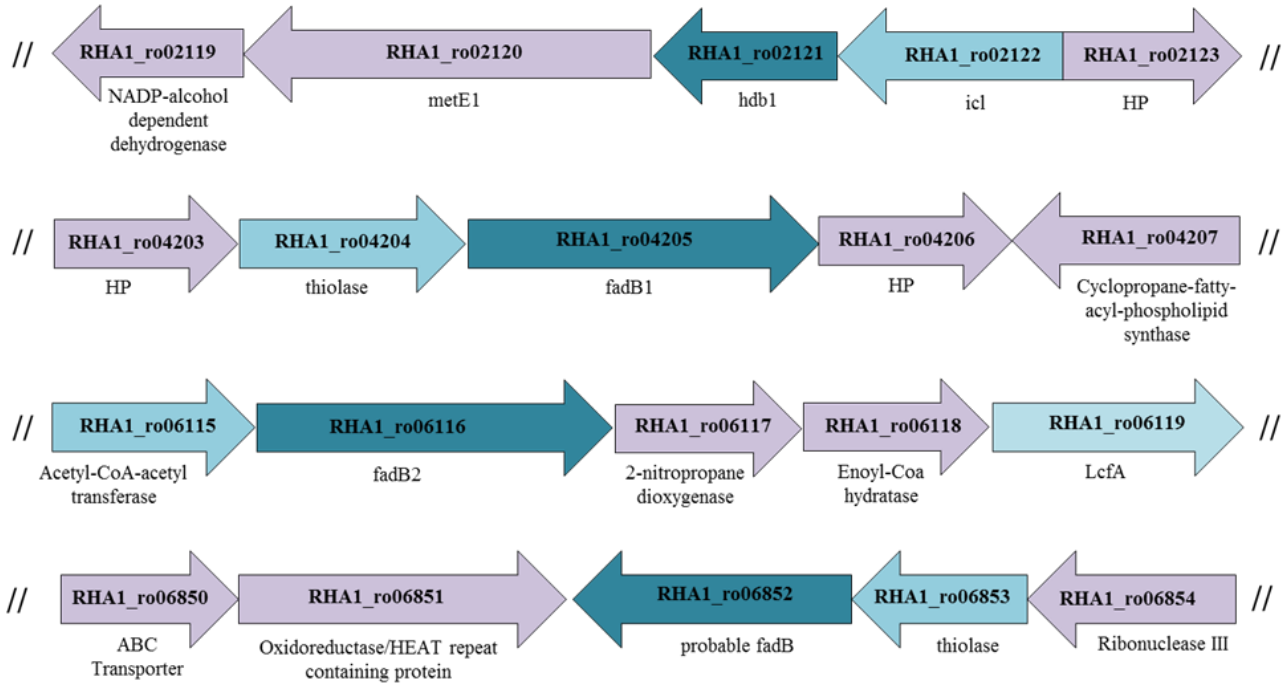

Figure 4. Organization of different genomic clusters occurring in *R. jostii* RHA1, containing putative genes involved in reactions of the β-oxidation pathway.

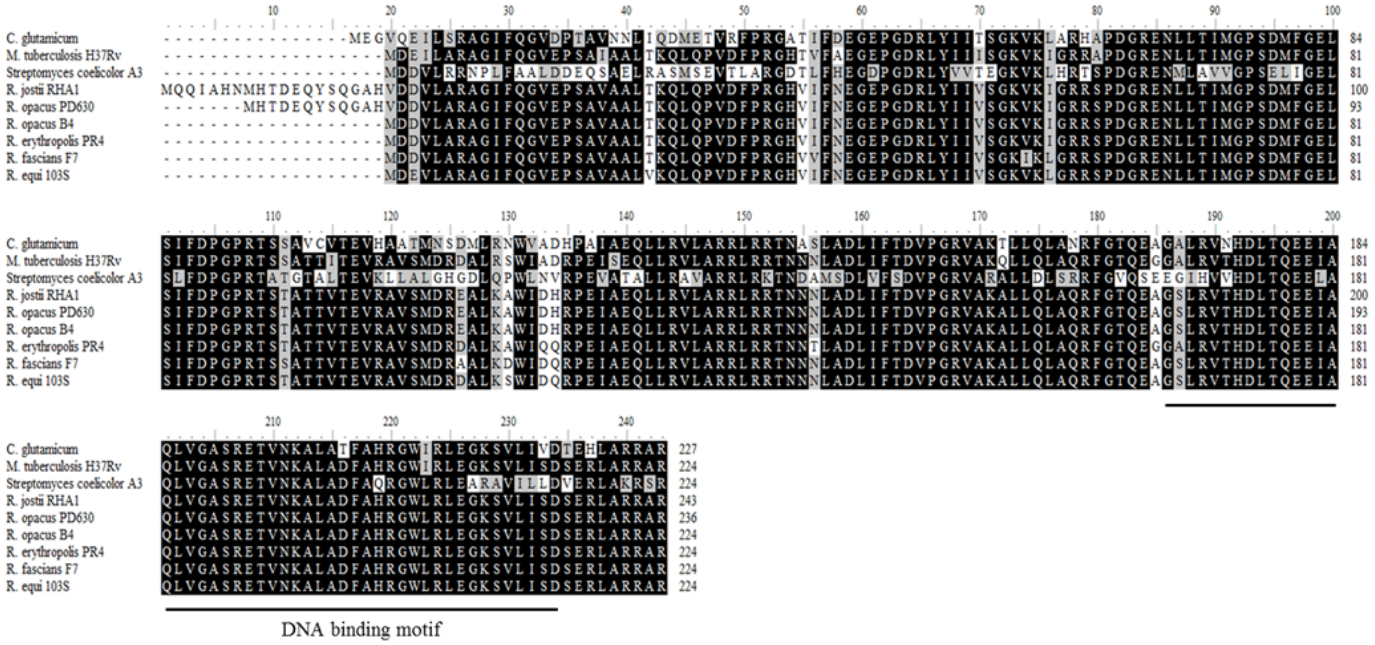

Figure 5. Alignments of *glxR* genes from diverse actinobacteria, including the six rhodococcal strains of this study. C., *Corynebacterium*; M., *Mycobacterium*; R., *Rhodococcus*.

acyl-CoA synthase activities, probably involved in both anabolism and catabolism of TAG in *Mycobacterium bovis* BCG (Low *et al.* 2010). Some lipolytic enzymes occurring in rhodococcal genomes may share similar properties with those present in mycobacteria. Further studies are necessary to identify and functionally characterize the rhodococcal lipolytic enzymes in order to understand their role in TAG metabolism.

The lipolytic enzymes hydrolyze TAG to yield nonesterified fatty acids, which may be derivatized to acyl-CoA. These products may subsequently be used in different metabolic pathways, such as the synthesis of phospholipids or re-esterification to TAG, depending on cellular necessities. Fatty acyl-CoA may eventually be degraded partially or completely by the β-oxidation pathway as shown in Figure 2. This catabolic route may serve in rhodococci not only for generating energy and precursors from lipids, but also as a source of fatty acids for TAG accumulation principally during growth on *n*-alkanes (Alvarez *et al.* 1997, Alvarez 2003). Some insights into the catabolism of fatty acids in rhodococci can be gained using a comparative genomic approach based on the available genome sequences. Our search of genomic databases suggests that rhodococci possess a complete and well conserved machinery for fatty acid oxidation (Figure 2), exhibiting a high number of genes involved in different reactions of the pathway (Table 2). Several paralogous genes may be involved in the β-oxidation of fatty acids in rhodococci with similar functionality but with differences in their spatial activities and substrate stereospecificity. Our sequence analysis indicates that gene content and organization in rhodococcal genomes are similar to that of mycobacteria. In contrast to *E. coli*, where the *fadA* and *fadB* genes form a single operon encoding the two proteins of the β-oxidation multienzyme complex (DiRusso 1990, Taylor *et al.* 2010), *M. tuberculosis* as well as rhodococci carry several *fadA* and *fadB* genes in their genomes. This enzyme redundancy occurring in actinobacteria may allow cells to accept a wide range of chain lengths as substrates, such as short-, medium- and long-chain molecules, and/or to activate fatty acid degradation under diverse environmental conditions. Further studies including gene cloning and enzyme purification and characterization are required to understand the specific role of each isoenzyme involved in fatty acid degradation in a physiological context in rhodococci. The *fadB2* gene of *M. tuberculosis* and other mycobacteria, which codes for a β-hydroxybutyryl-CoA dehydrogenase, is located in the genome adjacent to *icl1*, encoding the glyoxylate shunt enzyme isocitrate lyase (Taylor *et al.* 2010). The glyoxylate cycle allows cells to convert acetyl-CoA released by the β-oxidation pathway to succinate for the synthesis of carbohydrates, when cells are growing on fatty acids or acetate. A homologous gene was identified in *R. jostii* RHA1, which was annotated as a 3-hydroxybutyryl-CoA dehydrogenase (*hdb1*), showing 73% amino acid identity to Mt-*fadB2*. *hdb1* gene (*ro02121*) is situated in a conserved locus next to the isocitrate lyase gene, comparable with that of *M. tuberculosis* Mt-*fadB2* (Figure 4). The same gene organization also occurs in other rhodococci. Interestingly, Kelly *et al.* (2002) have reported that the

isocitrate lyase gene (*aceA*) and *fadB2* are co-transcribed into a 2.8 kb transcript during the growth of *R. equi* on acetate as its sole carbon source. A similar situation may occur in free-living rhodococci in order to couple the carbon flux through the β-oxidation pathway and glyoxylate shunt during fatty acid assimilation (Figure 2). On the other hand, the conserved clustered rearrangements of *fadB1* and *fadB2,* together with thiolase enzymes (*fadA)* in rhodococci suggests that those proteins may form a multienzymatic complex in a way similar to *E.coli* and *B. subtillis* (Figure 4). In these microorganisms, the β-oxidation pathway is globally regulated by *fadR*, which executes a negative control of catabolic genes in the absence of fatty acyl compounds. No homologous *fadR* genes were found in rhodococcal or mycobacterial genomes, suggesting that the regulation of the β-oxidation pathway is different in actinobacteria. Interestingly, Kim *et al.* (2004) identified the *glxR* gene involved in the regulation of the glyoxylate bypass in *Corynebacterium glutamicum*; a related mycolic acid-containing actinobacterium. The GlxR protein, which may form dimers, binds to the *aceB* promoter region in the presence of cAMP and repress the glyoxylate bypass genes (Kim *et al.* 2004). Bioinformatic analyses indicated that the *glxR* gene was highly conserved in the genome of all rhodococci of this study, as well as in other actinobacteria (Figure 5). This suggested that GlxR-like proteins may be one of the regulatory proteins involved in the degradation and assimilation of fatty acids in rhodococci. An unknown regulatory network must finely regulate TAG/fatty acid degradation and biosynthesis based on the availability of fatty acids. The key regulatory components and its organization, involved in the control of lipid homeostasis in rhodococci, remain to be elucidated.

Conclusions

Our study revealed additional insights of the distribution of genes involved in TAG accumulation and mobilization and some differences between the abundance of genes of the TAG metabolism among rhodococcal species. It is clear that *Rhodococcus* bacteria became specialists for TAG biosynthesis and accumulation during evolution. In this context, some rhodococcal species, such as *R. opacus* and *R. jostii*, were highly enriched with genes for the biosynthesis and degradation of fatty acids and TAG. Members of *R. erythropolis*, *R. equi* and *R. fascians* also have a complete set of genes/proteins for supporting TAG biosynthesis, accumulation and mobilization. However, these microorganisms seem to possess a more simplified configuration for TAG metabolism, in comparison to *R. opacus* and *R. jostii*. This study represents a starting framework, which may contribute to the development of a functional catalog of rhodococcal genes involved in TAG metabolism. This information will require revision and update as more genomic and functional genetics of rhodococci become available. In addition, the functional identification and characterization of the key genes involved in TAG metabolism, including those participating in regulatory mechanisms, will be one of the major challenges in this field.

Acknowledgements

The studies on this field were supported by the SCyT of the University of Patagonia San Juan Bosco, the Agencia Comodoro Conocimiento (MCR), the Oil m&s Company and the Project PFIP2008 CHU25. H.M. Alvarez and R.A. Silva are career investigators and M.A. Hernández a scholarship holder of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

Author's contributions

MSV participated in the acquisition of data, sequence analysis and alignments, interpretation of data, helped in the design of the study and drafted the manuscript. MAH and RAS participated in the sequence analysis and alignments, and interpretation of data. HMA conceived the study and participated in its design and coordination, interpretation of data, and helped to draft the manuscript. All authors read and approved the final manuscript.

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