RESEARCH LETTER

Glycogen formation by *Rhodococcus* species and the effect of inhibition of lipid biosynthesis on glycogen accumulation in *Rhodococcus opacus* PD630

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Abstract

Members of the genus *Rhodococcus* were investigated for their ability to produce glycogen during cultivation on gluconate or glucose. Strains belonging to *Rhodococcus ruber*, *Rhodococcus opacus*, *Rhodococcus fascians*, *Rhodococcus erythropolis* and *Rhodococcus equi* were able to produce glycogen up to 0.2–5.6% of cellular dry weight (CDW). The glycogen content varied from 0.8% to 3.2% of CDW in cells of *R. opacus* PD630, which is a well-known oleaginous bacterium, during the exponential growth phase, when cultivated on diverse carbon sources. Maltose and pyruvate promoted glycogen accumulation by cells of strain PD630 to a greater extent than glucose, gluconate, lactose, sucrose or acetate. This strain was able to produce triacylglycerols, polyhydroxyalkanoates and glycogen as storage compounds during growth on gluconate, although triacylglycerols were always the main product under the conditions of this study. Cerulenin, an inhibitor of *de novo* fatty acid synthesis, inhibited the accumulation of triacylglycerols from gluconate and increased the content of polyhydroxyalkanoates (from 2.0% to 4.2%, CDW) and glycogen (from 0.1% to 3.0%, CDW). An increase of the polyhydroxyalkanoates and glycogen content was also observed in two mutants of *R. opacus* PD630, which produced reduced amounts of triacylglycerols during cultivation of cells on gluconate.

Introduction

Members of the genus *Rhodococcus* are widely distributed in natural environments, such as soil, water and marine sediments (Warhurst & Fewson, 1994; Martínková *et al.*, 2009). They belong to the nonsporulating and mycolic acid-rich group within the actinomycetes, together with other related genera, including *Mycobacterium*, *Nocardia*, *Corynebacterium* and *Gordonia* (Gürtler *et al.*, 2004). *Rhodococcus* species are currently the subject of research in many countries of the world, and the number of publications and patents on rhodococci has intensified significantly in recent years. Several *Rhodococcus* genomic projects are now in progress through public and private efforts due to the increasing interest in their use for biotechnology, with potential applications in bioremediation, biotransformations, biocatalysis and other processes. In this context, oleaginous rhodococci [strains with the ability to accumulate $>$ 20% of the cellular dry weight (CDW) of triacylglycerols] may serve as sources of alternative oils and wax esters for industrial purposes. The applied potential of bacterial triacylglycerols and wax esters may be similar to that of vegetable sources, including use as feed additives, cosmetics, oleochemicals, lubricants and other manufactured products. In addition, bacterial oils could be used for biofuel production. The combination of fundamental knowledge of storage compound metabolism in rhodococci will contribute to the economic feasibility of bacterial oil production on an industrial scale and the potential for other applications.

The biosynthesis and accumulation of storage lipids, such as triacylglycerols and polyhydroxyalkanoates, is a well-established feature in *Rhodococcus* species (Alvarez *et al.*, 1996, 1997; Alvarez, 2003). In contrast, only recently it has been reported for the first time that a *Rhodococcus* strain, *Rhodococcus jostii* RHA1, can produce glycogen (Hernández *et al.*, 2008). Glycogen is a glucose polymer with α-1,4 and

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α-1,6 linkages, which is accumulated by several bacteria. The accumulation of glycogen has been reported previously for other related actinomycetes, such as strains of *Mycobacterium* (Belanger & Hatfull, 1999) and *Corynebacterium* (Seibold & Eikmanns, 2007; Seibold *et al.*, 2007). In a previous study, we demonstrated that *R. jostii* RHA1 possesses key genes for accumulation of diverse storage compounds, such as triacylglycerols, wax esters, polyhydroxyalkanoates, glycogen and polyphosphate (Hernández *et al.*, 2008). Under nitrogen-limiting conditions, lipids were the principal storage compounds accumulated by this strain. To examine whether the biosynthesis of glycogen is a common feature among *Rhodococcus* species, as is the production of triacylglycerols and polyhydroxyalkanoates, in this study, we analyzed diverse species belonging to this genus for their ability to synthesize and accumulate glycogen. In addition, we examined the potential interactions between pathways involved in the biosynthesis of storage compounds, such as triacylglycerols, polyhydroxyalkanoates and glycogen, in the oleaginous *Rhodococcus* research model, *R. opacus* PD630. The understanding of how cells coordinate the distribution of intermediates to distinct destinations and the partitioning of carbon between lipids and other alternative storage compounds is important for genetic and metabolic manipulations of selected microorganisms for biotechnological procedures. A better knowledge of the basic aspects of rhodococcal metabolism will also be useful for improving our understanding of the biology of these bacteria and their ability to interact with a diversity of natural environments.

Materials and methods

Bacterial strain and growth conditions

The bacterial strains used in the present study are listed in Table 1.

Rhodococcus strains were cultivated aerobically at 28 °C in nutrient broth (NB) medium or in mineral salts medium (MSM) according to Schlegel *et al.* (1961). Sodium gluconate, glucose, sucrose, maltose, lactose, sodium pyruvate, sodium citrate and sodium acetate were used as the sole carbon sources at a final concentration of 1% (w/v). When N-limiting conditions were specified, the concentration of ammonium chloride in MSM was reduced to 0.1 g L^{-1} (MSM0.1) to allow lipid accumulation. Cells were harvested during the exponential and stationary growth phases, washed with an NaCl solution (0.85%, w/v) and lyophilized for chemical analyses.

Table 1. Bacterial strains used in the present study

Bacterial species	Source or reference
Rhodococcus opacus PD630	DSMZ 44193, Alvarez *et al*. (1996)
Rhodococcus ruber	NCMIB40126, Haywood *et al*. (1991)
Rhodococcus fascians D 188-5	Desomer *et al*. (1990)
Rhodococcus equi	ATCC 6939
Rhodococcus erythropolis	DSMZ 43060
R. opacus PDM41	Institute for Molecular Microbiology and Biotechnology, WWU, Münster, Germany
*R. opacus atf1*ΩKm mutant	Alvarez *et al*. (2008)

Experiments with inhibitors

Cerulenin (Sigma, St. Louis, MO) was utilized for inhibition of fatty acid synthesis. Cells were cultivated on NB medium at 28 °C for 24 h, harvested, resuspended in nitrogen-free MSM (MSM0) containing sodium gluconate (1%, w/v) as the sole carbon source and 25 μg mL^{-1} of cerulenin, incubated at 28 °C for 24 h, harvested and lyophilized for chemical analyses.

Extraction and analysis of lipids

Freeze-dried cells were extracted with methanol–chloroform (MeOH–$CHCl_3$, 1 : 2, v/v). An aliquot of the whole-cell extract was analyzed by thin-layer chromatography (TLC) on 60F254 silica gel plates (Merck, Darmstadt, Germany) applying *n*-hexane–diethyl ether–acetic acid (80 : 20 : 1, v/v/v) as a solvent system. Lipid fractions were revealed using iodine vapor. Tripalmitin and cetylpalmitate (Merck) were used as standards.

Analysis of fatty acids and polyhydroxyalkanoates

For qualitative and quantitative determination of fatty acids and polyhydroxyalkanoates, 5–8 mg of lyophilized cells were subjected to methanolysis in the presence of 15% (v/v) sulfuric acid as described by Brandl *et al.* (1988), and the resulting acyl- and 3-hydroxyacyl-methylesters were analyzed by GC using an HP 5890 A gas chromatograph equipped with an InnoWAX capillary column (30 m × 0.53 mm × 1 μm) and a flame ionization detector. The injection volume was 0.2 μL, and helium (13 mm min^{-1}) was used as a carrier gas. The temperature of the injector and detector was 270 and 320 °C, respectively. A temperature program was used for efficient separation of the methyl esters (90 °C for 5 min, temperature increase of 6 °C min^{-1}, 220 °C for 15 min). For quantitative analysis, tridecanoic acid was used as an internal standard. All determinations were performed in triplicate experiments. The data were recorded as means and SDs.

Extraction, analysis and digestion of cellular polysaccharide

The polysaccharide was isolated from freeze-dried cells using the classical alkali treatment as has been reported previously (Elbein & Mitchell, 1973; Gunja-Smith *et al.*,

1977; Lillie & Pringle, 1980; Lou *et al*., 1997), and visualized using a TLC method (Seibold & Eikmanns, 2007). Total polysaccharide was determined using the phenol–sulfuric acid method (Dubois *et al*., 1956).

For quantitative analysis, the extracted polysaccharide was digested with α-amylase (; 10 IU mg^{-1} of dried polysaccharide; Sigma) and amyloglucosidase (; 20 IU mg^{-1} of dried polysaccharide; Sigma) in 50 mM sodium acetate buffer (pH 5) at 55 °C for 3–4 h and with gentle vortexing. Commercial glycogen standard (1 mg mL^{-1}) was used as a control for enzymatic hydrolysis. The amount of glucose formed under these conditions was taken as a measure of glycogen in cells. Glucose was determined using a specific glucose oxidase method (Keston, 1956). All determinations were performed in triplicate experiments. The data were recorded as means and SDs.

Results

Survey of key genes for glycogen metabolism in the available genomic databases

Various genomic databases of *Rhodococcus* strains are now available for public research. Among them, the genome sequence of *R. jostii* strain RHA1 has been the first sequence publicly available for the screening and identification of genes and metabolic pathways (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). Recently, we identified six putative genes (*glgA*, *glgB*, *glgC*, *glgE*, *glgP* and *glgX*) involved in glycogen biosynthesis and mobilization in a genome-wide bioinformatic study of the genomic database of strain RHA1 (Hernández *et al*., 2008). Using these RHA1 sequences, we performed a genome-wide examination of key genes involved in glycogen metabolism in the available databases of *R. opacus* B4, *Rhodococcus erythropolis* PR4 and *R. erythropolis* SK121. The degree of identity of full protein sequences of these species is shown in Table 2. In all cases, a high identity between orthologous proteins was observed. In general, we observed similar gene arrangements in all strains, with little differences. The *glgB*, *glgE* and *glgP* genes occurred in a cluster, whereas *glgA* and *glgC* were adjacent and clustered in the opposite orientation. Only in the *R. erythropolis* SK121 genome was a gene coding for a putative *O*-methyltransferase enzyme found between *glgA* and *glgC*. Finally, *glgX* was located in a separate cluster associated with another carbohydrate metabolism gene, which encodes a putative 1–4-α-D-glucan 1-α-D-glucosylmutase (also called maltooligosyl trehalose synthase) in the genome of all the strains studied.

These results suggested that the different strains possess the genetic potential to synthesize and mobilize glycogen.

Glycogen accumulation by different *Rhodococcus* species

The accumulation of glycogen by cells of various species of the genus *Rhodococcus* was analyzed. After cultivation of cells on minimal salts medium with gluconate, or glucose in the case of *Rhodococcus ruber* and *Rhodococcus equi*, because gluconate supported poor growth of cells, as the sole carbon source, a phenol–sulfuric acid-reactive material was detected in all bacteria investigated as revealed by TLC analysis. A commercial glycogen was used as a standard for TLC analysis (data not shown). Enzymatic analysis of the isolated polysaccharide after 24 h of growth indicated that in all cases, the material observed was a glucose polymer.

In general, the glycogen content amounted to approximately up to 5% of CDW in the strains studied as shown in Table 3. Among these microorganisms, *R. equi* produced higher amounts of glycogen than other bacteria, whereas *R. opacus* PD630 and *R. ruber* produced only scant amounts of glycogen under the culture conditions used in this study (Table 3). In all cases, no significant differences were observed (data not shown) between glycogen contents of the respective strains cultivated in a nitrogen-poor mineral medium and in a nitrogen-rich medium (NB medium). The results of the analyses of glycogen accumulation as well as those obtained in the survey of key genes for glycogen metabolism suggested that the ability to produce glycogen may be a common feature among *Rhodococcus* strains.

Glycogen accumulation by *R. opacus* strain PD630 grown on different carbon sources

Rhodococcus opacus PD630 is a triacylglycerol -accumulating specialist that has become a model among prokaryotes in

Table 2. Key proteins and amino acid identity (%I) of glycogen metabolism in *Rhodococcus* species

Enzyme name	*Rhodococcus jostii* RHA1*	*Rhodococcus opacus* B4 (%I)	*Rhodococcus erythropolis* SK121 (%I)	*Rhodococcus erythropolis* PR4 (%I)
Glycogen phosphorylase	Ro01447 (GlgP)	ROP_11560 (96%)	RHOER0001_5396 (85%)	RER_38880 (85%)
Glycosyltransferase	Ro05974 (GlgA)	ROP_60340 (98%)	RHOER0001_6257 (84%)	RER_41610 (84%)
Glycogen branching enzyme	Ro01449 (GlgB)	ROP_11580 (97%)	RHOER0001_5394 (83%)	RER_38900 (83%)
Glucose-1-phosphate adenylyltransferase	Ro05975 (GlgC)	ROP_60350 (99%)	RHOER0001_6259 (96%)	RER_41600 (96%)
Amylase/glucanase/maltosyltransferase	Ro01448 (GlgE)	ROP_11570 (96%)	RHOER0001_5395 (81%)	RER_38890 (83%)
Glycogen debranching enzyme	Ro01056 (GlgX)	ROP_07840 (96%)	RHOER0001_6363 (83%)	RER_35060 (81%)

*% identities are calculated relative to *Rhodococcus jostii* RHA1 protein sequences.

the lipid research area. The triacylglycerol content and composition of strain PD630 cultivated on a diversity of substrates has been reported previously (Alvarez *et al.*, 1996, 1997). Because the content and composition of accumulated triacylglycerols depend on the carbon source used for cell cultivation (Alvarez *et al.*, 1996, 1997), we investigated the influence of carbon sources on the glycogen accumulation in this oleaginous bacterium. Figure 1 shows the glycogen content of cells cultivated on different substrates, during the exponential and stationary growth phases. The glycogen content in the cells amounted to between 0.8 ± 0.3 (sucrose, fructose and gluconate) and $3.2 \pm 0.2\%$ CDW (maltose) after cultivation under nitrogen-limiting conditions. Maltose and pyruvate promoted glycogen accumulation to a level approximately threefold greater in comparison with the other substrates used, such as glucose, sucrose, acetate and lactose (Fig. 1). Interestingly, cells grown on maltose (34.1% CDW of triacylglycerols) and pyruvate (39.2% CDW of triacylglycerols) accumulated lower amounts of triacylglycerols in comparison with cells cultivated with gluconate (60.0% CDW of triacylglycerols), suggesting an inverse relationship between the triacylglycerols and glycogen contents in cells. The results indicated that the amount of glycogen accumulated by strain PD630 depends on the carbon source used for the cultivation of cells.

Table 3. Glycogen accumulation by different *Rhodococcus* strains cultivated at 28 °C in MSM0.1 with gluconate or glucose (1%, w/v) during the exponential and stationary growth phases

	% Glycogen (CDW)	
Bacterial strains	Exponential growth phase	Stationary growth phase
Rhodococcus opacus PD630	0.6 ± 0.05	1.2 ± 0.3
**Rhodococcus ruber*	ND	0.2 ± 0.09
Rhodococcus fascians D 188-5	2.3 ± 0.3	2.8 ± 0.7
Rhodococcus erythropolis	3.7 ± 0.4	5.2 ± 0.6
**Rhodococcus equi* ATCC 3936	5.6 ± 0.6	5.9 ± 0.8

*Cells were grown on glucose as the sole carbon source.
The rest of the strains (i.e. those not marked with *) were grown on gluconate.
ND, not detected.

Effect of the inhibition of lipid biosynthesis on glycogen accumulation by *R. opacus* PD630

In order to study the potential metabolic relationship between triacylglycerols and glycogen biosynthesis, cells of *R. opacus* PD630 were incubated in a nitrogen-free mineral medium (MSM0) containing gluconate as the sole carbon source and in the presence of an inhibitor of lipid metabolism. Cerulenin inhibits the *de novo* fatty acid biosynthesis pathway by binding to the active sites of ketoacyl synthases I and II (Funabashi *et al.*, 1989). We used gluconate as a carbon source for cultivation experiments because this substrate supports higher amounts of triacylglycerol accumulation by cells of strain PD630 than other carbon sources (Alvarez *et al.*, 1996). Thus, the inhibition of lipid biosynthesis may have a significant effect on glycogen accumulation. Cerulenin ($25\ \mu g\ mL^{-1}$) inhibited fatty acid biosynthesis and subsequent accumulation of triacylglycerols as is shown in Fig. 2. The triacylglycerol content of cells cultivated in the presence of the inhibitor was likely to have been produced by cells during their preculture in NB as suggested by the results shown in Fig. 2b. In addition, the total amounts of glycogen and polyhydroxyalkanoates were also affected by cerulenin as shown by an approximately twofold and 30-fold increase in the polyhydroxyalkanoates and glycogen contents, respectively.

Glycogen accumulation by mutants of *R. opacus* PD630

Glycogen accumulation was also studied in two mutants of *R. opacus* PD630 defective in triacylglycerol accumulation (Table 4). Mutant PDM41 was obtained by chemical mutagenesis with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, whereas an *atf1*ΩKm mutant was constructed by a specific kanamicyn cassette disruption of the *atf1* gene of strain

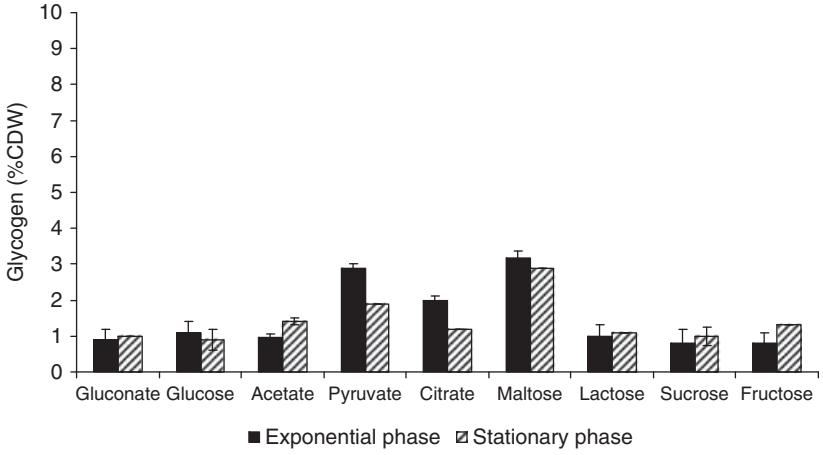
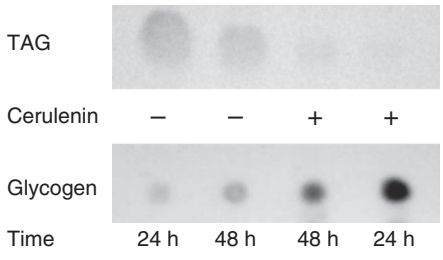


Fig. 1. Glycogen accumulation by *Rhodococcus opacus* PD630 grown on MSM0.1 with different carbon sources during the exponential and stationary growth phases.

(a)



(b)

Strain and growth conditions	% TAG*	% PHA*	% Glycogen*
R. opacus PD630 (NB medium) Preculture cells	23.4	2.5	0.5
R. opacus PD630 (MSM0 medium). Gluconate 1% without cerulenin	65.8	2.0	0.1
R. opacus PD630 (MSM0 medium). Gluconate 1% with cerulenin (25 μg mL^{-1})	16.6	4.2	3.0

Fig. 2. Effect of cerulenin on storage compound accumulation by *Rhodococcus opacus* PD630. Cells were cultivated on NB medium at 28 °C for 24 h (preculture cells), harvested and then resuspended in nitrogen-free MSM with gluconate (1%, w/v) and in the presence of cerulenin. (a) Semiquantitative TLC analysis of triacylglycerols and glycogen in strain PD630 in the presence and absence of cerulenin; (b) quantitative analysis of triacylglycerols, polyhydroxyalkanoates and glycogen in strain PD630 in the presence and absence of cerulenin after 24 h of incubation at 28 °C.

Table 4. Glycogen, PHA and TAG accumulation by *Rhodococcus opacus* PD630 and its mutants PDM41 and *atf1*ΩKm

Bacterial strain	TAG (% CDW)	PHA (% CDW)	Glycogen (% CDW)
Rhodococcus opacus PD630 WT	63.8 ± 0.5	2.0 ± 1.1	0.6 ± 0.04
Rhodococcus opacus PDM41	9.1 ± 1.5	4.1 ± 1.2	4.3 ± 0.04
*Rhodococcus opacus atf1*ΩKm	39.2 ± 2.9	2.4 ± 0.74	1.3 ± 0.03

Cells were cultivated on NB medium at 28 °C for 24 h, harvested and then resuspended and incubated in nitrogen-free MSM with gluconate (1%, w/v) as the sole carbon source for 24 h at 28 °C.

PD630 (Alvarez *et al.*, 2008). The *atf1* gene is one of the genes involved in the biosynthesis of triacylglycerols in *R. opacus* PD630 as has been reported previously (Alvarez *et al.*, 2008). Atf proteins (diacylglycerol acyltransferase enzyme) catalyze the condensation of acyl-CoA and diacylglycerol with the formation of triacylglycerols. Other isoenzymes besides Atf1 must contribute to triacylglycerol accumulation in strain PD630, because disruption of the *atf1* gene resulted in a decrease in the cellular triacylglycerol content, but not in the absence of triacylglycerols in cells (Alvarez *et al.*, 2008). Triacylglycerol synthesis of both mutants used in this study was reduced as compared with the wild type as shown in Table 4. The *atf1*ΩKm mutant showed an approximately twofold increase in the glycogen content in comparison with the wild type, whereas the polyhydroxyalkanoate content in both strains was similar (Table 4). Interestingly, mutant PDM41, which, from gluconate, accumulated 9.1% triacylglycerols compared with 63.8% in the wild type, exhibited an approximately twofold and sevenfold increase in the polyhydroxyalkanoates and glycogen content, respectively, in comparison with the wild type (Table 4).

Discussion

In this study, we analyzed the genetic potential and the physiological ability of diverse species of the genus *Rhodococcus* to synthesize glycogen from gluconate or glucose as the sole carbon sources. Our results suggested that the ability to produce glycogen may be widespread among several rhodococcal species. The total amounts of glycogen produced by cells seem to be a strain-dependent feature and in no case amounted to more than 60 mg g^{-1} of dry cells (6% of CDW). In related bacteria such as *Corynebacterium glutamicum* and *Mycobacterium smegmatis*, values of glycogen between 90 and 186 mg g^{-1} of dry cells (9–18.6% of CDW) during cultivation on minimal medium with glucose, sucrose or fructose, have been reported (Elbein & Mitchell, 1973; Seibold *et al.*, 2007). Nitrogen starvation did not seem to stimulate glycogen biosynthesis in the strains studied as has been reported for *M. smegmatis* (Elbein & Mitchell, 1973), because the glycogen content in cells cultivated in nitrogen-poor and nitrogen-rich media was rather similar.

As reported for *R. jostii* RHA1 (Hernández *et al.*, 2008), glycogen accumulation in all the strains studied started during the exponential growth phase. Glycogen accumulation during the exponential growth phase has been also observed in other actinomycetes, such as *M. smegmatis* (Belanger & Hatfull, 1999) and *C. glutamicum* (Seibold *et al.*, 2007). Glycogen may play a role as a metabolic intermediate because it is accumulated during the exponential growth phase by cells and may be mobilized later in the stationary phase; thus, glycogen has been proposed as a carbon capacitor for glycolysis during exponential growth (Belanger & Hatfull, 1999). Glycogen may be a part of a mechanism for controlling excess sugar in *Rhodococcus*, or may act as part of a sensing/signalling mechanism as has been proposed previously (Hernández *et al.*, 2008).

Rhodococcus opacus PD630, which is a well-known oleaginous bacterium, was able to produce glycogen during growth on different carbon sources, in addition to producing triacylglycerols and polyhydroxyalkanoates. The content of glycogen in cells depended on the carbon source used for growth. In general, the total content of glycogen in strain PD630 varied from 0.8% to 3.2% of the CDW in the exponential growth phase and 0.9% to 2.9% of CDW during the stationary phase. Cells cultivated on pyruvate and maltose accumulated around

3% (CDW), whereas the glycogen content in cells grown on gluconate, lactose and sucrose was no greater than 1% (CDW). The lower content of triacylglycerols of cells grown on pyruvate and maltose in comparison with cells cultivated on gluconate might be related to the higher glycogen accumulation. Recently, Seibold *et al.* (2010) reported that two transcriptional regulators (RamA and RamB) are involved in the carbon source-dependent regulation of glycogen content and in the control of the expression of *glgC* and of *glgA* in *C. glutamicum*. Whether a similar carbon source-dependent regulation mechanism of glycogen biosynthesis is present in *R. opacus* PD630 must be investigated in the future.

The results of this study demonstrated that it is possible to modify the relative amounts of triacylglycerols, polyhydroxyalkanoates and glycogen and the carbon distribution through metabolism using metabolic inhibitors and by mutation of the genes involved in storage compound synthesis and accumulation. In general, inhibition of fatty acid biosynthesis by the addition of cerulenin to the medium caused an increase in the polyhydroxyalkanoates and glycogen content in cells. The mutants affected in triacylglycerol accumulation used in this study also produced increased amounts of glycogen and eventually of polyhydroxyalkanoates during cultivation on gluconate in comparison with the wild type. This effect was more evident in the mutant PDM41 than in the *atf1*ΩKm mutant. When the biosynthesis of triacylglycerols is impaired by inhibition of the *de novo* fatty acid biosynthesis pathway or the disruption of a gene involved in triacylglycerol accumulation, carbon distribution through metabolism changes and intermediates become more available for the synthesis of glycogen and polyhydroxyalkanoates in cells.

These approaches contribute toward a better understanding of storage compound metabolism and the interaction of pathways in *Rhodococcus* species, which could be of interest for planning further metabolic manipulation of cells for biotechnological purposes.

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