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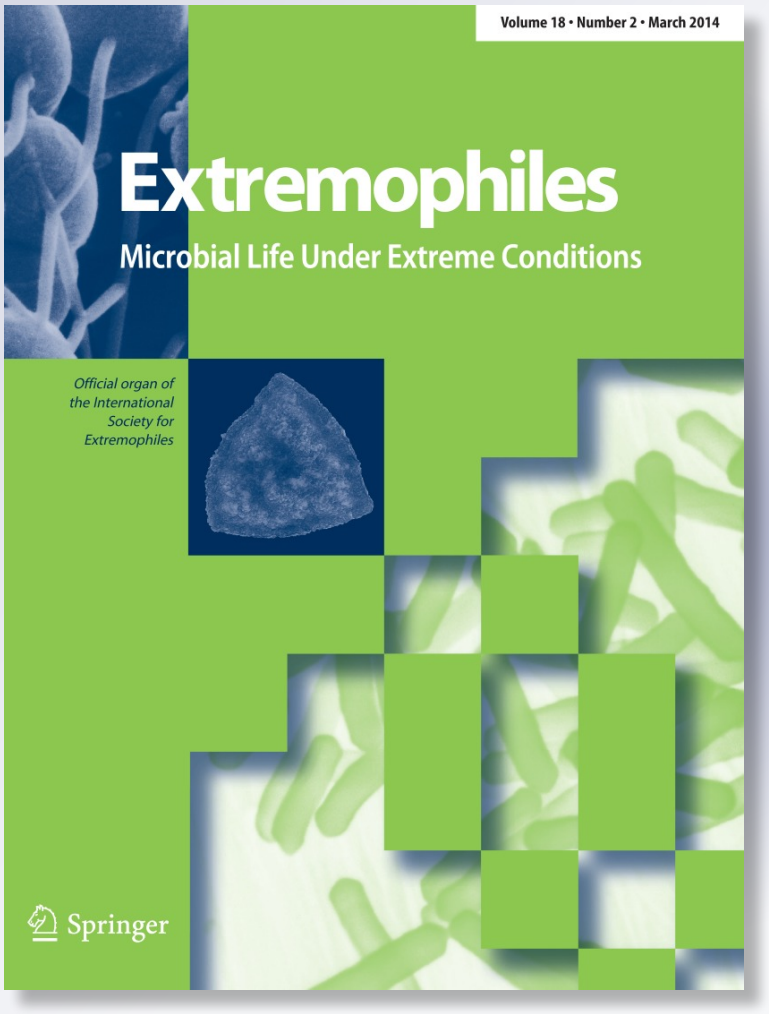

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ORIGINAL PAPER

Triacylglycerol accumulation and oxidative stress in *Rhodococcus* species: differential effects of pro-oxidants on lipid metabolism

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Abstract In general, members of *Rhodococcus* genus are highly resistant to desiccation. Desiccation is a complex process which includes the formation of reactive oxygen species that results in significant damage to cells. In this study, we demonstrate that extremophile actinobacterial strains isolated from diverse environments, mainly belonging to *Rhodococcus* genus, exhibited high tolerance to the pro-oxidants hydrogen peroxide (H_2O_2) and methyl viologen (MV). In addition, we investigated the possible interconnections between the responses of the oleaginous *Rhodococcus opacus* PD630 to oxidative stress and lipid metabolism, since both processes demand a metabolic reorganization of cells. Experiments with metabolic inhibitors showed differential effects of both pro-oxidants on lipid metabolism in PD630 cells. The inhibition of carotenoid biosynthesis by the addition of diphenylamine to the media negatively affected the tolerance of cells to H_2O_2, but not to MV. The inhibition of triacylglycerol (TAG) biosynthesis and accumulation in PD630 did not affect the tolerance of cells to H_2O_2 and MV; whereas, the blockage of lipolysis decreased the tolerance of cells to H_2O_2 (but not MV) under carbon-starvation conditions. Interestingly, the addition of MV to the media (but not H_2O_2) induced a reduction of TAG accumulation by cells. Resuming, results of this study revealed metabolic connections between lipid metabolism and oxidative stress responses in *R. opacus* PD630, and probably in other extremophile TAG-accumulating rhodococci.

Keywords Physiology · Metabolism · *Rhodococcus*

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Introduction

The members of the genus *Rhodococcus* are aerobic and non-sporulating bacteria, usually found in a wide diversity of environments, such as tropical and arctic soils, deserts as well in marine and deep-sea sediments (Whyte et al. 1999; Heald et al. 2001; Peressutti et al. 2003; Luz et al. 2004; Peng et al. 2008). Some members of this genus have been isolated from environments with extreme conditions, such as the semiarid Patagonia and the Andean Puna located in South and North of Argentina, respectively (Ordoñez et al. 2009; Silva et al. 2010; Bequer Urbano et al. 2013). The huge metabolic repertoire of these microorganisms as well as their capability to adapt their metabolism to a wide range of nutritional conditions, are in part responsible for the occurrence of these actinobacteria in different natural ecosystems. One interesting property of these microorganisms, which allows

withstanding the fluctuating nutritional conditions found in natural environments, is their ability to produce variable amounts of storage compounds, such as polyhydroxyalkanoates (PHA), triacylglycerols (TAG), glycogen and polyphosphate (Hernández et al. 2008). The accumulation of different storage compounds by rhodococci probably permits cells to respond rapidly to changes in nutritional state and to balance metabolism under different environmental conditions. In general, rhodococci accumulate TAG as main storage compounds, whereas PHA and glycogen represent only minor components of cells (Hernández and Alvarez 2010). Some strains can be considered as oleaginous bacteria since they accumulate more than 20 % of their biomass as lipids. *Rhodococcus opacus* PD630, which is the best known TAG-accumulating member of the *Rhodococcus* genus, is able to accumulate very high levels of TAG in cells after cultivation on gluconate and other substrates (Alvarez et al. 1996). The accumulation of significant amounts of TAG by rhodococci is a carbon-intensive and energy-demanding process, which compete with cell growth. Thus, the occurrence of such storage compounds in microorganisms which habits energy-poor environments must be an important feature in their physiology. Recently, we demonstrated that TAG metabolism is relevant for the adaptation and survival of the extremophile *Rhodococcus* sp. A5 under carbon starvation, osmotic stress and UV irradiation, and essential under desiccation conditions (Bequer Urbano et al. 2013). The mobilization of TAG was also essential for survival of *R. opacus* PD630 cells under desiccation conditions (Alvarez et al. 2004). Desiccation is a complex process enclosing more than one stress, including starvation, osmotic stress and oxidative stress, among others. For this reason, rhodococci living in arid environments may face the challenge of desiccation, developing physiological and metabolic adaptations to withstand these periods and ensure survival. Desiccation in cells causes overproduction of reactive oxygen species (ROS) that results in significant damages to cell structures (Clement et al. 1999; Pereira et al. 2003; França et al. 2005, 2007; Contreras-Porcia et al. 2011). Since rhodococci have been demonstrated to be highly resistant to desiccation (Alvarez et al. 2004; LeBlanc et al. 2008; Bequer Urbano et al. 2013), being frequently isolated from arid and desert soils (Okoro et al. 2009; Bhatnagar and Bhatnagar 2005; Connon et al. 2007), they must possess efficient oxidative defense mechanisms. However, as far as we know, oxidative stress responses of rhodococcal microorganisms have not been investigated previously.

Metabolic adjustment may play a critical role in proper execution of oxidative defense responses. Since NADPH is the essential cofactor for several antioxidant enzymes, cells need to finely regulate homeostasis of NADPH pool to enable proper deployment of defensive responses (Mailloux et al. 2011; Singh et al. 2008). Interestingly, the massive biosynthesis and accumulation of TAG by oleaginous rhodococci is also an NADPH-consuming process. Thus, an oleaginous microorganism must be able to maintain a high carbon flux and reducing equivalents generation toward the lipid production pathways. In this context, rhodococcal cells must finely reorganize their metabolism and alter the homeostasis of cytoplasmic metabolites during biosynthesis, accumulation and mobilization of TAG in the presence of oxidative stress. Such processes and challenges may occur simultaneously in the environment. In order to enhance our understanding of rhodococcal biology, we investigated the responses of different rhodococcal strains to oxidative stress and its effects on TAG metabolism using the oleaginous *R. opacus* PD630 as research model.

Materials and methods

Bacterial strains and culture media

In this study, the following bacterial strains were used: *Rhodococcus* sp. A5, *Rhodococcus* sp. CH13 and *Dietzia* sp. A12, which were previously isolated from Andean Puna (North of Argentina) (Ordoñez et al. 2009); *Rhodococcus fascians* F7, *Rhodococcus fascians* S1.17b and *Rhodococcus jostii* 602, which were isolated from soil samples in Patagonia (south of Argentina) (Silva et al. 2010); *Rhodococcus jostii* RHA1 (Masai et al. 1995) and *Rhodococcus opacus* PD630 (Alvarez et al. 1996) were used as model strains. *Escherichia coli* DH5α strain was used as a control strain for in situ superoxide dismutase (SOD) inhibition assay as described below.

Cells were grown aerobically at 28 °C in nutrient broth medium or in mineral salts medium (MSM) according to Schlegel et al. (1961) with sodium gluconate (1 %, w/v) or glucose (1 %, w/v), as sole carbon sources. To allow the accumulation of lipids, the concentration of ammonium chloride in the MSM was reduced from 1.0 to 0.1 g/l (nitrogen-limiting conditions). To obtain solidified media, 1.8 % (w/v) agar was added.

Sensitive to pro-oxidants in agar plate

Bacterial cultures were grown in NB overnight. After that, cultures were harvested in exponential growth phases, washed once with sterile saline solution (0.85 %, w/v), resuspended to an OD_{600} of 0.8 and subjected to serial dilutions. Aliquots of 10 µl were then loaded onto NB agar plate, supplemented with MV or H_2O_2 at different concentrations.

Antioxidant enzyme activity

Cells grown to exponential phase were disrupted by French Press in lysis buffer (20 mM Tris–HCl containing 5 mM EDTA, 100 mM NaCl, 10 % glycerol, 0.1 mM phenylmethylsulfonyl fluoride and 14 mM β-mercaptoethanol, pH 7). Lysates were cleared by centrifugation and protein concentration was estimated in the supernatant by a binding assay (Bradford 1976) using bovine serum albumin as standard. SOD activity was visualized in situ after electrophoresis of the corresponding cellular lysates in non-denaturing polyacrylamide gels 12 % as described previously (Beauchamp and Fridovich 1971) using inhibition by H_2O_2 and KCN to determine the metal identity in the enzyme (Donahue et al. 1997). Catalase activity was visualized in situ after electrophoresis in non-denaturing polyacrylamide gels 10 %, as described previously (Scandalios 1968).

Lipid extraction and thin layer chromatography (TLC)

The semi-quantitative analyses of intracellular lipids in cells were performed by TLC. For this, 1 ml of cell cultures at OD_{600} of 2.0 (±0.1) was extracted with 300 µl chloroform/methanol (2:1, v/v) for 1 h and shaking every 15 min. The supernatant was subjected to TLC on 60F254 silica gel plates (Merck, Darmstadt, Germany) applying the following solvent system: hexane–diethyl ether–acetic acid (80:20:1 v/v/v) (Alvarez et al. 1996). Lipid fractions were visualized after brief exposure to iodine vapor. Tripalmitin (Merck, Darmstadt, Germany) was used as reference substance for TLC.

Tolerance to pro-oxidants of *R. opacus* PD630 in nitrogen-poor and nitrogen-rich solid media

NB bacterial cultures collected at an OD_{600} of 0.8 (exponential growth phase) were subjected to serial dilutions. Aliquots of 10 µl were then loaded onto solid MSM0.1 and MSM1 (nitrogen-poor and nitrogen-rich media, respectively) with gluconate as carbon source, supplemented with different concentrations of H_2O_2 and MV.

Tolerance to pro-oxidants of R. opacus PD630 during TAG biosynthesis and accumulation

Strain PD630 was grown with shaking at 28 °C overnight in NB medium. Then, culture was collected, washed once with sterile saline solution and resuspended to an OD_{600} of 8.0 (±0.1) in nitrogen-free MSM medium (MSM0), in order to promote lipid accumulation, with sodium gluconate as carbon source (1 %) in the presence/absence of 10 mM MV and 20 mM H_2O_2. Cerulenin (Sigma, St. Louis, MO, USA) was utilized for inhibition of fatty acid synthesis at concentration of 25 µg/ml. On the other hand, diphenylamine (DPA) was used for inhibition of carotenoid pigments biosynthesis. For this, cells were grown in NB agar supplemented with 0.05 mM of at 28 °C for 5 days. After growth, cells were suspended in saline solution at an OD_{600} of 8.0, and 1 ml of this cell suspension was used to inoculate 50 ml of MSM0 (without any nitrogen source) with gluconate and in presence/absence of 10 mM MV and 20 mM H_2O_2.

All cultures were collected after 20 h of incubation at 28 °C and viable counts (CFU) determined. TAG accumulation was analyzed by semi-quantitative TLC as described above. All determinations were done in triplicate experiments. The data were recorded as means and standard deviations; and differences in count of CFU after application of each stress condition and the inhibitor were determined by analysis of simple variance with software Statistica 7.0. ANOVA variance analysis was used with a probability level of $p < 0.05$.

Tolerance to pro-oxidants of *R. opacus* PD630 during TAG mobilization

To assess the importance of TAG mobilization in cells challenged with MV or H_2O_2, Orlistat was used as an inhibitor of lipases (Low et al. 2009). With this treatment, TAG mobilization during incubation of cells under oxidative stress conditions, can be prevented.

Cells were first cultivated under nitrogen-limiting conditions to obtain the maximum storage (MSM0.1) with 1 % (w/v) gluconate, harvested, washed in sterile NaCl solution (0.85 %, w/v), and resuspended in MSM medium containing 1 g/l ammonium chloride but lacking an available carbon source, in duplicated cultures. 100 µM of Orlistat was added to one culture, whereas the other one without inhibitor was used as control. Samples were withdrawn after 24 h in the presence/absence of 10 mM MV and 20 mM H_2O_2, and viable counts (CFU) were determined. TAG accumulation was analyzed by semi-quantitative TLC as described above. All determinations were done in triplicate experiments. The data were recorded as means and standard deviations; and differences in count of CFU after application of each stress condition and the inhibitor were determined by analysis of variance with software Statistica 7.0. ANOVA variance analysis was used with a probability level of $p < 0.05$.

Results

Antioxidant enzyme activity

Superoxide dismutase and catalase are central antioxidant enzymatic scavengers. These antioxidant activities were

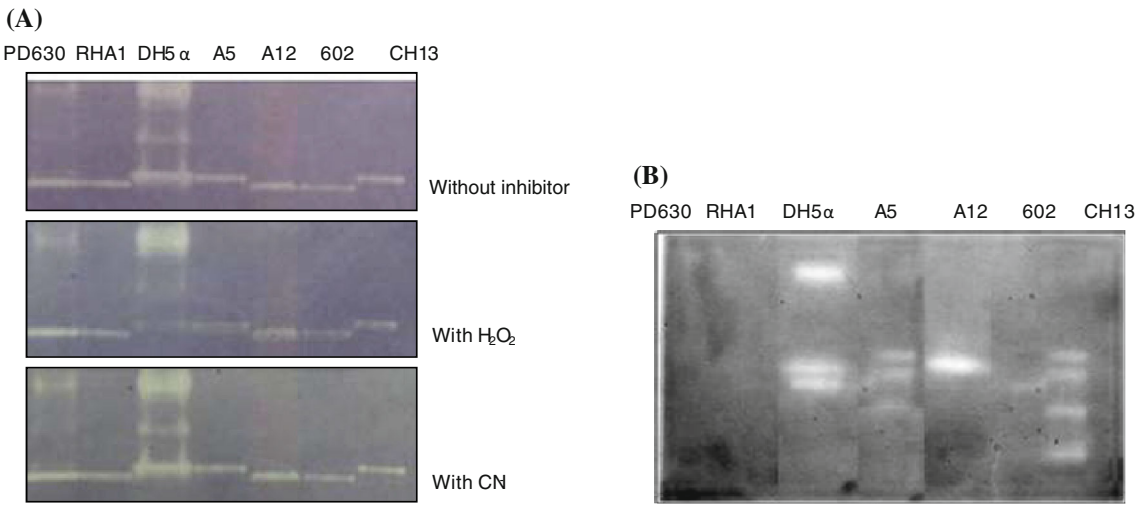

Fig. 1 SOD and catalase activity measurements in strains PD630, RHA1, A5, A12, 602 and CH13. Aliquots of soluble cell extracts of the indicated strains corresponding to approximately 10 μg of protein were resolved by non-denaturing PAGE and subsequent in situ staining for SOD (**a**) and catalase (**b**) activity. The inhibitory effect of 2 mM H_2O_2 or 2 mM KCN on SOD activity was also evaluated (**a**). *Escherichia coli* DH5α was used as control strain

visualized in situ after native polyacrylamide gel electrophoresis (PAGE) of soluble extracts from different extremophile rhodococci taken at exponential growth in NB medium. A single SOD activity band was visualized in all studied actinobacteria being non-sensitive to KCN and H_2O_2, behaving as Mn-SOD (Fig. 1a). A single catalase electrophoretic species was detected in *Dietzia* sp. A12, three in *Rhodococcus* sp. A5 and four isoenzymes in *Rhodococcus* sp. CH13, whereas no catalase active band was detectable in the soluble extracts of strains PD630, RHA1 and 602 (Fig. 1b).

Sensitive to pro-oxidants in agar plates

In order to analyze the tolerance of rhodococcal cells to pro-oxidants agents, we investigated three extremophile strains from the high altitude environments of North Argentina (Puna) (strains A5, A12 and CH13); three strains from Patagonia in South Argentina (strains F7, S1.17b and 602); and two model strains, PD630 and RHA1, which were originally isolated from soil samples in Germany and Japan, respectively. Tolerance to pro-oxidant agents was tested by placing culture serial dilution drops of the studied strains on NB agar plates supplemented with the previously mentioned concentrations of H_2O_2 and MV. *Dietzia* sp. A12 and *Rhodococcus* sp. CH13 were more tolerant to MV challenge than the other studied actinobacteria (Fig. 2). Both strains were able to grow in the presence of 5.0 mM MV at the 10^{-5} and 10^{-3} dilutions, respectively; whereas *R. opacus* PD630, *R. jostii* 602 and *R. jostii* RHA1 showed limited growth at the 10^{-1} dilution at the same concentration. On the other hand, strains *Rhodococcus* sp. A5, *R. fascians* F7 and *R. fascians* S1.17b were the most sensitive strains during exposure to MV (Fig. 2b). All studied actinobacteria showed good tolerance to H_2O_2 even at 0.5 mM concentration, with exception of strains A5 and RHA1, which were the most sensitive microorganisms (Fig. 2a).

Relationship between oxidative stress and triacylglycerol accumulation in *R. opacus* PD630

The response to oxidative stress and TAG accumulation, promote metabolic changes in rhodococcal cells since both processes need a reductive cellular environment to work efficiently (Alvarez and Steinbüchel 2010; Krapp et al. 2011). In addition, oxidative stress might alter the activity of some proteins involved in TAG biosynthesis and accumulation in these oleaginous microorganisms. For this reason, we investigated the influence of oxidative stress on TAG metabolism (biosynthesis and mobilization) in *R. opacus* strain PD630, which is usually used as a research model in lipid field (Alvarez and Steinbüchel 2010).

Tolerance to pro-oxidants in nitrogen-rich and nitrogen-limiting culture conditions

The cultivation of cells in mineral salts medium in the presence of an excess of the carbon source under nitrogen-limiting conditions (MSM0.1) promotes a massive accumulation of TAG by oleaginous *Rhodococcus*, such as strain PD630 (Alvarez et al. 1996). In contrast, when cells are cultivated in nitrogen-rich media (MSM1) in the presence of a carbon source, TAG biosynthesis and accumulation is not induced (Alvarez et al. 2000). In order to

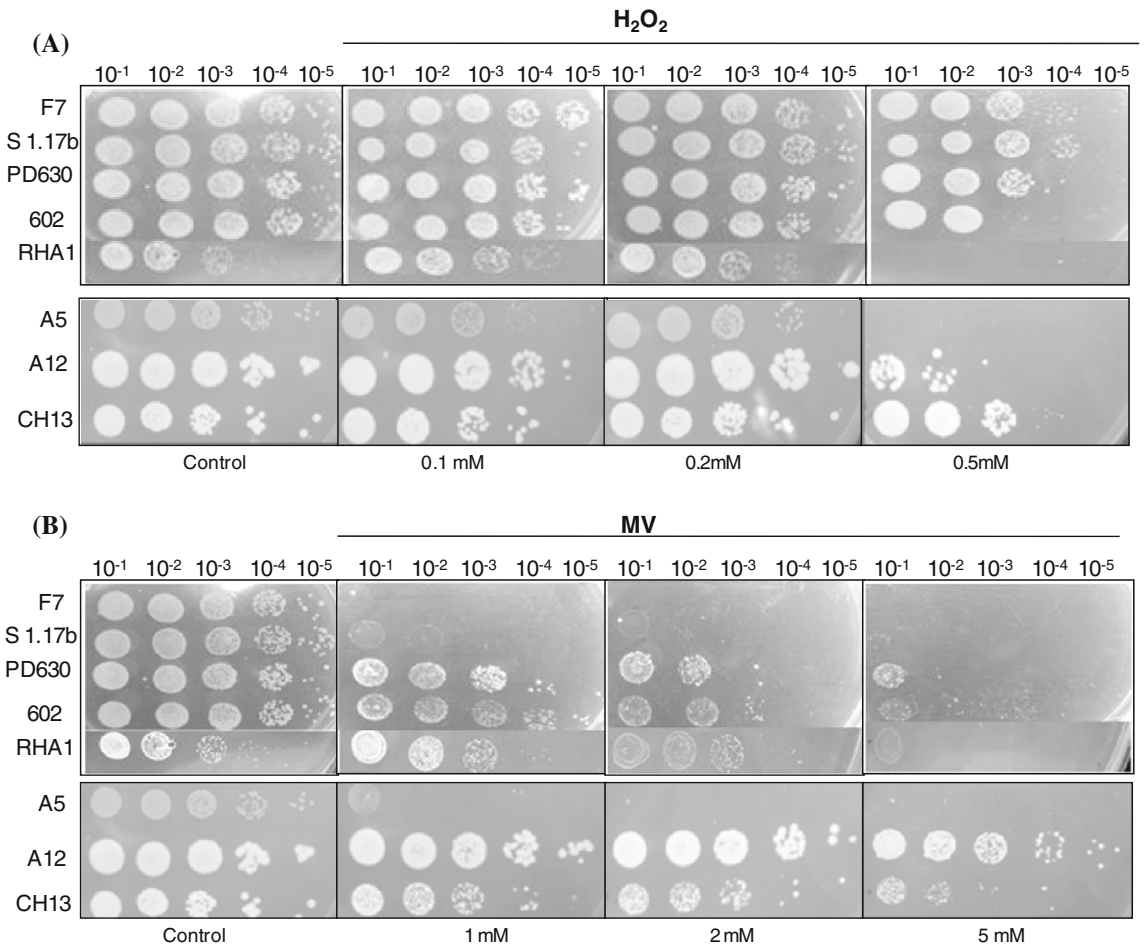

Fig. 2 Sensitivity to oxidative stress generated by H_2O_2 (**a**) and MV (**b**) in different rhodococcal isolates grown on NB media

analyze the relationship between the oxidative stress responses and TAG accumulation, cells of PD630 were cultivated in MSM0.1 and MSM1 with gluconate as sole carbon source, and simultaneously exposed to pro-oxidant agents. Cells were more tolerant to H_2O_2 challenge when cultivated under TAG accumulation conditions (Fig. 3a). When 0.2 mM H_2O_2 was present in the culture medium, only cells cultivated in MSM0.1 with gluconate were able to grow at 10^{-4} dilutions (Fig. 3a). No growth was observed by cells cultivated under nitrogen-rich medium at the same H_2O_2 concentration. Interestingly, the tolerance to MV was similar after cell cultivation in MSM0.1 and MSM1 with gluconate. Cells grown in MSM1 were slightly more tolerant than those cultivated under nitrogen-limiting conditions as is shown in Fig. 3a. Cells cultivated in MSM1 grew better than those of MSM0.1 at 10^{-2} dilutions when 3 and 4 mM MV were present in the media. In addition, we found other differential effect of H_2O_2 and MV treatments on lipid accumulation, when we analyzed the TAG content in cells in MSM0.1 media by semi-quantitative TLC (Fig. 3b). The treatment of cells with MV caused a decrease in TAG content, in contrast to cells treated with H_2O_2.

Interactions between oxidative stress and TAG biosynthesis and accumulation by R. opacus PD630

To analyze the interaction between the response of cells to oxidative stress and lipid metabolism, lipid-accumulating cells were treated with H_2O_2 and MV in the presence/absence of an inhibitor of the de novo fatty acid biosynthesis (cerulenin), which also avoids TAG accumulation; and of an inhibitor of the biosynthesis of carotenoid pigments (DPA). Figure 4a shows that the treatment with both pro-oxidant agents during TAG accumulation by *R. opacus* PD630 produced a significant decrease ($p < 0.05$) of cell counts in comparison to cells cultivated without H_2O_2 and MV. In addition, the presence of MV in the culture medium negatively affected TAG accumulation as revealed by semi-quantitative TLC (Fig. 4b). In contrast, cells treated with H_2O_2 accumulated comparable amounts of TAG than those cultivated in the absence of this oxidant agent. When fatty

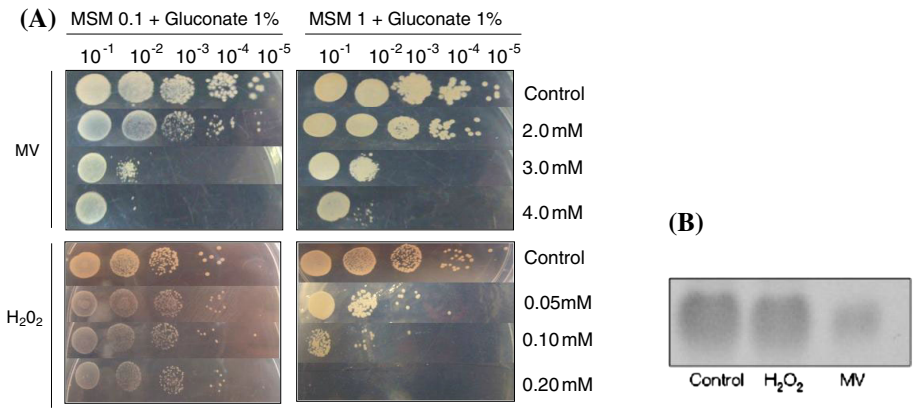

Fig. 3 Sensitivity of strain PD630 to oxidative stress generated by H_2O_2 and MV in nitrogen-rich and nitrogen-limiting culture conditions (**a**) and TAG accumulation after TLC analysis (**b**)

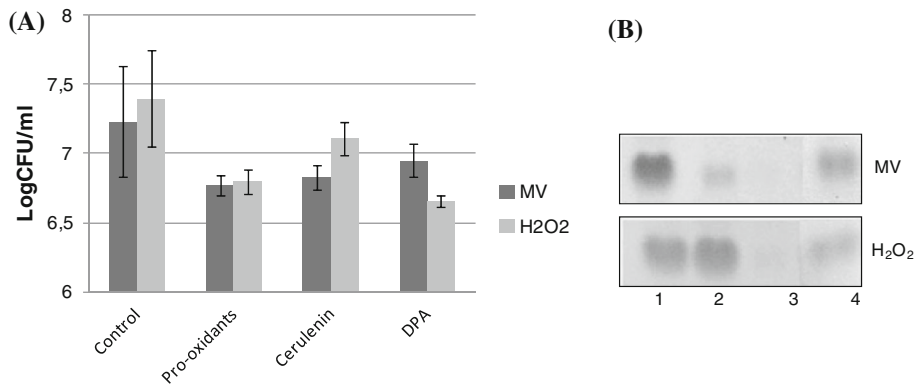

Fig. 4 Effect of cerulenin and DPA on the responses of PD630 cells to pro-oxidants. **a** CFU counts, and **b** TLC analysis showing TAG content of cells. References: *1* control, *2* MSM0 + pro-oxidants, *3* MSM0 + pro-oxidants + cerulenin, *4* MSM0 + pro-oxidants + DPA

acids and TAG biosynthesis were inhibited by cerulenin, no significant differences ($p > 0.05$) in cell number were observed after treatment with MV, in comparison to the culture without the lipid inhibitor (Fig. 4a). However, cells treated with H_2O_2 in the presence of cerulein were significantly more resistant than those cultivated in the absence of the lipid inhibitor (Fig. 4a). The dramatic decrease of TAG accumulation as result of the inhibition of the de novo fatty acid biosynthesis pathway is shown in (Fig. 4b). When cells were treated with DPA for inhibiting carotenoid biosynthesis, a significant decrease ($p < 0.05$) of cell counts occurred in the presence of H_2O_2, but not in presence of MV (Fig. 4a). A slight decrease in TAG content was observed in cells treated with H_2O_2 and DPA, compared with cells cultivated with H_2O_2 in the absence of the inhibitor (Fig. 4b). In contrast, cells treated with MV and DPA accumulated higher amounts of TAG than cells cultivated with MV in the absence of the inhibitor (Fig. 4b).

Interactions between oxidative stress and TAG mobilization by R. opacus PD630

Since *R. opacus* PD630 is able to accumulate significant amounts of TAG during growth on gluconate, the interaction between the response of cells to oxidative stress and the mobilization of TAG, was studied with or without the addition of the lipase inhibitor, Orlistat. It is important to remark that although Orlistat inhibited TAG degradation during cultivation of PD630 cells under conditions that promote lipid mobilization, it did not affect their growth or cell survival when cultivated in NB medium or MSM0.1 with gluconate as sole carbon source, media which promote growth and TAG biosynthesis, respectively (data not shown).

When cells were cultivated under carbon-starvation conditions, they mobilized the endogenous TAG in the absence of Orlistat, as revealed by TLC (Fig. 5b). The inhibition of lipid degradation caused a significant decrease of cell counts ($p < 0.05$) compared to that obtained in the absence of Orlistat (Fig. 5a). The addition of the lipase inhibitor did not produce any effect on the response of the cells to MV (Fig. 5a). Accordingly, inhibition of TAG mobilization by Orlistat during treatment of cells with MV under carbon-starvation conditions was confirmed by TLC (Fig. 5b). Interestingly, the inhibition of TAG mobilization produced a significant decrease in cells counts ($p < 0.05$) during treatment of cells with H_2O_2, in comparison to the culture in the absence of Orlistat (Fig. 5a). Surprisingly, TAG were not mobilized, but instead a slight increase in their content was observed in TLC analysis, when cells

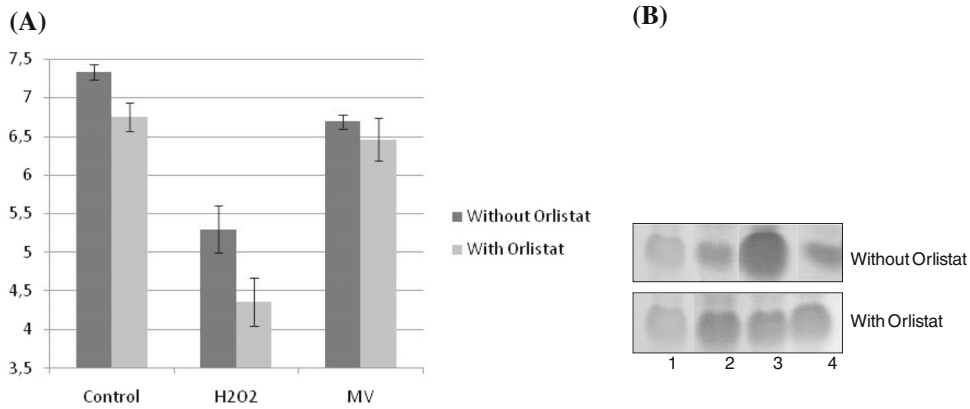

Fig. 5 Effect of Orlistat (inhibitor of lipase) on the responses of PD630 cells to pro-oxidants. **a** CFU counts, and **b** TLC analysis showing TAG content of cells. References: *1* MSM0.1 + gluconate, *2* MSM1 without carbon source and without oxidative stress, *3* MSM1 without carbon source + H_2O_2, *4* MSM1 without carbon source + MV

were cultivated under carbon-starvation conditions in the presence of H_2O_2 and in the absence of Orlistat (Fig. 5b). This unexpected effect was always reproducible when the same experiment was repeated several times.

Discussion

Despite the importance of rhodococci in the environment, the tolerance to oxidants and their protective mechanisms against this stress have been only poorly studied. In this work, we investigated the oxidative stress tolerance of 8 actinobacteria strains (most of them belonging to *Rhodococcus* genus), including bacteria isolated from extreme environments, such as the Andean Puna and Patagonia located in North and South Argentina, respectively. Additionally, we used two rhodococcal strains (*R. opacus* PD630 and *R. jostii* RHA1) considered as model microorganisms by diverse studies. All investigated strains, independent of the geographical regions from which they were originally isolated, were more resistant to the H_2O_2 and MV than Gram-negative extremophiles belonging to *Acinetobacter* genus, investigated previously under similar conditions used in this study (Di Capua et al. 2011). This difference in the resistance to oxidants showed by Gram-positive actinobacteria compared to Gram-negative members may be explained, at least in part, by the complex structure of their cellular envelope, which is dominated by the presence of large branched mycolic acids (Sutcliffe et al. 2011). The cellular envelope configuration of rhodococci makes the entry of pro-oxidants into the cell difficult. On the other hand, Di Capua et al. (2011) reported that extremophile *Acinetobacter* strains isolated from the Andean Puna (Argentina) were more tolerant to H_2O_2, MV and UV exposure than collection strains of the same genus. Results of the present work suggested that the tolerance to pro-oxidants in rhodococci was more related to the species than the geographical origin of strains.

In general, *R. fascians* strains isolated from soil samples in Patagonia were the most sensitive microorganisms to MV treatment, and the group of *R. jostii*/*R. opacus* the most tolerant. In contrast, *R. fascians* strains were among the most resistant microorganisms in the presence of H_2O_2. These differences in the responses to pro-oxidants may be explained by the different biochemical and genetic endowment of each rhodococcal species and/or the efficiency of the respective protective mechanisms.

In aerobic organisms, ROS are produced as by-products of normal oxygen respiration. Enzymatic analysis after native PAGE indicated that all studied strains express at least one SOD and several catalase active species during exponential growth phase. The absence of detectable catalase activity in soluble extracts of *R. opacus* PD630 and *Rhodococcus* sp. 602 is quite intriguing considering the high tolerance to H_2O_2 shown by these strains. In addition to catalases, peroxidases can also serve as scavengers of H_2O_2 in bacteria (Mishra and Imlay 2012). On the other hand, the usual difficulty for disrupting rhodococcal cells and/or the inactivation of catalase activity in cellular extracts of these microorganisms may be other reasons, which explains the obtained results. The inhibition test (resistance to H_2O_2 and KCN) in PAGE demonstrated that an Mn-SOD type was expressed by all strains used in this study during exponential growth phase.

Since the response of cells to oxidative stress challenge and the accumulation of TAG by rhodococci require a reconfiguration of their metabolism, which may include a fine regulation of the homeostasis of the NADPH pool and the activation of diverse genes/proteins involved in lipid biosynthesis, among other metabolic interactions, we investigated the tolerance of *R. opacus* PD630 to H_2O_2 and MV exposure during accumulation and mobilization of TAG using different inhibitors of lipid metabolism. Figure 6 shows the main metabolic pathways involved in lipid metabolism and antioxidant responses (Alvarez et al. 1997; Ghosal et al. 2005). Results of this study demonstrated metabolic connections between TAG metabolism and

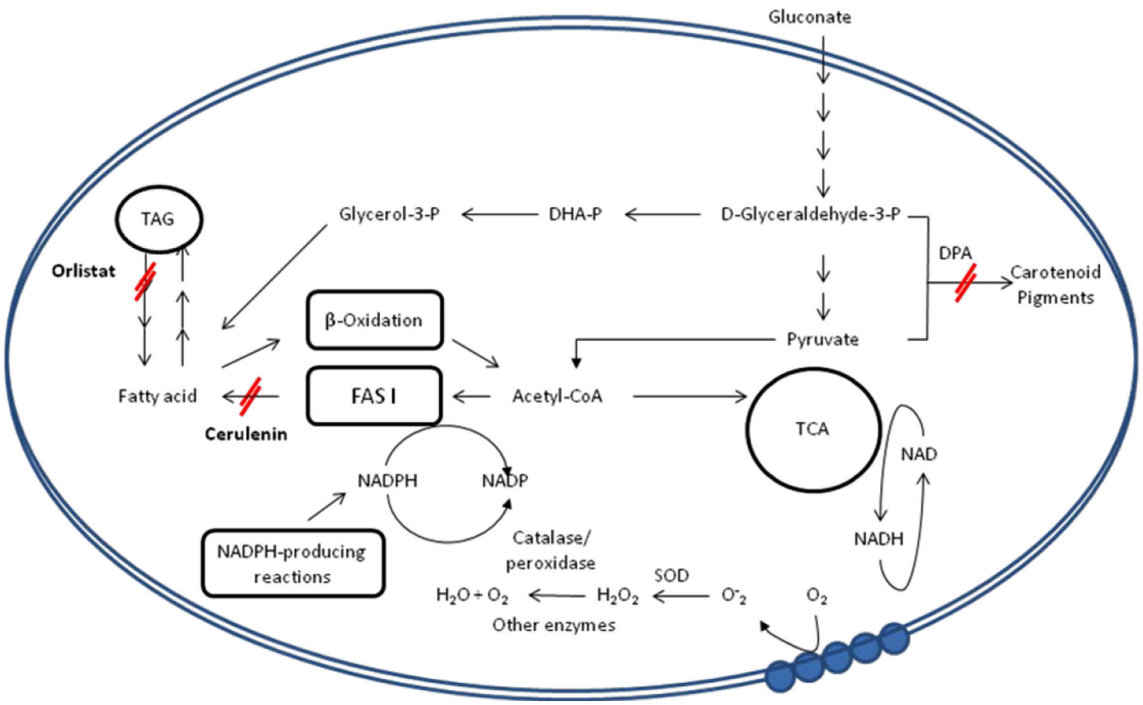

Fig. 6 Main metabolic reactions occurring in TAG-accumulating cells and antioxidant responses (adapted from Alvarez et al. 1997). *DHA-P* dihydroxyacetone-phosphate, *DPA* diphenylamine, *FAS I* fatty acid synthase system I, *SOD* superoxide dismutase, *TAG* triacylglycerols, *TCA* tricarboxylic acid cycle

oxidative stress responses in the oleaginous *R. opacus* PD630. Biomolecules undergo different vulnerability to H_2O_2 and MV, which usually reacts with O_2 generating the production of O_2^- (Hassan and Fridovich 1978). Moreover, H_2O_2 oxidizes most target molecules slower than O_2^- does (Imlay 2003). For these reasons, H_2O_2 and MV may produce different impacts in cells and differential metabolic interactions during TAG biosynthesis and mobilization by strain PD630. MV negatively affected lipid biosynthesis and accumulation in strain PD630, producing a dramatic decrease of TAG accumulation in cells cultivated under nitrogen-limiting conditions, which promoted lipid production. In addition, cells cultivated under non-accumulating conditions seemed to be more resistant to MV than TAG-accumulating cells, whereas the tolerance of cells to MV was not negatively affected when TAG accumulation was impaired by the addition of cerulenin. Some of the genes or proteins of the lipid biosynthesis and accumulation machinery may be sensitive to the redox changes occurring in PD630 cells in the presence of MV. Alternatively, the increase of NADPH pool which supports TAG accumulation under nitrogen-limiting conditions may promote oxidative stress in the presence of MV. It is known that NADPH, in contrast to NADH, transmits electrons to MV, generating the production of O_2^- which does not cross the cell envelope (Hassan and Fridovich 1979). On the other hand, cells of strain PD630 treated with MV were able to mobilize intracellular TAG under carbon-starvation conditions, and the inhibition of TAG degradation had no effect on tolerance of cells to MV. This result suggested that the responsive mechanisms against the pro-oxidant MV are executed independently of the TAG and fatty acid catabolism in cells of PD630, and they are not altered by a change of lipid mobilization under the used conditions (Fig. 6). Carotenoid pigments do not seem to be relevant for the antioxidant responses of cells to MV, since the inhibition of carotenoid biosynthesis by the addition of DPA to the culture media did not affect cell survival during MV treatment. Interestingly, the inhibition of pigments biosynthesis produced an increase of TAG accumulation during MV treatment. A possible explanation for this effect is that the inhibition of carotenoid synthesis pathway, which sequesters D-glyceraldehyde-3-phosphate and pyruvate from central metabolism in rhodococci, produces an increase of the carbon flux through the fatty acids and TAG biosynthesis pathways (Fig. 6).

In contrast to MV, the response to H_2O_2 seemed to be synergic (to some extent) with the TAG accumulation process in PD630, since TAG-accumulating cells were more resistant to H_2O_2 than those cultivated under non-accumulating conditions. Moreover, the addition of H_2O_2 to the media did not affect TAG accumulation under nitrogen-limiting conditions. Interestingly, carotenoid pigments seemed to play an important role in cell protection against H_2O_2, in contrast to MV. The inhibition of carotenoid

biosynthesis by the presence of DPA affected cell counts and the ability of cells to accumulate TAG, particularly during treatment with H_2O_2, which is lipid soluble, in strain PD630. Carotenoid pigments are non-enzymatic antioxidants, which protect lipids from peroxidation reactions by scavenging reactive free radicals (Liebler and McClure 1996). These results are in concordance with those reported by Tian et al. (2007). The authors demonstrated that a colorless mutant strain of *Deinococcus radiodurans* was more sensitive than the wild type able to synthesize carotenoid pigments during exposure to H_2O_2 and desiccation. On the other hand, Osawa et al. (2011) identified novel carotenoids with potent antioxidative activities in *Rhodococcus* sp. strain CIP, which was taxonomically close to *R. fascians*. The occurrence of similar carotenoid species in *R. fascians* strains isolated from soil samples in Patagonia may explain to some extent their high tolerances to H_2O_2 observed in this study. Our results showed that cells treated with H_2O_2 in the presence of cerulenin, which inhibits TAG accumulation, were significantly more resistant than those cultivated in the absence of the lipid inhibitor. Carotenoid biosynthesis may be also induced during TAG accumulation in PD630 cells during cultivation under nitrogen-limiting conditions, as a protective mechanism for lipid-rich cells against oxidative stress. Thus, the increase of carotenoid concentration in cells, which may occur in the presence of cerulenin, may enhance the responses of cells to H_2O_2 under lipid-accumulation conditions.

On the other hand, the treatment with H_2O_2 seemed to represent a more restrictive challenge than the addition of MV during incubation of PD630 cells under carbon-starvation conditions, which promotes TAG mobilization. The inhibition of TAG mobilization by the addition of Orlistat produced a significant decrease in cell survival during treatment of cells with H_2O_2, in comparison to the culture in the absence of the inhibitor. Surprisingly, the treatment of cells with H_2O_2 produced an increase of TAG accumulation after incubation of cells under carbon-limiting conditions in the presence of Orlistat. The inhibition of TAG degradation by the addition of Orlistat may produce a metabolic imbalance specifically during the response to H_2O_2, but not to MV, which caused a decrease of cell survival in the presence of H_2O_2 under carbon-starvation conditions. The slight increase of TAG content in cells treated with H_2O_2 under carbon-starvation conditions when TAG mobilization was inhibited may represent a lateral effect of the metabolic imbalance between lipid catabolism and antioxidant responses against H_2O_2. NADPH formation seems to be stimulated when bacteria are exposed to H_2O_2 (Brumaghim et al. 2003; Krapp et al. 2011), thus an extra NADPH pool might be available for TAG biosynthesis by strain PD630 treated with H_2O_2 when TAG mobilization is inhibited by Orlistat under carbon-limiting conditions. Turnover of biomolecules may provide carbon for TAG biosynthesis under such conditions, to some extent (Fig. 6).

Results of this study using *R. opacus* PD630 may be extrapolated to other rhodococci usually occurring in extreme environments (Burchell et al. 2004; Ordoñez et al. 2009; Bequer Urbano et al. 2013). The ability to accumulate TAG (Bequer Urbano et al. 2013) and to withstand oxidative stress (Ordoñez et al. 2009) seem to be usual features in extremophile rhodococci, which facilitated their colonization of harsh terrestrial environments. The better knowledge of metabolic interactions between different cellular processes in rhodococci may enhance our understanding on the responses of such relevant microorganisms in extreme environments.

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