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Lipid storage in high-altitude Andean Lakes extremophiles and its mobilization under stress conditions in *Rhodococcus* sp. A5, a UV-resistant actinobacterium

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**Abstract** The production of triacylglycerols (TAG) or wax esters (WS) seems to be a widespread feature among extremophile bacteria living in high-altitude Andean Lakes (HAAL), Argentina. Twelve out of twenty bacterial strains isolated from HAAL were able to produce TAG or WS (between 2 and 17 % of cellular dry weight) under nitrogen-limiting culture conditions. Among these strains, the extremophile *Rhodococcus* sp. A5 accumulated significant amounts of TAG during growth on glucose (17 %, CDW) and hexadecane (32 %, CDW) as sole carbon sources. The role of accumulated TAG in the response to carbon starvation, osmotic stress, UV-radiation and desiccation was investigated in *Rhodococcus* sp. A5 using an inhibitor of TAG degradation. Cells degraded TAG during these stresses in the absence of the inhibitor. The inhibition of TAG mobilization affected cell survival during osmotic stress only during the initial growth stage. Little or no surviving cells were observed after carbon starvation, UV-treatment and desiccation, when TAG mobilization was inhibited. These results suggested that TAG metabolism is relevant for the adaptation and survival of A5 cells under carbon starvation, osmotic stress and UV irradiation, and essential under desiccation conditions, which prevail in HAAL environments.

**Keywords** HAAL · Extremophiles · Triacylglycerol · *Rhodococcus*

**Introduction**

High-altitude Andean Lakes (HAAL) are systems of shallow lakes distributed across the Puna at altitudes varying from 3,000 to 6,000 m above sea level (Albarracín et al. 2012; Di Capua et al. 2011; Flores et al. 2009; Ordoñez et al. 2009). These ecosystems present extreme environmental conditions such as high levels of ultraviolet radiation (UVR), hypersalinity, periodical desiccation, low nutrient concentration, high concentration of heavy metals and metalloids (mainly arsenic) and wide daily temperature fluctuations (≈30 °C). The combination of all these factors makes HAAL ecosystems a unique natural laboratory for exploring and monitoring interactions between the geophysical environment and its biodiversity dynamics (Dib et al. 2009; Seufferheld et al. 2008).

Despite the harsh conditions, there is a high microbial diversity in the community present at the HAAL (Flores...
et al. 2009; Ordoñez et al. 2009). Moreover, recent evidence indicates that those microorganisms present at extreme environments displayed efficient survival strategies being able to produce biomolecules adapted to their unusual living conditions: i.e., photolyases for repairing strong DNA damage (Albarracín et al. 2012) and efficient enzymatic mechanisms against oxidative stress (Di Capua et al. 2011). In this context, screening and characterization of HAAL extremophiles able to produce triacylglycerols (TAG) and/or wax esters (WS) in response to nutritional stress conditions is of main interest for deeper understanding of physiology on extremophiles. The accumulation of these lipids may be part of a strategy of cells for balancing their metabolism under environmental fluctuating conditions. Storage lipids may play key roles in microbial activity and survival in such environment as the HAAL, where steady states as well as fluctuating extreme conditions are the rule.

During starvation periods, bacteria usually accumulate polyhydroxyalkanoates (PHA) as storage lipids, which serve later as endogenous carbon sources (Steinbüchel 1991). However, TAG and WS accumulation are restricted to some Gram-negative genera, such as Acinetobacter, Marinobacter and Alcanivorax (Alvarez et al. 1997a; Holtzapple and Schmidt-Dannert 2007; Kalscheuer et al. 2007; Makula et al. 1975; Rontani et al. 1999) and Gram-positive actinobacteria belonging to Rhodococcus, Nocardia, Mycobacterium and Streptomycetes genera (Alvarez 2003; Alvarez et al. 1996, 1997b; Arabolaza et al. 2008; Daniel et al. 2004; Olukowski and Packter 1994). TAG are excellent reserve materials due to their extremely hydrophobic properties, which allow their accumulation in large amounts in cells without changing the osmolarity of cytoplasm. In fact, their degradation produces the maximum yields of energy in comparison with other storage compounds such as carbohydrates and polyhydroxyalkanoates, since the carbon atoms of acyl moieties of TAG are in their most reductive form (Alvarez 2006; Alvarez and Steinbüchel 2002). The storage lipids in bacteria may be important not only for their energy potential but also as a reservoir of metabolic water under desiccation conditions, since fatty acid oxidation releases large amounts of metabolic water (Alvarez et al. 2004). Moreover, TAG may possess other important functions in bacterial cells, such as the regulation of the fatty acid composition of membrane lipids, as a sink for reducing equivalent in cells during oxygen-limiting conditions, as precursor source for mycolic acid biosynthesis during adaptation of mycolic acid-producing actinobacteria to environmental stresses, among other possible functions (Alvarez and Steinbüchel 2010).

Till present, microbes able to produce TAG or WS have been isolated mainly from oligotrophic environments, such as marine sediments or arid soil but few reports of these biotechnological interesting microbes have been made from aquatic bacteria suffering life-threatening conditions. For these reasons, screening of TAG biosynthesis and accumulation in bacteria from HAAL is a novel task and may prove to be a relevant feature for organisms living in such an inhospitable setting with changing environmental conditions. In this work, twenty extremophilic strains previously isolated from HAAL were studied for their ability to produce TAG and/or WS in response to nutritional stress. The most fitted strain for accumulating lipids was selected and further characterized as Rhodococcus sp. A5. The possible role of TAG in the response and survival to stress conditions of this novel extremophile is, accordingly, discussed.

Materials and methods

Bacterial strains, media and growth conditions for lipid determination

Twenty bacterial strains belonging to the LIMLA-PROIMI Extremophilic Strain Collection previously isolated and taxonomically identified from five lakes located at the HAAL were used in the present study. Collection strains Rhodococcus corynebacteroides DSM 20151 and Rhodococcus kroppenstedtii DSM 44908, as well as a spontaneous mutant of A5 strain detected after UVR-treatment and further isolated were used as controls. Original wild-type strain is called as A5WT (red-original strain) while the mutant was called as A5W1 (white-mutant strain). A5WT strain is available to the scientific community worldwide at the LIMLA-PROIMI Extremophilic Strain Collection (http://www.limla.com.ar).

Cells were grown aerobically at 28 °C in nutrient broth medium (0.8 %, w/v) or in mineral salts medium (MSM) according to Schlegel et al. (1961) with sodium gluconate (1 %, w/v), glucose (1 %, w/v), sodium acetate (0.2 %, w/v) or glycerol (0.3 %, 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 (lipid-storage/promoting conditions, hereafter LSP conditions). To obtain solidified media, 1.8 % (w/v) agar was added. Cells were grown aerobically at 28 °C overnight in 10-ml nutrient broth medium on a rotary shaker. After growth, cells were harvested, washed once with sterile saline solution and inoculated in 50-ml MSM0.1 containing sodium gluconate, glucose (1 %, w/v) or sodium acetate (0.2 %, w/v) as sole carbon sources. These cultures were incubated at 28 °C on a rotary shaker. After 48 or 72 h of incubation to allow lipid accumulation, cells were harvested, washed twice with sterile saline solution, lyophilized and analyzed for their lipid content.
Treatment of cells under stress conditions

To assess the importance of TAG in cells challenged with different stresses, Orlistat was used as an inhibitor of lipases (Low et al. 2009). With this treatment, TAG mobilization during incubation of cells under stress conditions, such as carbon starvation, osmotic stress, UVR and desiccation can be prevented.

In order to study the response of cells to carbon starvation, cells were first cultivated under LSP conditions to obtain the maximum storage (MSM 0.1 %) with 1 % (w/v) glucose, 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 at different times (after 1, 3 and 5 days of incubation) and analyzed for the content of TAG. Aliquots of 100 μl were removed from each sample and microbial growth was assessed by counting the number of colony-forming units (CFUs) after 48 h of incubation at 28 °C in NB agar.

For osmotic stress challenge, cells were cultivated under LSP conditions to obtain the maximum content of the storage compounds, harvested, washed in phosphate buffer (pH 7), and resuspended in MSM medium containing 1 g/l ammonium chloride and 1 % (w/v) glucose as sole carbon source with the addition of NaCl to a final concentration of 2 % (w/v), in the presence and absence of Orlistat (100 μM), respectively. Samples were withdrawn during exponential (24 h of incubation) and stationary growth (48 h of incubation) phases and analyzed for the content of TAG. CFU counts during osmotic stress were determined by plating 100-μl aliquots after appropriate dilutions. A control culture not subjected to saline stress was included.

For UV challenge, cells were cultivated under LSP conditions to obtain the maximum content of the storage compounds. Cells were harvested, washed once with sterile NaCl solution (0.85 %, w/v) and suspended at an OD600nm of 1.3 in sterile NaCl solution. Cell suspension was divided into two aliquots, and deposited onto disposable covered Petri plate; one half in the presence of the inhibitor of lipase (100 μM) and the other without inhibitor, for 60 min. Next, both aliquots were exposed to UV-B treatment for 90 min. After exposure, culture aliquots were subjected to serial dilution for later counting of CFU and collected for TAG analysis.

In order to determine the response of cells to desiccation, cells were grown aerobically at 28 °C overnight in 10-ml nutrient broth medium on a rotary shaker. After growth, cells were harvested, washed once with sterile NaCl solution (0.85 %, w/v) and resuspended to reach an OD600 of 2 (±0.1) in sterile NaCl solution. Approximately 20 aliquots (7 μl each) from this cell suspension were spotted onto 0.45-μm filters (Sartorius, Göttingen, Germany). These filters were placed onto MSM0.1 agar plates containing 1 % (w/v) glucose as carbon source (LSP conditions) and were incubated at 28 °C for 5 days. After growth of the colonies, one set of filters was removed from the agar plates and impregnated with solution of 100 μM of Orlistat for 60 min prior to drying. After the incubation of the inhibitor, both set of filters (treated and untreated with the inhibitor) were dried by incubation in empty sterile plates at 28 °C and 18 % relative humidity for 7 days, and the viable count (CFU) was determined at different times. The filters, on which the colonies were grown, were cut into small pieces containing only a single colony and were added to sterile Eppendorf microcentrifuge tubes. The cells from each colony were resuspended separately, and the CFU was determined before and after desiccation treatment. The other colonies were used to analyze TAG by semi-quantitative thin layer chromatography (TLC).

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 $p < 0.05$.

Lipid extraction and TLC

The qualitative and semi-quantitative analyses of intracellular lipids in cells were performed by TLC. For this, 2 mg of lyophilized cells was extracted with 200 μl chloroform/methanol (2:1, v/v) for 2 h and shaking every 15 min. The supernatant was then concentrated and 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 and cetylpalmitate (Merck, Darmstadt, Germany) were used as reference substances for TAG and WS, respectively.

Analysis of total fatty acids content

The quantification of the total fatty acids is considered as a good estimator of TAG content, since 98 % of the total fatty acids in the cell are accumulated as TAG. For this purpose the fatty acids were transformed to the constituent fatty acid methyl esters (Alvarez et al. 1996) and analyzed by gas chromatography (GC). In brief, 7–10 mg of lyophilized cells was subjected for 3 h to methanolation at 100 °C in the presence of 15 % (v/v) sulfuric acid in methanol. The resulting methyl esters were analyzed using a HP5890A gas chromatograph equipped with a InnoWAX capillary
column (30 m × 0.53 mm × 1 μm) and a flame ionization detector, using helium as carrier gas (13 ml min⁻¹). The temperature of the injector and detector was 270 and 320 °C, respectively. The oven temperature was maintained at 90 °C for 5 min; then programmed at 6 °C increase per minute. When the temperature reached 240 °C, it was held for 15 min. For quantitative analyses, tridecanoic acid was used as an internal standard. The data represent the mean of two independent experiments.

Phenotypic and genotypic characterization of strain A5

Morphological and physiological characteristics of A5 strain were observed on various media: yeast extract/malt extract agar (ISP 2), Luria–Bertani agar (LB), and Tryptein soy agar (TSB) incubated for 4 days at 30 °C (Albarracin et al. 2010). Gram and acid–alcohol-fast stains were carried out on a 3-day-old culture as described by Doetsch (1981). Cell morphology was visualized using a Nikon optic microscope. For scanning electron microscopy, the strain was grown on ISP 2 agar. The agar blocks were cut from the growth medium and fixed (glutaraldehyde 4.25 % in phosphate buffer 0.1 M pH 7.4) for 3 h at 4 °C followed by dehydration in a graded acetone series. The critical drying point was obtained by exchanging the acetone through liquid CO₂. The samples were covered by gold and visualized using a Zeiss Supra 55VP (Carl Zeiss NTS GmbH, Germany) scanning electron microscope (LAMENOACCT-CONICET).

A fingerprint of enzymatic activities was obtained using API 20E, API ZYM and API Coryne test strips (bioMérieux). The temperature for growth was tested at 25, 30, 37 and 45 °C (Albarracin et al. 2010). Cell morphology was visualized using a Nikon optic microscope. For scanning electron microscopy, the strain was grown on ISP 2 agar. The agar blocks were cut from the growth medium and fixed (glutaraldehyde 4.25 % in phosphate buffer 0.1 M pH 7.4) for 3 h at 4 °C followed by dehydration in a graded acetone series. The critical drying point was obtained by exchanging the acetone through liquid CO₂. The samples were covered by gold and visualized using a Zeiss Supra 55VP (Carl Zeiss NTS GmbH, Germany) scanning electron microscope (LAMENOACCT-CONICET).

DNA was prepared from cells of A5 strain that were grown on LB broth for 48 h at 30 °C and harvested by centrifugation (3,000 g for 10 min at 4 °C). The pellets were washed twice with distilled water. Total genomic DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer’s recommendations.

For taxonomic identification, PCR amplifications were performed in 25-μl reaction volume using universal 16S rRNA gene oligonucleotide primers: 27f and 1492r (Heuer et al. 1997) and ITS (internal transcribed spacers) primers (Daffonchio et al. 1998) in a thermal cycler (Perkin-Elmer, model 9700). PCR products were run in 0.8 % (16S rRNA genes) or 2 % (ITS genes) agarose gel, stained with SYBR Green and visualized using a Gel DocTM XR+ with Image LabTM software (BioRad). Purification of DNA from gel slabs was performed using QIAquick Gel Extraction Kit (Qiagen), and DNA sequencing was performed by the dyeoxy chain termination method with an ABI Prism 3730XL DNA analyzer, using the ABI Prism BigDye terminator cycle sequencing ready reactions kit (PE Biosystems) according to the manufacturer protocol. The 16S rRNA sequence reported in this paper was deposited in GenBank under accession number DQ112024.

Multiple alignments of A5 strain 16S rRNA gene sequence (1,372 nt) and reference sequences from the NCBI/EMBL databases were performed using the CLUSTALW program (Thompson et al. 1994). Evolutionary relationships of 16S rRNA sequences from strain A5 and the closest 15 organisms within the genus Rhodococcus were inferred using the neighbor-joining method (Saitou and Nei 1987). Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is
drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 1361 positions in the final dataset. *Milsia brevis* was used as outgroup. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011).

**Results**

Screening for strains able to produce storage lipids among HAAL extremophiles

From the entire large collection of HAAL extremophile strains available in LIMLA, we selected a set of twenty strains isolated from diverse lakes located above 4,000 m, which exhibit extreme environmental conditions. Among them, twelve strains showing interesting polyextremophilic profiles, i.e., multiple resistances to antibiotics, high salt concentration, UVR and arsenic, were able to synthesize and accumulate storage lipids to some extent. Most of them belonged to Gram-negative gammaproteobacteria genera such as *Acinetobacter* and *Marinobacter*, and to actinobacterial genera, i.e., *Nocardia*, *Dietzia* and *Rhodococcus*. In addition, four extremophile strains taxonomically assigned to genera in which biosynthesis of neutral lipids was not reported previously were able to produce TAG or WS; the Gram-negative *Janthinobacterium* sp. V21 (betaproteobacteria) and the Gram-positive strains *Cohnella* sp. CH7 (Firmicutes) and *Agrococcus* sp. CH9 (Actinobacteria) produced WS (R<sub>WS</sub> 0.73) and TAG (R<sub>TAG</sub> 0.44) whereas the Gram-negative strain *Stenotrophomonas* sp. Ver10 (gammaproteobacteria) only produced TAG, as revealed by TLC analyses (data not shown). However, none of them were able to produce more than 7.3 % (CDW) of neutral lipids after cultivation of cells under nitrogen-limiting conditions, which promote lipid accumulation.

Characterization of the extremophile *Rhodococcus* sp. A5

Due to its ability to accumulate significant amounts of lipids, the actinobacterium strain A5 was used as model organism for studying lipid storage and mobilization in HAAL extremophiles. Accordingly, we performed a more detailed taxonomical assignment which includes phenotypic and genotypical characterization.

Comparison of partial sequence of 16S rRNA-coding gene in the Eztaxon database (eztaxon-e.ezbiocloud.net/) demonstrated a closer relation of A5 with members of the family Pseudonocardiae (between 99.5 and 95 % identity). The phylogenetic analyses with corresponding nucleotide sequences of representatives of this family showed that the organism belongs to the genus *Rhodococcus* (Fig. 1). In fact, the strain forms a distinct monophyletic clade together with *Rhodococcus kroppenstedtii* and *R. corynebacteroides*. This relationship is supported by the 100 % bootstrap value recorded using the neighbor-joining method.

Strain A5 showed morphological properties consistent with its assignment to the genus *Rhodococcus*; it is an aerobic organism that forms smooth, soft, redish-pigmented colonies on LB, TSB or ISP2 media. The cells are rod-coccoid-like, Gram-positive and acid–alcohol-fast (Fig. 2). It was able to grow in NaCl (up to 7.5 %) and in a wide temperature ranges (25–45 °C). While performing the preliminary UV resistance assays for this paper a mutant strain of A5, which had lost its original red color, was observed in the agar plates where the survival population was grown. The mutant was confirmed by molecular methods, i.e., ITS and ARDRA, which showed identical profiles in both strains (data not shown), herein named as A5<sub>WT</sub> (red-original strain) and A5<sub>WH</sub> (white-mutant strain). Apart from the pigmentation, we could not detect any difference in morphology by optical and scanning electron microscopy (Fig. 2). Only slight differences in physiology, such as optimum growing temperature, NaCl resistance profile (up to 5 %) and some enzymatic activities (alkaline phosphatase and pyrrolidonilarilamidase), were observed among both strains.

Resistance profiles for UVR of A5<sub>WT</sub>, A5<sub>WH</sub>, DSM 20151, DSM 44908 were tested on LB agar inoculated with serial dilutions (10<sup>-1</sup>–10<sup>-5</sup>) of an initial cell suspension and exposed to increasing doses of UV-B irradiation. A5<sub>WT</sub> was the most resistant strain, as it endures more successfully up to the highest irradiation dose (Fig. 3). Its mutant, A5<sub>WH</sub>, revealed a similar resistance profile indicating that the loss of pigments, most probable carotenoids (data not shown), did not affect the fitness of the strain under UVR. As expected, the reference strains, *R. corynebacteroides* and *R. kroppenstedtii*, exhibited the highest inhibition of growth under UVR (Fig. 3), being the latter completely sensitive, even to the lower UV-B dose tested (15 kJ m<sup>-2</sup>). These results suggested that the UVR resistance trait in A5 is more likely related to its extreme environmental origin than to a species-related attribute.

Analysis of lipid accumulation by *Rhodococcus* sp. A5

*Rhodococcus* sp. A5<sub>WT</sub> was able to accumulate TAG and PHA from glucose as well as from hexadecane, as sole carbon sources, although PHA were found in minor
quantities than TAG in all cases (Table 1). Similar behavior was observed for the mutant strain A5WH and *Rhodococcus corynebacteroides* DSM 20151. In general, hexadecane supported higher TAG accumulation than glucose in all three strains: i.e., 1.6-fold more for A5WT, 1.8-fold more for A5WH and twofold more for DSM 20151 in stationary phase (Table 1). Under the same culture conditions, the extremophlic strain A5 produced higher amounts of TAG on glucose (up to 4.6-fold more in stationary phase) and hexadecane (up to twofold more in stationary phase) than the related reference strain, *R. corynebacteroides* DSM 20151. In turn, similar lipid accumulation was found between the mutant and the wild type A5 strains.
Mobilization of triacylglycerols in response to stress by Rhodococcus sp. A5

Since Rhodococcus sp. A5 accumulated significant amounts of TAG during growth on glucose, the role of TAG in overcoming different stresses occurring naturally at the HAAL 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 A5 WT 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 glucose as sole carbon source, media which promote growth and TAG biosynthesis, respectively (data not shown).

Intracellular deposits of TAG were diminished during incubation of A5 WT cells under carbon starvation conditions (Fig. 4b), suggesting their utilization as endogenous carbon and energy source. Controls (not inhibited but starved) showed a significant decrease in the number of CFU with time (Fig. 4a). But this reduction was even more drastic (ca. one order of magnitude) \((p < 0.05)\) when cells were starved and simultaneously treated with Orlistat. Accordingly, inhibition of TAG mobilization by Orlistat during carbon starvation conditions was confirmed by TLC (Fig. 4b).

TAG may serve as a source of intermediates and/or reducing power in rhodococci for the biosynthesis of diverse compatible solutes during osmotic stress (Alvarez et al. 2004). Taking this into account, a likely role of TAG on osmotic stress tolerance of strain A5 was tested. Cells were pre-cultivated in MSM0.1 plus glucose to promote TAG accumulation, harvested and washed, and finally resuspended in MSM1 plus glucose in the presence of 2 % (w/v) of NaCl. In these conditions, a significant decrease in CFU counts \((p < 0.05)\) from both treatments (with and without Orlistat) in comparison to the control not exposed to saline stress was observed during the exponential, but not in the stationary growth phase (Fig. 4c), despite that inhibition of TAG mobilization by Orlistat under saline stress was observed in both, exponential and stationary phase (Fig. 4d). It was clear that the inhibition of TAG

### Table 1

Lipid content in strains A5 WT, A5 WH and R. corynebacteroides DSM 20151 after cultivation on different carbon sources

| Bacterial and carbon source | Growth phase | PHA (%w/w) | Fatty acids (%w/w) |
|-----------------------------|--------------|------------|--------------------|
| **Rhodococcus sp. A5 WT**   | Exponential  | 2.9        | 11.0               |
| Glucose 1 %                 | Stationary   | 2.0        | 17.6               |
| Hexadecane 0.1 %            | Exponential  | 1.3        | 32.1               |
|                            | Stationary   | 1.2        | 28.3               |
| **Rhodococcus sp. A5 WH**   | Exponential  | 2.8        | 9.4                |
| Glucose 1 %                 | Stationary   | 2.4        | 11.5               |
| Hexadecane 0.1 %            | Exponential  | 1.9        | 35.3               |
|                            | Stationary   | 1.4        | 21.3               |
| **R. corynebacteroides DSM 20151** | Exponential | 2.4        | 9.2                |
| Glucose 1 %                 | Stationary   | 2.9        | 3.8                |
| Hexadecane 0.1 %            | Exponential  | 0.5        | 17.9               |
|                            | Stationary   | 1.9        | 13.7               |
Fig. 4  Effect of Orlistat (inhibitor of TAG degradation) on the response of the extremophile *Rhodococcus* sp. A5 to carbon starvation (a, b), osmotic stress (c, d), UVR (e, f) and desiccation conditions (g, h). a, c, e, g CFU counts; b, d, f, h TLC analysis showing TAG contents of cells.
mobilization resulted in a reduction of A5 cells number under osmotic stress during exponential growth phase, since significant differences in CFU counts (ca. 10 %) in comparison with cells cultivated in the absence of the inhibitor (Fig. 4c, d) were observed.

The influence of TAG in the resistance of A5WT cells to UVR was tested by assessing the survival rates and TAG accumulation after treatment with or without Orlistat. Indeed, TAG mobilization seems to be necessary to endure the UV radiation exposure (Fig 4e). Although UVR treatment negatively affected cell survival in approximately 3 magnitude orders, cells treated with Orlistat were significantly more UV-sensitive than untreated cells (ca. 25 %) (Fig. 4e, f). Since this experiment involved a short-time challenge treatment of cells (90 min), the difference between TAG contents in cells treated with or without Orlistat was not evident after TLC analysis (Fig. 4f).

Finally, the influence of TAG in desiccation tolerance by strain A5WT was assessed by comparing survival of TAG-accumulating cells subjected to desiccation with or without Orlistat. A higher percentage of cells survived after 7 days of desiccation (approximately 79 % of cell survival) when cells were able to mobilize accumulated TAG (Fig. 4g). In contrast, no surviving cells were observed when TAG degradation was inhibited in cells cultivated under the same conditions (Fig. 4g, h).

Discussion

Among twenty extremophiles isolated from high-altitude Andean Lakes, twelve produced variable amounts of triacylglycerols (TAG) and/or wax esters (WS) (between 2 and 17 %, CDW) under nitrogen-limiting culture conditions, suggesting that accumulation of storage lipids is a widespread feature in such extreme environments. The most fitted strain for accumulating lipids was the UV-resistant Rhodococcus sp. A5, which was fully characterized in this study. Interestingly, A5WT proved to rely on TAG mobilization for successfully overcoming four types of stress: i.e., carbon starvation, osmotic stress, UV-radiation and desiccation, suggesting that these lipids play an important role in balancing cell metabolism in its original extreme environment.

TAG mobilization seemed to be necessary but not essential for the adaptation of strain A5WT cells to carbon starvation, since the inhibition of TAG degradation affected survival of cells under this stress condition. However, the effect of TAG degradation inhibition may be compensated to some extent by the endogenous use of additional storage compounds such as PHA or glycogen. In a previous study, we demonstrated that the production of glycogen is a common feature among rhodococci (Hernández and Alvarez 2010). R. opacus PD630 is able to mobilize storage lipids during carbon starvation (Alvarez et al. 2000), as well as under desiccation conditions (Alvarez et al. 2004). TAG/WS may serve as an endogenous energy source or as a source of carbon and intermediates for the biosynthesis of compounds needed for survival of cells under different stress conditions; such as compatible solutes, extracellular polymeric substances, lipids of the cellular envelope, among others. Similar functions have been proposed for PHA and glycogen in bacteria, which allow the successful adaptation to sudden carbon starvation (de Eugenio et al. 2010; Kaduri et al. 2005; Wang and Wise 2011).

Under saline stress, rhodococcal cells may achieve an osmotic adjustment mostly during the initial stages, which may include the synthesis of compatible solutes, among other processes. Previous studies reported the ability of R. opacus PD630 and R. jostii RHA1 to produce a diversity of osmolytes, such as trehalose, ectoine and hydroxyectoine, during desiccation conditions (Alvarez et al. 2004; LeBlanc et al. 2008). In this study, the inhibition of TAG mobilization by A5WT cells when cultivated under osmotic stress conditions produced a decrease of CFU counts in comparison to cells grown in the absence of the inhibitor. This effect was observed only during the first hours of cultivation, whereas no differences in CFU counts were detected in the late stage of cultivation. These results suggested that TAG mobilization may participate in the metabolic and osmotic adjustment of A5WT cells during the initial stages of saline stress that usually occur in the HAAL environments. However, TAG degradation seemed to be not essential for the adaptation of A5WT cells to osmotic stress.

On the other hand, the inhibition of TAG mobilization by the addition of Orlistat produced a dramatic effect on the response of A5WT cells to UV radiation and principally to desiccation. UV radiation causes a different but overlapping type of damage. UV-A radiation causes indirect damage to cellular DNA, proteins and lipids, by catalyzing the intracellular formation of chemical intermediates such as reactive oxygen species (ROS). In contrast, UV-B radiation causes direct DNA damage by inducing the formation of DNA photoproducts (Agogué et al. 2005). Since UV radiation increases by 19 % for every 1,000 m of altitude, bacteria living in high-altitude environments like Rhodococcus sp. A5 are expected to show a wide range of resistance mechanism to this challenge (Fernandez Zenoff et al. 2006). In our study, UV treatment was made by dark recuperation where the principal mechanisms of defense are the same to oxidative stress: catalases, peroxidases and superoxide dismutase enzymes, among other. Decrease in count of CFU in the presence of UVR and the inhibition of TAG degradation may indicate that TAG are contributing to withstand this stress. The degradation of fatty acids from
TAG may contribute to the increase of NADPH generation in cells. The effectiveness of oxidative stress-protecting enzymes, as the scavengers of ROS, depends on the availability of NADPH and a reductive environment promoted by this cofactor. Thus, a normal functioning cell should have adequate levels of NADPH and a small amount of NADH (Singh et al. 2007). In this sense, the impossibility of strain A5_wt to degrade TAG by the addition of Orlistat may generate an imbalance of the NADPH/NADH ratio, which affects the functionality of defense mechanisms against oxidative stress.

Cells of Rhodococcus sp. A5 were not able to survive under desiccation conditions when TAG degradation was inhibited by the lipase inhibitor. Similar results were obtained when cells of R. opacus PD630 were incubated under desiccation conditions after treatment with acrylic acid, which inhibited the β-oxidation pathway (Alvarez et al. 2004). These results indicated that TAG mobilization and β-oxidation pathway (lipid metabolism) remain active during desiccation in rhodococcal cells. Desiccation is a complex process enclosing more than one stress, including starvation, oxidative, osmotic and hydric stresses, among others. The accumulation of TAG may be one of the key features that allow rhodococci to withstand arid environments. In this context, TAG may be required for the maintenance of cellular viability and integrity during desiccation. The biosynthesis and accumulation of TAG before the stress may be an important survival factor in these microorganisms and necessary for the successful adaptation of cells to desiccation (LeBlanc et al. 2008). The turnover of these storage lipids may permit cells to remain metabolically active and viable for prolonged periods of time under desiccation conditions. Internal storage lipids may serve not only for generating energy, but also as source of precursors for the biosynthesis and/or turnover of essential lipids, such as membrane lipids and lipids from the cellular envelope as mycolic acids, and for generating metabolic water and NADPH, to maintain a reductive environment in cells, by degradation of fatty acids.

The results presented in this study highlight the role of TAG as part of a strategy on a model lipid-accumulating actinobacterium to cope with the extreme conditions found in the HAAL environments. The production of TAG may be part of complex strategic survival mechanisms in HAAL microbes which allow them to colonize and thrive in extreme environments, especially when multiple stresses occur simultaneously. Further work is being conducted to elucidate metabolic pathways related with this interesting ability, which otherwise offers a great biotechnological potential.

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