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Susana Bequer Urbano, Virginia H. Albarracín, Omar F. Ordoñez, María E. Farías & Héctor M. Alvarez

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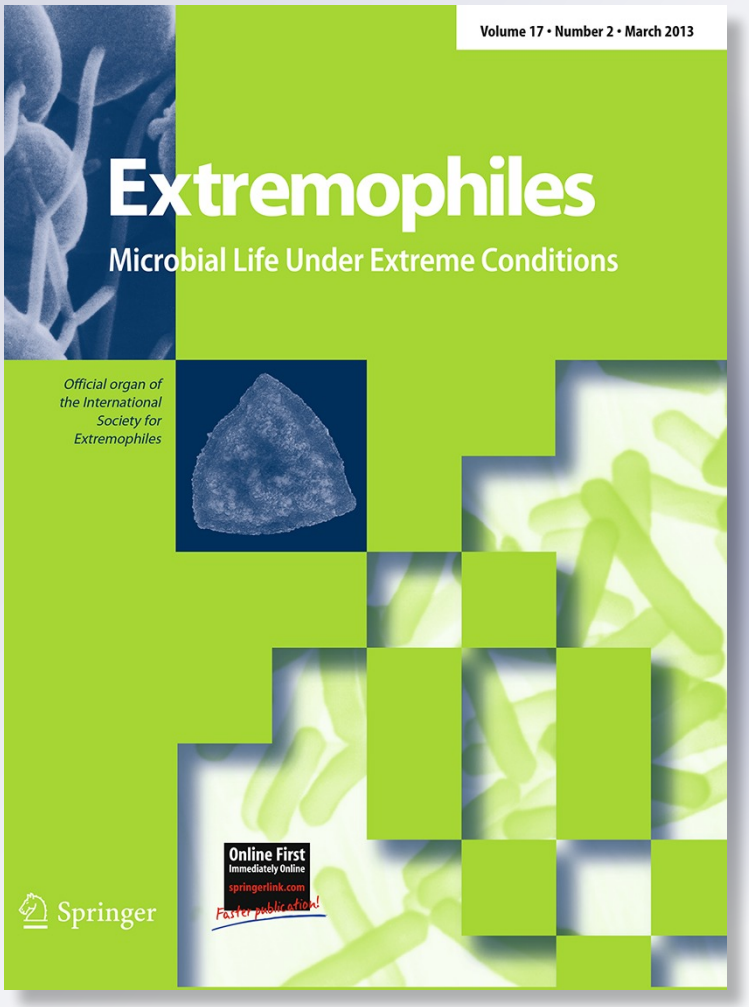

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ORIGINAL PAPER

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*

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S. Bequer Urbano · H. M. Alvarez (✉)
Facultad de Ciencias Naturales, Centro Regional de Investigación Y Desarrollo Científico Tecnológico (CRIDECIT), Universidad Nacional de la Patagonia San Juan Bosco, Km 4-Ciudad Universitaria, 9000 Comodoro Rivadavia, Chubut, Argentina
e-mail: halvarez@unpata.edu.ar

V. H. Albarracín · O. F. Ordoñez · M. E. Farías
Laboratorio de Investigaciones Microbiológicas de Lagunas Andinas (LIMLA), PROIMI Planta Piloto de Procesos Industriales Microbiológicos, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. Belgrano y Pasaje Caseros, 4000 San Miguel de Tucumán, Argentina

V. H. Albarracín
Facultad de Ciencias Naturales e Instituto Miguel Lillo, Universidad Nacional de Tucumán, 4000 San Miguel de Tucumán, Tucumán, Argentina

V. H. Albarracín
Max-Planck-Institute for Chemical Energy Conversion, Stiftstrasse 34-36, 45470 Mülheim, Germany

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 (~Δ30 °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 *Streptomyces* genera (Alvarez 2003; Alvarez et al. 1996, 1997b; Arabolaza et al. 2008; Daniel et al. 2004; Olukoshi 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 compound 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 $A5_{WT}$ (red-original strain) while the mutant was called as $A5_{WH}$ (white-mutant strain). $A5_{WT}$ 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 OD_{600nm} of 1.3 in sterile NaCl solution. Cell suspension was divided in 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 OD_{600} 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 methanolysis 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 (Albarracín 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 critic drying point was obtained by exchanging the acetone through liquid CO_2. The samples were covered by gold and visualized using a Zeiss Supra 55VP (Carl Zeiss NTS GmbH, Germany) scanning electron microscope (LAMENOA-CCT-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 on ISP 2 media. Resistance profile to increasing NaCl concentrations (0, 2.5, 5, 7.5 and 10 %) was tested; for this purpose, A5 strain was grown on ISP 2 broth at 30 °C. The described procedures were similarly performed using the $A5_{WH}$ mutant.

Resistance profile in UVR was tested on LB agar. For this purpose, mid-exponential phase cells were harvested in the mid-exponential phase by centrifugation at 8,000 rpm for 30 min at 4 °C. The pellets were washed twice in 0.9 % NaCl and cells were diluted in NaCl 0.9 % solution till an OD_{600} of 0.6 was reached. Serial dilutions were performed in NaCl 0.9 % solution and 5-μl aliquots of each dilution were inoculated on LB agar plates. The inoculated plates were immediately exposed to UV-B irradiation (Vilbert Lourmat VL-4; maximum intensity at 312 nm) for 15 min (15 kJ m^{-2}), 30 min (30 kJ m^{-2}), and 45 min (45 kJ m^{-2}), and were then incubated in the dark to prevent photoreactivation for 72 h at 30 °C. UV-B irradiance was quantified with a radiometer (Vilbert Lourmat model VLX-3W) coupled with a UV-B sensor (Vilbert Lourmat model CX-312). The minimal intensity measured was 0.244 mW cm^{-2} while maximal intensity was 0.282 mW cm^{-2}. Controls of unexposed samples were run simultaneously in darkness. For comparison, the described procedures were similarly performed using the $A5_{WH}$ mutant and the closest taxonomical strains *Rhodococcus corynebacteroides* DSM 20151 and *Rhodococcus kroppenstedtii* DSM 44908. Microbial growth was recorded with four positive signs (++++) when it was similar to the growth in the dark, with three positive signs (+++) when it was slightly different from the growth in the dark, with two positive signs (++) when it was significantly different from the growth in the dark, with one positive sign (+) when the growth was too low that allowed colony counting, with a negative sign (−) when it was no growth at all. For plotting the data depicted in Fig. 3, four, three, two or one signs where taken as 4, 3, 2 or 1 units of growth for each dilution and then summed up together to reach a unique value. Negative signs counted as null unit of growth.

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 dideoxy 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. *Milisia 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_f 0.73) and TAG (R_f 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 Pseudonocardiaceae (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 kroppenstedii* 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 $A5_{WT}$ (red-original strain) and $A5_{WH}$ (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 $A5_{WT}$, $A5_{WH}$, DSM 20151, DSM 44908 were tested on LB agar inoculated with serial dilutions (10^{-1}–10^{-5}) of an initial cell suspension and exposed to increasing doses of UV-B irradiation. $A5_{WT}$ was the most resistant strain, as it endures more successfully up to the highest irradiation dose (Fig. 3). Its mutant, $A5_{WH}$, 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^2). 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. $A5_{WT}$ was able to accumulate TAG and PHA from glucose as well as from hexadecane, as sole carbon sources, although PHA were found in minor

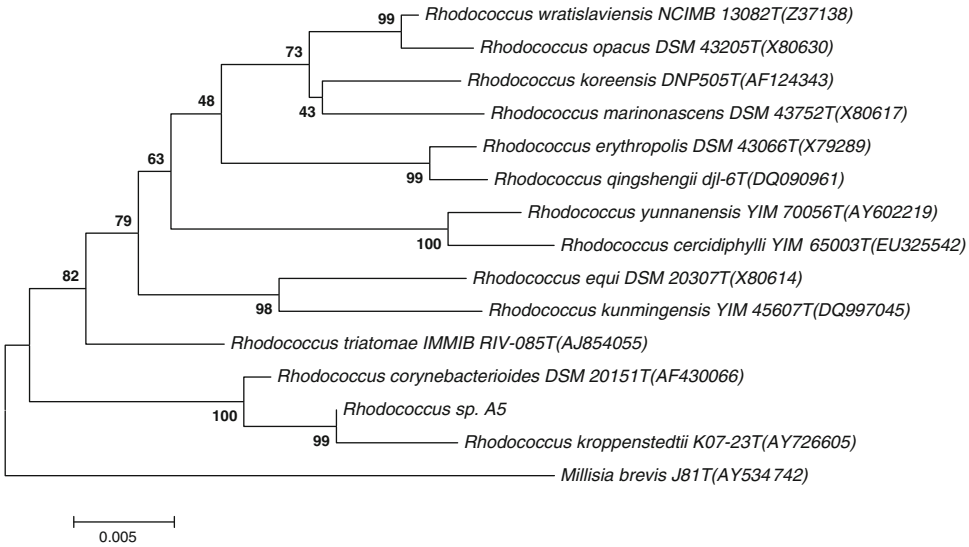

Fig. 1 Evolutionary relationships of 16S rARN sequences from strain A5 and closest 15 organisms within the genus *Rhodococcus* were inferred using the neighbor-joining method (Saitou and Nei, 1987). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances 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, using the maximum composite likelihood method (Tamura et al. 2004) and 500 resamplings for bootstrap analyses. All studies were carried out with the MEGA5 Program (Tamura et al. 2011)

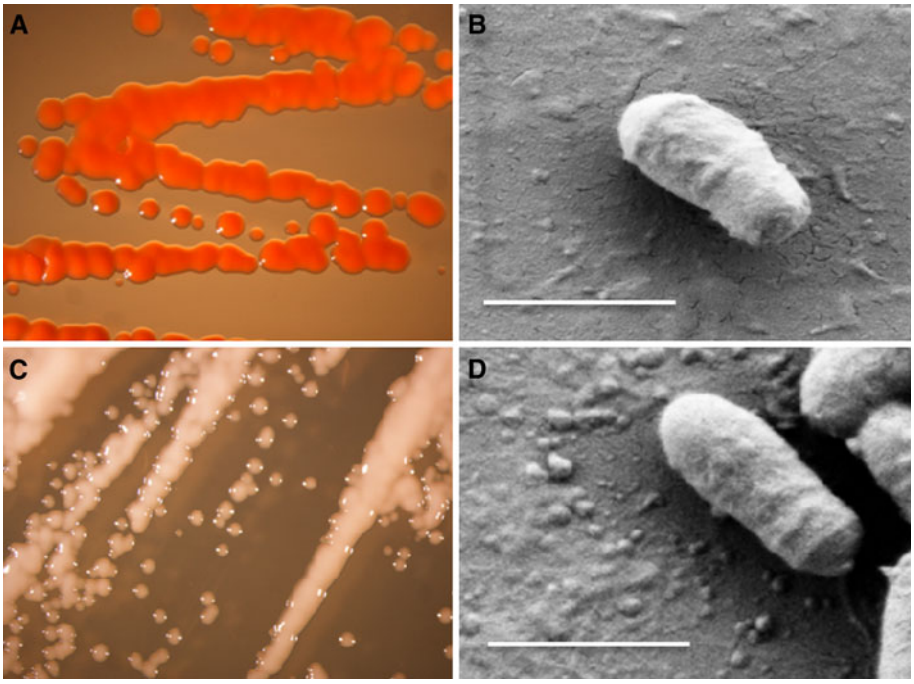

Fig. 2 Stereoscopic (**a**, **c**) and SEM (**b**, **d**) images of wildtype (**a**, **b**) and mutant (**c**, **d**) strains of *Rhodococcus* sp. A5. The *scale bars* correspond to 1 μm

quantities than TAG in all cases (Table 1). Similar behavior was observed for the mutant strain $A5_{WH}$ 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 $A5_{WT}$, 1.8-fold more for $A5_{WH}$ and twofold more for DSM 20151 in stationary phase (Table 1). Under the same culture conditions, the extremophilic 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.

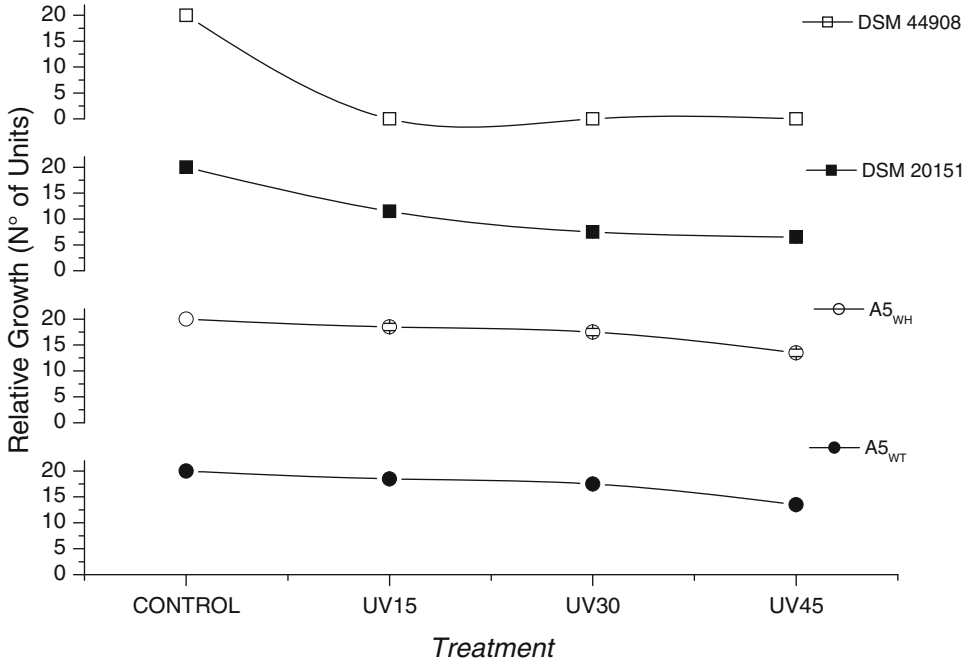

Fig. 3 Resistance profile in UVR of $A5_{WT}$, $A5_{WH}$, DSM 44908 and DSM 20151 strains were tested on LB agar inoculated with serial dilutions (10^{-1}–10^{-5}) of an initial cell suspension and exposed to UV-B irradiation for 15 min (15 kJ m^{-2}), 30 min (30 kJ m^{-2}), and 45 min (45 kJ m^{-2})

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. $A5_{WT}$			
Glucose 1 %	Exponential	2.9	11.0
	Stationary	2.0	17.6
Hexadecane 0.1 %	Exponential	1.3	32.1
	Stationary	1.2	28.3
Rhodococcus sp. $A5_{WH}$			
Glucose 1 %	Exponential	2.8	9.4
	Stationary	2.4	11.5
Hexadecane 0.1 %	Exponential	1.9	35.3
	Stationary	1.4	21.3
R. corynebacteriodes DSM 20151			
Glucose 1 %	Exponential	2.4	9.2
	Stationary	2.9	3.8
Hexadecane 0.1 %	Exponential	0.5	17.9
	Stationary	1.9	13.7

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

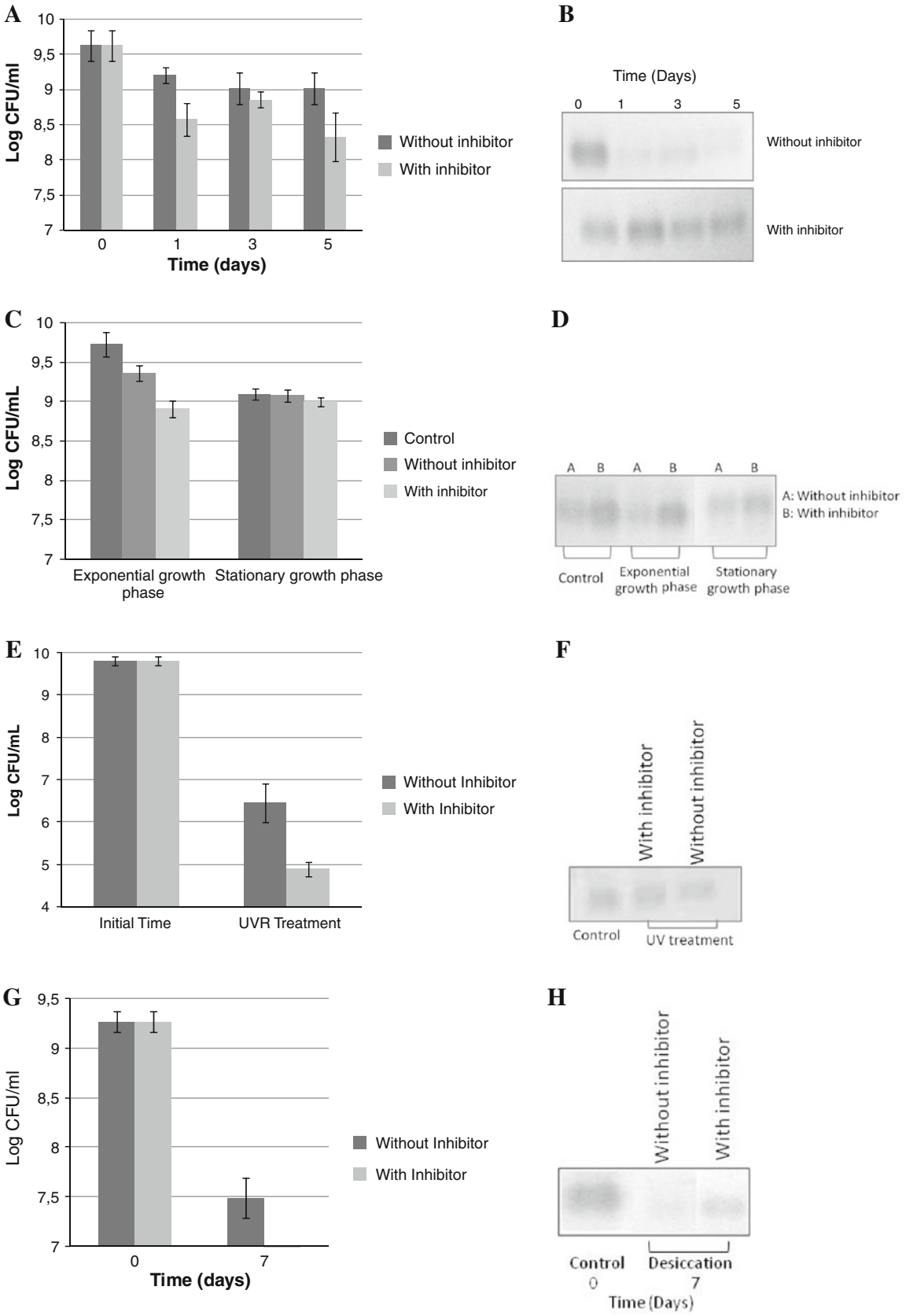

Fig. 4 Effect of Orlistat (inhibitor of TAG degradation) on the response of the extremophile *Rhodococcus* sp. A5 to carbon starvation (**a**, **b**), osmotic stress (**c**, **d**), UVR (**e**, **f**) and desiccation conditions (**g**, **h**). **a**, **c**, **e**, **g** CFU counts; **b**, **d**, **f**, **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 $A5_{WT}$ 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 $A5_{WT}$ 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, $A5_{WT}$ 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 $A5_{WT}$ 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 $A5_{WT}$ 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 $A5_{WT}$ 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 $A5_{WT}$ 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 $A5_{WT}$ 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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References

Agogué H, Joux F, Obernosterer I, Lebaron P (2005) Resistance of marine bacterioneuston to solar radiation. Appl Environ Microbiol 71:5282–5289

Albarracin VH, Alonso-Vega P, Trujijjo ME, Amoroso MJ, Abate CM (2010) *Amycolatopsis tucumanensis* sp nov., a copper-resistant actinobacterium isolated from polluted sediments. Int J Syst Evol Microbiol 60:397–401

Albarracín VH, Pathak GP, Douki T, Cadet J, Borsarelli CD, Gärtner W, Farías ME (2012) Extremophilic *Acinetobacter* strains from high-altitude lakes in Argentinean Puna: remarkable UV-B resistance and efficient DNA damage repair. Orig Life Evol Biosph 42:201–221

Alvarez HM (2003) Relationship between β-oxidation pathway and the hydrocarbon-degrading profile in actinomycetes bacteria. Inter Biodeter Biodegr 52:35–42

Alvarez HM (2006) Bacterial triacylglycerols. In: Welson LT (ed) Triglycerides and cholesterol research. Nova Science Publishers Inc., New York, pp 159–176

Alvarez HM, Steinbüchel A (2002) Triacylglycerols in prokaryotic microorganisms. Appl Microbiol Biotechnol 60:367–376

Alvarez HM, Steinbüchel A (2010) Physiology, biochemistry and molecular biology of triacylglycerol accumulation by *Rhodococcus*. In: Alvarez HM (ed) Biology of *Rhodococcus*., Microbiology monographs seriesSpringer, Berlin, pp 263–290

Alvarez HM, Mayer F, Fabritius D, Steinbüchel A (1996) Formation of intracytoplasmic lipid inclusion by *Rhodococcus opacus* PD630. Arch Microbiol 165:377–386

Alvarez HM, Kalscheuer R, Steinbüchel A (1997a) Accumulation of storage lipids in species of *Rhodococcus* and *Nocardia* and effect of inhibitors and polyethylene glycol. Fett/Lipid 99:239–246

Alvarez HM, Pucci OH, Steinbüchel A (1997b) Lipid storage compounds in marine bacteria. Appl Microbiol Biotechnol 47:132–139

Alvarez HM, Kalscheuer R, Steinbüchel A (2000) Accumulation and mobilization of storage lipids by *Rhodococcus opacus* PD630 and *Rhodococcus ruber* NCIMB 40126. Appl Microbiol Biotechnol 54:218–223

Alvarez HM, Silva RA, Cesari AC, Zamit AL, Peressutti SR, Reichelt R, Keller U, Malkus U, Rasch C, Maskow T, Mayer F, Steinbüchel A (2004) Physiological and morphological responses of the soil bacterium *Rhodococcus opacus* strain PD630 to water stress. FEMS Microbiol Ecol 50:75–86

Arabolaza A, Rodriguez E, Altabe S, Alvarez HM, Gramajo H (2008) Multiple pathways for triacylglycerol biosynthesis in *Streptomyces coelicolor*. Appl Environ Microbiol 74:2573–2582

Daffonchio D, Borin S, Frova G, Manachini PL, Sorlini C (1998) PCR fingerprinting of whole genomes: the spacers between the 16S and 23S rRNA genes and of intergenic tRNA gene regions reveal a different intraspecific genomic variability of *Bacillus*

cereus and *Bacillus licheniformis*. Int J Syst Bacteriol 48:107–116

Daniel J, Deb C, Dubey VS, Sirakova TD, Abomoelak B, Morbidoni HR, Kolattukudy PE (2004) Induction of a novel class of diacylglycerol acyltransferases and triacylglycerol accumulation in *Mycobacterium tuberculosis* as it goes into a dormancy-like state in culture. J Bacteriol 186:5017–5030

de Eugenio LI, Escapa IF, Morales V, Dinjaski N, Galán B, García JL, Prieto MA (2010) The turnover of medium-chain-length polyhydroxyalkanoates in *Pseudomonas putida* KT2442 and the fundamental role of PhaZ depolymerase for the metabolic balance. Environ Microbiol 12:207–221

Di Capua C, Bortolotti A, Farías ME, Cortez N (2011) Ultraviolet-resistant *Acinetobacter* sp. isolates from Andean Wetlands display high catalase activity. FEMS Microbiol Lett 317:181–189

Dib JR, Weiss A, Neumann A, Ordoñez O, Estévez MC, Farías ME (2009) Isolation of bacteria from remote high altitude Andean lakes able to grow in the presence of antibiotics. Recent Pat Antiinfect Drug Discov 4:66–76

Doetsch RN (1981) Determinative methods of light microscopy. In: Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Phillips GB (eds) Manual of methods for general bacteriology. American Society for Microbiology, Washington, pp 21–33

Fernandez Zenoff V, Ziñeriz F, Farías ME (2006) Diverse responses to UV-B radiation and repair mechanisms of bacteria isolated from high-altitude aquatic environments. Appl Environ Microbiol 72:7857–7863

Flores MR, Ordoñez FO, Farías ME (2009) Isolation of UV-B resistant bacteria from two Andean wetlands (4,400 m) with saline and hypersaline conditions. J Appl Gen Microbiol 55:447–458

Hernández MA, Alvarez HM (2010) Glycogen formation by *Rhodococcus* species and the effect of inhibition of lipid biosynthesis on glycogen accumulation in *Rhodococcus opacus* PD630. FEMS Microbiol Lett 312:93–99

Heuer H, Krsek M, Baker P, Smalla K, Wellington EMH (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl Environ Microbiol 63:3233–3241

Holtzapple E, Schmidt-Dannert C (2007) Biosynthesis of isoprenoid wax ester in *Marinobacter hydrocarbonoclasticus* DSM 8798: identification and characterization of isoprenoid coenzyme A synthetase and wax ester synthases. J Bacteriol 189:3804–3812

Kadouri D, Jurkevitch E, Okon Y, Castro-Sowinski S (2005) Ecological and agricultural significance of bacterial polyhydroxyalkanoates. Crit Rev Microbiol 31:55–67

Kalscheuer R, Stöveken T, Malkus U, Reichelt R, Golyshin PN, Sabirova JS, Ferrer M, Timmis KN, Steinbüchel A (2007) Analysis of storage lipid accumulation in *Alcanivorax borkumensis*: evidence for alternative triacylglycerol biosynthesis routes in bacteria. J Bacteriol 189:918–928

LeBlanc JC, Gonçalves ER, Mohn WW (2008) Global response to desiccation stress in the soil actinomycete *Rhodococcus jostii* RHA1. Appl Environ Microbiol 74:2627–2636

Low KL, Rao PS, Shui G, Bendt AK, Pethe K, Dick T, Wenk MR (2009) Triacylglycerol utilization is required for regrowth of in vitro hypoxic nonreplicating *Mycobacterium bovis* bacillus Calmette-Guerin. J Bacteriol 191:5037–5043

Makula RA, Lockwood PJ, Finnerty WR (1975) Comparative analysis of the lipids of *Acinetobacter* species grown on hexadecane. J Bacteriol 121:250–258

Olukoshi ER, Packter NM (1994) Importance of stored triacylglycerols in *Streptomyces*: possible carbon source for antibiotics. Microbiology 140:931–943

Ordoñez OF, Dib JR, Flores MR, Farías ME (2009) Extremophile culture collection from Andean lakes. Extreme pristine environments that host a wide diversity of microorganisms with tolerance to UV radiation. Microb Ecol 58:461–473

Rontani JF, Bonin PC, Volkman JK (1999) Production of wax esters during aerobic growth of marine bacteria on isoprenoid compounds. Appl Environ Microbiol 65:221–230

Saitou N, Nei M (1987) The neighbour-joining method: a new method for constructing phylogenetic trees. Mol Biol Evol 4:406–425

Schlegel HG, Kaltwasser H, Gottschalk G (1961) Ein Submersverfahren zur Kultur wasserstoffoxidierender Bakterien: wachstumsphysiologische Untersuchungen. Arch Mikrobiol 38:209–222

Seufferheld M, Alvarez HM, Farías ME (2008) Polyphosphates as microbial modulators of environmental stress. Appl Environ Microbiol 74:5867–5874

Singh R, Mailloux R, Puiseux-Dao S, Appanna V (2007) Oxidative stress evokes a metabolic adaptation that favors increase NADPH synthesis and decrease NADH production in *Pseudomonoas fluorescens*. J Bacteriol 189:6665–6675

Steinbüchel A (1991) Polyhydroxyalkanoic acids. In: Byrom D (ed) Biomaterials. MacMillan, London, pp 123–213

Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA 101:11030–11035

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Thompson JD, Higgins D, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Wang L, Wise MJ (2011) Glycogen with short average chain length enhances bacterial durability. Naturwissenschaften 98:719–729