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Article type : Original Article

Insights into the mechanisms of desiccation resistance of the Patagonian PAH-degrading strain *Sphingobium* sp. 22B

Headline: Desiccation resistance in *Sphingobium* sp. 22B

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Summary

Aim: To analyze the physiological response of *Sphingobium* sp. 22B to water stress.

Methods and results: The strain was grown under excess of carbon source and then submitted to low (60RH) and high (18RH) water stress conditions for 96 h. Quantification of trehalose, glycogen, polyhydroxybutyrate (PHB), and transmission electron microscopy (TEM) was studied. Genes linked with desiccation were searched in *Sphingobium* sp. 22B and *Sphingomonas* "*sensu latu"* genomes and their transcripts were quantified by Real-Time PCR. Results showed that, in absence of water stress, strain 22B accumulated 4.76± 1.41% of glycogen, 0.84± 1.62% of trehalose and 44.9± 6.4% of PHB per cellular dry weight. Glycogen and trehalose were mobilized in water stresses conditions, this mobilization was significantly higher in 60RH in comparison to 18RH. Gene *treY* was upregulated 6-fold change in 60RH relative to 18RH. TEM and quantification of PHB revealed that PHB was mobilized under 60RH condition accompanied by the downregulation of the *phbB* gene. TEM images showed an extracellular amorphous matrix in 18RH and 60RH. Major differences were found in the presence of *aqpZ* and trehalose genes between strain 22B and *Sphingomonas* genomes.

Conclusion: Strain 22B showed a carbon conservative metabolism capable of accumulation of three types of endogenous carbon sources. The strain responds to water stress by changing the expression pattern of genes related with desiccation, formation of an extracellular amorphous matrix and mobilization of the carbon sources according to the degree of water stress. Trehalose, glycogen and PHB may have multiple functions in different degrees of desiccation. The robust endowment of molecular responses to desiccation shown in *Sphingobium* sp. 22B could explain its survival in semiarid soil.

Significance and Impact of the study: Understanding the physiology implicated in the toleration of the PAH-degrading strain *Sphingobium* sp 22B to environmental desiccation may improve the bioaugmentation technologies in semiarid hydrocarbons contaminated soils.

Key words: PAH-degrading *Sphingobium*; *Sphingobium* sp 22B; desiccation; water stress; Patagonia; PHB; glycogen; trehalose

Introduction

Patagonia is a semiarid scrub plateau located at the southern end of South America, shared by Argentina and Chile. It constitutes a vast area of steppe and desert that extends south from latitude 37° to 51° S occupying 673,000 square kilometers. This region has low precipitation concentrated in winter, strong water deficits in spring and summer, and persistent and intense western winds (Paruelo *et al.* 1998).

Indigenous microorganisms are regularly exposed to desiccation, this being the limiting factor of life in semiarid Patagonia. Desiccation in cells causes DNA and protein destabilization by oxidation and browning reactions (Potts and Webb 1994), and a transition to the gel phase in cell membranes (Ramos *et al.* 2001). Several stresses occur simultaneously during desiccation, since in this process cells are losing intracellular water which also generates carbon starvation, osmotic and oxidative stresses. Therefore, the specific and common factors for each of these stresses are difficult to determine (Ramos *et al.* 2001).

Resistance mechanisms for environmental factors have been studied extensively in different bacteria genera. In *Bacillus subtilis*, *E. coli* and other Gram negative bacteria, a general response to stress has been described overlapping the osmotic shock responses, responses to starvation, and desiccation tolerance, where the same mechanism is induced in these

unfavorable conditions. This mechanism involved the expression of *rpoS* gene, which encodes a putative sigma factor (σ^S) that confers to RNA polymerase the affinity for specific promoters of genes associated with adverse environmental conditions (Helmann and Chamberlin 1991). The increase tolerance of microorganisms to desiccation has been linked with the ability to synthesize compatible solutes (Crowe *et al.* 1987; Welsh 2000; Alvarez *et al.* 2004), sporulation process in *Bacillus* (Setlow 1995), modifications in the composition of fatty acids in the biological membranes (Halverson and Firestone 2000) and exopolysaccharides synthesis (Roberson and Firestone 1992; Ophir and Gutnick 1994). The formation of biofilms (Tribelli and López 2011) and the accumulation of endogenous carbon sources, such as polyhydroxyalkanoates (PHA) (Matin *et al.* 1979; Ayub *et al.* 2004), have also been associated with the ability to survive and tolerate unfavorable environmental conditions like desiccation.

The *Sphingobium* sp. strain 22B was isolated in a previous study from hydrocarbon contaminated soil in the semiarid Patagonia (Madueño *et al.* 2011). The persistence exhibited by strain 22B during inoculation in autochthonous bioaugmentation technology (Madueño *et al.* 2015) demonstrated its ability to tolerate the environmental desiccation of the region.

Microorganisms of the genus *Sphingomonas* are Gram negative, rod-shaped, chemoheterotrophic and strictly aerobic bacteria broadly distributed in nature. *Sphingomonas ("sensu latu")* present four subgenera proposed by Takeuchi *et al.* (2001) called *Sphingobium*, *Novosphingobium*, *Sphingopyxis* and *Sphingomonas ("sensu stricto")*. This genus comprises PAH-degrading microorganisms present in soil, which are frequently isolated from PAH-enriched cultures (Baraniecki *et al.* 2002; Festa *et al.* 2013). *Sphingomonas* species are characterized by their large catabolic diversity, being able to degrade a wide range of natural and anthropogenic aromatic compounds (Stolz 2009). Members of this group are broadly distributed in nature and have been isolated from a variety

of environments such as pristine (Lin *et al.* 2012) and contaminated (Vacca *et al.* 2005) soil, in cold (Margesin *et al.* 2012), extreme (Farias *et al.* 2011) and arid environments (Reddy and Garcia-Pichel 2007). Although there are some reports on physiology and responses to environmental factors in bacteria of the genus *Sphingomonas* (Fegatella and Cavicchioli 2000; Fida *et al.* 2012), little is known about the responses used by these microorganisms against the fluctuating availability of water in semi-arid Patagonia.

In this work, we researched some physiological properties that allow strain 22B to survive to the environmental conditions of Patagonia. The occurrence in strain 22B of mechanisms involved in the tolerance of cells to desiccation reported for other bacteria, such as the ability to produce reserve compounds, compatible solutes, and extracellular polymeric substances (EPS) were examined. In addition, the recently obtained genomic information of strain 22B (Madueño *et al.* 2016) provided the possibility of analyzing the occurrence, expression and distribution of key genes involved in those physiological processes.

MATERIALS AND METHODS

Culture conditions

Sphingobium sp. 22B (DDBJ/ENA/GenBank under the accession number LTAB00000000) was grown in liquid mineral medium (LMM) with 1 % glucose at 28 °C and 150 rpm for 48 h. The concentration of ammonium chloride in LMM was reduced to 0.1 g l^{-1} to allow the accumulation of reserve compounds (5 g l^{-1} ClNa, 1 g l^{-1} K_2PO_4H, 1.7 g l^{-1} $(NH_4)_2H_2PO_4$, 0.1 g l^{-1} $(NH_4)_2SO_4$, 0.2 g l^{-1} SO_4Mg). Cells were harvested at 48 h, washed with NaCl solution (0.85 %, w/v) and filtered or lyophilized for subsequence analyses.

Water stress resistance test in *Sphingobium* sp. 22B

Sphingobium sp. 22B was cultivated for 48 h with an excess of carbon source (culture conditions) in 3 Erlenmeyer flasks. Total culture volume (400 ml) of each flask was filtered in sterile conditions in fractions of 20 ml onto 0.45 μm and 60 mm pore-size nitrocellulose filters (Millipore) by a vacuum pump. The filters with harvested cells on their surface were submitted to the following conditions:

- High water stress Condition (18RH): 20 filters were placed separately in independent sterile petri dishes and laid together into desiccator at 18 % of relative humidity (RH). RH was measured with a thermohygrometer with Max/Min function (Gesa) for 96 h at 28°C. Finally, all filters were taken by tweezer in sterilized conditions and resuspended in 400 ml of PS. Different fractions of this volume were used for cell survival determination, extraction and quantification of intracellular PHB, glycogen and trehalose, and for transmission electron microscopy (see below).
- Low water stress Condition (60RH): 20 filters were placed separately in independent sterile petri dishes and, afterwards, all of them placed together into a container at 60 % RH measured with a thermohygrometer with Max/Min function (Gesa) for 96 h at 28°C. Finally, all filters were taken by tweezer in sterilized conditions and resuspended in 400 ml of PS. Different fractions of this volume were used for cell survival determination, extraction and quantification of intracellular PHB, glycogen and trehalose, and for transmission electron microscopy.
- Control: 20 filters were obtained and resuspended all together immediately in 400 ml sterile physiological solution (PS) without stress condition.

Independent Erlenmeyer flasks were used for each condition. The assay was performed in triplicate.

Determination of cell survival

The survival of the strain 22B in each condition was performed by viable count (cfu) with R2 medium (Reasoner and Geldreich 1985) using 1/10 serial dilutions of 1ml of cell suspension obtained after water stress treatments. Survival rates were calculated as [log (cfu in low (60RH) or high (18RH) water stress condition)] / [log (cfu in the control condition)] x100 (Alvarez *et al.* 2004). The percentage of lost water was calculated weighting filters before and after being subjected to stress condition.

Transmission Electron Microscopy

1.5 ml of cell suspension in PS was spun down at 800 g for 5 min and fixed in 2 % glutaraldehyde in phosphate buffer 0.2 M (pH 7.2-7.4) for 2 h at 4 °C. Secondary fixation was performed using osmium tetroxide 1 % for 1 h at 4 °C and subsequently, the samples were dehydrated in a growing series of alcohols and embedded in epoxy resin. Ultrathin sections (90 nm) were contrasted with uranyl acetate and lead citrate and 10 to 15 fields were examined in each condition in a transmission electron microscope JEM 1200 EX II (JEOL Ltd., Tokyo, Japan) and photographed with a camera Erlangshen ES1000W, Model 785 (Gatan Inc., Pleasanton, California, USA) in Servicio Central de Microscopía Electrónica de la Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata.

Extraction and quantification of intracellular PHB

For quantitative determination of PHB, 5-10 mg of lyophilized cells were subjected to methanolysis in the presence of 15 % (v/v) sulphuric acid, and 3-hydroxybutyrate-methylester was analyzed by HP 5890 gas chromatograph (GC) with flame ionization detector (FID) and VF-23ms column 30 mx 0.25 mm x 0.39 mm (Varian). The injection

volume was 0.2 µl. Helium (13 mm/min) was used as carrier gas. The temperature of the injector and detector was 270 °C. Tridecanoic acid was used as internal standard.

Extraction and quantification of intracellular Glycogen

Total polysaccharide was extracted from lyophilized cells by classical alkaline hydrolysis (Elbein and Mitchell 1973). Glycogen quantification was performed by enzymatic hydrolysis. Alkaline extracts were digested with 2 µl of alpha-amylase (519 IU) and 15 µl of amyloglucosidase (129 IU) in 50 mM sodium acetate buffer pH 5 in final volume of 1 ml at 55 °C for 4 h. Glucose was determined by a specific glucose oxidase method (Hernández *et al.* 2008)

Extraction and quantification of intracellular trehalose

Trehalose was extracted from 10 to 30 mg of lyophilized cells with 15 ml of 80 % ethanol for 3 h at 65 ºC. After centrifugation, each pellet was washed with 5 ml of 80 % ethanol and centrifuged. Both supernatants were combined and vacuum-dried. The residue was dissolved in 0.5-1 ml water (Zhang and Yan 2012). The water-soluble residue containing trehalose was analyzed by isocratic HPLC. Trehalose was separated on a Carbopac PA1 (Dionex) column (4 x 250 mm) and NaOH/AcNa (0.2 M / 0.3 M) pH 12 was used as eluent and detected with electrochemical PAD (Pulse Amperometric Detector) Water 2465.

Primer design, RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

The sequences of single-copy *Sphingobium* sp. 22B genes (DDBJ/ENA/GenBank; LTAB00000000) (Table 3) were used as template for the design of primers related with physiological key genes by Primer 3 Software (version 0.4.0). Specificity of primers was verified *in silico* by RAST (Rapid Annotation using Subsystem Technology version 2.0) and

by Artemis Software and by PCR with the same program detailed below, followed by gel electrophoresis. The used primers are shown in Table 1. Cell cultures for RNA extraction were obtained, as detailed before, in water stress resistance test, using filter area representing approximately 5 ml of culture for each sample, which was resuspended in RNA later® (Sigma Aldrich) solution and stored at -80°C to prevent RNA degradation. RNA extraction was carried out with RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. For cDNA synthesis, 1 mg of each RNA sample was treated with DNase I (Promega) for 1 h at 37 °C. The DNA-free RNA was then used as template to synthesize the cDNA with M-MLV Reverse Transcriptase (Invitrogen) and Random Hexamer Primer (Thermo Scientific) following the manufacturer's instructions. RNA extraction and cDNA synthesis were carried out in triplicate from independent cultures of each condition. The efficiencies of the selected primer pairs (Table 1) were checked by real time PCR (Stratagene Mx3000P) with serial dilutions of an equimolar mixture of cDNA of the three conditions tested in this work as templates. PCR efficiency was near 1. The reaction mix contained 1 µl of DNA template, 1 µM of the forward and reverse primer, 0.2 µl of BSA (Sigma) 2× SYBR Green PCR Master Mix (Promega). Total reaction volume of 10 µl was reached with PCR-grade water. The program started with a hold at 95 ºC for 10 min, followed by 45 cycles of denaturation at 95 ºC for 10 s, annealing at 55 ºC for 15 s and elongation at 72 ºC for 15 s. qPCR assays were performed in three technical replicates on samples and negative controls. The negative controls consisted of PCR blanks with only the reaction mix and PCR blanks containing the mix and 1 µl of PCR-grade water. Threshold cycles (Ct) were measured in separate tubes, in triplicate. Identity and purity of the amplified product were checked by analyzing the melting curve at the end of amplification. The differences between Cts were calculated in every sample for each gene of interest as follows: Ct gene of interest - Ct 16SrRNA gene as reference gene marker. Relative changes in the expression level of one specific gene (ΔΔCt)

were calculated by the ΔCt method. The expression ratios for the different genes were obtained dividing the normalized power values in desiccation (18RH) and humid (60RH) conditions calculated from the ΔΔCt method, using the 16s as reporter gene.

"In silico" search of stress-related genes in draft-genome of *Sphingobium* sp. 22B and in complete genomic projects in *Sphingomonas* strains

RAST (SEED Viewer version 2.0) and NCBI annotation server was used for the selection of genes related with osmotic stress, trehalose and glycogen biosynthesis and utilization, PHB metabolism and exopolysaccharide synthesis in *Sphingobium* sp. 22B. The sequence of the coding proteins of each gene of the strain 22B (DDBJ/ENA/GenBank; LTAB00000000) was compared in november 2017 by BLAST genome (http://www.ncbi.nlm.nih.gov/genome) with 10 strains of *Sphingobium* and *Novosphingobium* with complete genome projects available at that moment (Table 4). In addition, each gene name and EC number was searched in *Sphingomonas* complete genome projects.

Statistical Analysis

All experiments in this study were performed in triplicate. Results are expressed as mean values ± S.E.M. Data were analyzed by ANOVA (one-way) followed by Fisher´s test. In all cases, P values were calculated with Student's t test and those lower than 0.05 were considered statistically significant. All statistical tests were performed using GraphPad Prism 6.0.

RESULTS

Physiological responses of strain *Sphingobium* sp. 22B to water stress conditions

The physiological responses of the strain 22B to water stress were compared during incubation of cells under high water stress condition (18RH) and low water stress condition (60RH). In agreement with previous results (Madueño *et al.* 2011), *Sphingobium* sp. 22B exhibited high cell culturability (80.6 ± 2.6 %) after 96 h under 18RH (with 94.5 ± 0.85 % water loss). A cell culturability of 99.2 ± 0.45 % was observed in 60RH where 58.8 ± 5.1 % of water loss occurred.

Sphingobium sp. 22B was able to produce and accumulate 44.9 ± 6.4 % of PHB, 4.8 ± 1.4 % of glycogen and 0.84 ± 0.16 % of trehalose per cellular dry weight (CDW) during its growth with an excess of carbon source after 48 h of incubation at 28° C (Table 2) (control condition). The content of trehalose and glycogen varied significantly ($P < 0.05$) during cell incubation under 18RH and 60RH conditions. In comparison to the control condition, after 96 h under 18RH, cells mobilized approximately 77 % of glycogen (2.0 ± 0.3 %) and 65 % of trehalose (0.28 ± 0.1) content, but PHB was not mobilized (45.7 ± 28.4) (Table 2). In contrast, under 60RH, cells mobilized the three store carbon compounds studied in this work, approximately 50 % of PHB (22.1 ± 15.9), 85 % of glycogen (0.7 ± 0.3) and 88 % of trehalose (0.1 ± 0.04) (Table 2).

The ultrastructure of *Sphingobium* sp. 22B cells was analyzed by transmission electron microscopy (TEM) in control, 18RH and 60RH conditions (Fig. 1). Transmission electron micrographs of cells in the 60RH condition (Fig. 1 A) showed intracytoplasmic inclusion bodies with smaller size in comparison with those shown in control cells and cells under the 18RH condition (Fig. 1 C, E). Interestingly, TEM showed the production of an amorphous matrix which could represent an extracellular polymeric substance around the cells after 96 h

of incubation under 60RH (Fig. 1 B) and 18RH (Fig. 1 D) conditions, in contrast to control cells and cells grown in R3 broth (Fig. 1G).

Genomic features linked to survival under desiccation

The availability of the *Sphingobium* sp. 22B genome (DDBJ/ENA/GenBank; LTAB00000000) (Madueño *et al.* 2016) allowed us to analyze the occurrence of genes hypothetically involved in water stress response. Since 22B strain was able to produce PHB, glycogen, trehalose and probably an EPS, and because there are many reports that showed the relationship of these compounds with desiccation, we searched for putative genes related to their metabolism. As it is a draft-genome, still incomplete, we regarded the apparent absence or low copy number of a given gene with caution. Results of this search are summarized in Table 3.

Gene *aqpZ* is present in the genome of strain 22B (Table 3) and encodes a water channel belonging to the major intrinsic protein family (MIP) (King *et al.* 2004) and has been involved in environmental stress response in bacteria (Wang 2002; Sinetova *et al.* 2015; Wood 2015). Putative genes for two different pathways of trehalose biosynthesis (*otsAB* and *treYZ)* and two genes for its degradation (*tre, tp*) were found in *Sphingobium* sp. 22B (Table 3). As expected, 22B strain genome contained genes for the entire glycogen biosynthesis and degradation pathways, such as *glgC*, *glgA*, *glgB*, *glgP*, and *glgX*, (Table 3). Multiple genes involved in PHB metabolism were identified with at least 3 of them coding for polyhydroxyalkanoic acid synthase enzymes, 1 for acetoacetyl-CoA reductase and 5 for 3-ketoacyl-CoA thiolase enzymes. The presence of diverse genes for transferases and glycosyltransferases (*rfbP, epsF*, *lgt1*, *lgt2*) related with the synthesis of extracellular polysaccharides were also found in 22B strain genome (Table 3).

Comparative analyses of stress-related genes in draft-genome of *Sphingobium* sp. 22B and in *Sphingomonas* complete genomic projects

The genetic endowment of *Sphingobium* sp. 22B related with the responses to environmental stresses was compared to those of other 10 *Sphingomonas* complete genome projects available in National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) (Table 4).

In most of the studied strains, the complete pathway for biosynthesis and degradation of glycogen and for biosynthesis of trehalose from glucose (*otsAB*) were found (Table 4). An aquaporin family gene related with osmotic stress (*aqpZ*) and *epsF* gene involved in the biosynthesis of an EPS were found in *Sphingobium* sp. 22B and also in 4 other *Sphingobium* sp. strains (Table 4). Major differences between *Sphingomonas ¨sensu latu¨* strains and strain 22B were found in trehalose metabolism. Gene *tre*, which encodes trehalase, and *treY* and *treZ* genes for the biosynthesis of trehalose from glycogen, were only found in two *Sphingomonas* strains (*Novosphingobium* sp. PP1Y, *Sphingobium* sp. YBL2 for *tre* gene, and *Sphingobium* sp. SYK-6 Ac, *Sphingobium* sp. YBL2 *treY* and *treZ* genes) (Table 4), and also *tp* gene which encodes trehalose phosphorylase enzyme was found in 3 *Sphingomonas* complete genome projects studied (*Sphingobium* sp. YBL2, *Sphingobium* sp. EP60837, *Sphingobium* sp. MI1205).

Quantitative Real-Time PCR (qRT-PCR)

To analyze the expression of genes hypothetically related with water stress response in *Sphingobium* sp. 22B specific primers were designed. Those primers were related to single copy genes, involved in osmotic stress (*aqpZ*), trehalose biosynthesis (o*tsA, otsB, treY, treZ),* trehalose utilization (*tre, ga,*), glycogen biosynthesis and utilization (*glgA, glgB, glgC glgP*), PHB (*phbB*) and exopolysaccharide biosynthesis (*epsF, lgt1*). Only those that showed clear specificity and efficiencies near 1 were used in this assay. Figure 2 shows the fold changes in mRNA levels of the genes *glgC, glgP, aqpZ, phbB, otsA, treY, lgt1* in 18RH, 60RH and

control conditions calculated from the ΔΔCt method. *AqpZ* gene expression showed no difference between 18RH, 60RH and control conditions. The glycogen degradation and biosynthesis genes *glgC* and *glgP* were significantly downregulated in 18RH and 60RH conditions in comparison to control, although no differences in the expression under both water stress conditions studied were found (Figure 2). The expression of *phbB* gene, which is involved in poly-hydroxybutyrate biosynthetic process, was downregulated in 60RH and was not significantly different in 18RH in comparison to the control condition (Figure 2). Putative gene *lgt1*, encoding a glycosyl transferase involved in EPS biosynthesis, and *otsA* gene which encodes for an alpha,alpha-trehalose-phosphate synthase, were downregulated in 60RH in comparison with the control, and not detected in the high water stress condition (18RH). The gene *treY* which encodes a malto-oligosyltrehalose synthase was 6-fold upregulated in the low water stress condition (60RH) in comparison with the control condition (Figure 2).

Discussion

Microorganisms have the capacity to utilize a huge variety of nutrients and adapt to continuously changing environmental conditions. Many microorganisms, including yeast and bacteria, accumulate carbon and energy reserves to cope with starvation conditions temporarily present in the environment (Wilson *et al.* 2010). *Sphingobium* sp. 22B strain shows a carbon conservative metabolism which allows the cells to store carbon and energy within different compounds, such as PHB, glycogen, and trehalose (Table 2). These compounds may play different roles in cells and may be part of the complex metabolic network present in strain 22B to cope with the adaption to environmental stresses.

The synthesis and accumulation of PHB is a known and widely distributed property in prokaryotes and depends on either the type of strain or the carbon source used in the process (Verlinden *et al.* 2007; Getachew and Woldesenbet 2016). PHB seems to be relevant for 22B strain physiology, since it accumulates significant amounts of these lipids (Table 2, Fig.1 E)

and has in its genome a robust endowment of genes/proteins for their metabolism (Table 3). As a comparison with strain 22B, who stores 45 % CDW of PHB in the control condition, other bacteria like *Bacillus cereus* CFR06 and *Caulobacter crescentus* accumulate 50 % and 18 % CDW of PHB respectively from glucose as carbon source (Qi and Rehm 2001; Halami 2008). In particular other strains of *Sphingomona*s showed an accumulation of polyhydroxyalkanoates from glucose in percentages that ranged from a 2.9 to 70.2 % (w/w) (Godoy 2003). In strain 22B, PHB may serve as an endogenous carbon and energy source, providing a temporal nutritional independence from the environment and the maintenance of the metabolic activity during starvation under low water stress conditions (60RH) (Table 2). The results obtained in this study, however, showed that under the high water stress condition (18RH), *Sphingobium* sp. 22B maintained the same intracellular PHB concentration as the control (Table 2), kept the integrity of intracytoplasmic inclusion bodies (Fig.1) and showed the same expression level of *phbB* gene in 18RH condition in comparison with the control condition (Figure 2). Therefore, the endogenous mobilization of PHB did not seem to be a process related to high water stress condition (18RH) in strain 22B. Recently Goh *et al.* (2014), provided evidence that in *Delftia acidovorans* the enhancement of stress tolerance can be achieved without mobilization of previously accumulated PHA. The authors argue that intracytoplasmic PHA granules could act as a specific site for binding a stress-resistant protein reducing the stress-resistant protein cytoplasmic concentration and consequently the expression of more proteins. Obruca *et al.* (2016), analyzed the PHB monomer 3-hydroxybutyrate (3HB) as a chemical chaperone capable of protecting model enzymes from different environmental stresses finding that 3HB exhibits a greater protective effect than that of the trehalose. In concordance with those authors' arguments, PHB might play diverse roles under different degrees of water stress: it can serve as an endogenous carbon source under 60RH and can protect cells under 18RH.

Cells of 22B strain actively metabolized carbohydrates in both conditions of water stress tested in this work, suggested by the mobilization of intracellular glycogen and trehalose (Table 2), the upregulation of *treY* gene in low water stress condition, the absence of significant differences between the expression of the glycogen synthesis and degradation genes *glgC* and *glgP*, and the downregulation of *otsA* gene in water stress conditions (Fig. 2). Glycogen biosynthesis is another mechanism for carbon storage in bacteria and *Sphingobium* sp. 22B accumulates approximately 5 % CDW (Table 2) under excess of the carbon source. Beer *et al*., in 2004 indirectly suggested that members of the genus *Sphingomonas* may have the ability to produce glycogen, and are able to reach a biomass glycogen level of 5 to 24 (% w/w). Glycogen has been detected in higher concentrations in other bacteria such as 18 % of the dry weight in Cyanobacteria after nitrogen withdrawal (Klotz and Forchhammer 2017) and up to 60 CDW % in Clostridia just prior to sporulation (Preiss *et al*. 1983). According to our knowledge this is the first report of the production of glycogen in a strain of *Sphingobium*. Under the water stress conditions studied in this work, endogenous mobilization of glycogen in strain 22B occurs probably for attending cells in carbon starvation (Table 2). In concordance with the greatest mobilization of glycogen in the 60RH condition (Table 2), *treY* gene, who is involved in the conversion of endogenous glycogen to trehalose, was upregulated (Fig. 2). However, the concentration of trehalose was less (0.1 ± 0.04 % CDW) in the 60RH condition in comparison with 18RH (0.28 ± 0.1 % CDW). Some authors have reported that trehalose genes were overexpressed in osmotic stress and desiccation (Cytryn *et al*. 2007; Johnson *et al*. 2011) and assigned to trehalose different biological roles such as carbon source or compatible solute under osmotic-shock conditions (Omar *et al*. 2014; Shleeva *et al*. 2017). In *Rhodococcus opacus* PD630 the accumulation of trehalose was 0.48 % CDW from gluconate as carbon source after seven days in dehydration (Alvarez *et al.* 2004). Comparative genome analysis revealed that strain 22B could be

synthesizing trehalose through two different pathways (*otsAB* and *treYZ*) while only in 2 strains of the others *Sphingomonas "sensu latu"* included in this study (*Sphingobium* sp. SYK-6 and YBL2) the *treYZ* pathway was found to be present. Diverse trehalose synthesis pathways could provide *Sphingobium* sp. 22B the necessary metabolic flexibility to respond to environmental stress with this disaccharide, giving to the strain competitive advantages over other bacteria under desiccation. Additionally, glycogen and trehalose in *Sphingobium* 22B may serve as an endogenous source of carbon and energy and may provide sugar residues for the biosynthesis of extracellular polymer, which is produced during 60RH and 18RH condition, as the TEM analysis revealed (Fig. 1B, D).

Sphingobium sp. 22B has at least 5 putative genes that encode for glycosyltransferases involved in the synthesis of extracellular polymeric substances (EPS) (*lgt1*, *lgt2*, *epsF*, *rfbP*) (Table 4). EPS has been associated with the responses of *Sphingomonas wittichii* RW1 to osmotic stress, since a transcriptomic study demonstrated the induction of genes involved in the polysaccharide synthesis, assembly and export of EPS (Roggo *et al.* 2013). Although in strain 22B submitted to water stress conditions, an extracellular amorphous matrix was seen in TEM, *lgt1* gene expression (Figure 2) was downregulated indicating that this gene may not be involved in the synthesis of EPS. The production of an extracellular amorphous matrix by strain 22B in response to water stress may offer a significant mechanical protection to cells, preventing intracellular water loss (Ophir and Gutnick 1994), and allowing the cells to make metabolic adjustments to survive to water stress (Roberson and Firestone 1992; Tribelli and López 2011).

In 4 of the 10 *Sphingomonas "sensu latu"* complete genomes and in *Sphingobium* sp. 22B genome, the gene *aqpZ* coding for aquaporin protein, was present (Table 4). Aquaporins are water channels mediating transmembrane water flux in *E. coli* (Calamita 2000) and the absence or even mutations of this gene in many living microorganisms indicates that

aquaporins may have nonessential functions and are not always related to microbial survival (Tanghe *et al.* 2006). Many authors studying aquaporins found inconsistencies (Tanghe *et al.* 2006) in results during hypoosmotic (Booth and Louis 1999) and hyperosmotic stress in bacteria (Hernández-Castro *et al.* 2003).The results obtained in this study suggested that aquaporins has no evident function in *Sphingobium* sp. 22B under water stress conditions, since *aqpZ* was not differentially expressed between the water stress conditions studied (Figure 2).

ACKNOWLEDGMENTS

This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica (PICT 2013-0103). Part of this work was performed by the grant "UNLP Jóvenes Investigadores 2014" obtained for Madueño L. In the frame of a doctoral fellowship to Madueño L. part of this work was supported by CONICET and Oil M&S Company (R 2422). Madueño L. is research member of CONICET. Festa S has a posdoctoral cofounded fellowships supported by CONICET-FYPF. Morelli I. S. is research member of CIC-PBA and Coppotelli B. M and Alvarez H. M are research members of CONICET.

NOTES

We thank to the technical support of CONICET, B. Esteban Manuel González and Dr. Maria Guillermina Zubiría for their help with the quantification of intracellular trehalose and Quantitative Real-Time PCR.

Conflict of Interest

The authors have no conflict of interest to declare.

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FIGURE LEGENDS

Table 1: qPCR Primers used in this study

Table 2: Intracellular percentage of glycogen, trehalose and PHB per cellular dry weight (CDW) in the strain *Sphingobium* sp. 22B in control, 18RH (18 % of relative humidity) and 60RH (60 % of relative humidity) conditions. Values are means ± SEM.

Table 3: Putative genes hypothetically linked to survival under desiccation in *Sphingobium* sp. 22B draft-genome (http://www.ncbi.nlm.nih.gov/genome). Genes showed are related with

osmotic stress, trehalose and glycogen biosynthesis and utilization; PHB and exopolysaccharide biosynthesis in *Sphingobium* sp. 22B genome**.**

Table 4: Comparative genome analysis between *Sphingobium* sp. 22B draft-genome and *Sphingomonas ¨sensu latu¨* complete genome projects showing the presence of at least one (+) of putative genes linked with osmotic stress; trehalose and glycogen biosynthesis and utilization; PHB and exopolysaccharide biosynthesis. NF: Putative genes not found.

Figure 1: Transmission electron micrographs of *Sphingobium* sp. 22B. Ultrathin sections (A) and negative stained (B) transmission electron micrographs in 60RH condition (60 % of relative humidity). Ultrathin sections (C) and negative stained (D) transmission electron micrographs in 18RH condition (18 % of relative humidity). Ultrathin sections (E) and negative stained (F) of the strain grown on glucose for 48 h at 28°C (without stress condition). Negative stained transmission electron micrographs of strain growing in R3 broth for 48 h (G) (without stress condition).

Figure 2: Gene expression of key genes/markers (*glgP, glgC, aqpZ, phbB, igt1, otsA and treY*) regulated during 18RH (high water stress) and 60RH (low water stress) conditions. Values expressed as fold change are means ± SEM of three independent RNA preparations and indicate the change in the mRNA levels of genes studied in comparison with the control condition (Value of 1). The significance of gene expression between conditions are shown with different letters (a,b) and was determined by Student's t test using a P value of ± 0.05 as the threshold.

Primer name	EC number	Sequence (5'-3')	Pb	Reference
glgC_F	2.7.7.27	5'GTCCATGGGCATCTACGTCT3'	244	This work
glgC_R		5'GTCGGTCAGGTCGATATTGG3'		This work
glgP_F	2.4.1.1	5'ATCTGGTGCAGGGCATCTAC3'	187	This work
glgP_R		5'GTCGCATATTCCCTGATCGT3'		This work
AqpZ_F	--	5'CCGTCACGCTGGTTATGC3'	150	This work
AqpZ_R		5'GGATCTTGGCCGACATCTG3'		This work
phbB_F	1.1.136	5'GACGACTGGAACGAGGTGAT3'	231	This work
phbB_R		5'ATTGACGGTGACGCCATATT3'		This work
lgt1_F	--	5'TTCAACGTCCACAGCATGAC3'	188	This work
lgt1_R		5'GTGCCCCAATAATGGATGTC3'		This work
OtsA_F	--	5'GTCATTTCGAACCGGGTCAG3'	163	This work
OtsA_R		5'CTTCATCCTCCGAAAAGCCG3'		This work
treY_F	5.4.99.15	5'GCCAATGACCTGCTGAACTG3'	250	This work
treY_R		5'TCTCCACCACGATATAGGCG3'		This work
1055f	----	5' ATGGCTGTCGTCAGCT 3'	337	Harms, 2003
1392r		5' ACGGGCGGTGTGTAC 3'		Harms. 2003

Table 1: qPCR Primers used in this study

Conditions	Glycogen % CDW	Trehalose % CDW	PHB % CDW
Control	4.8 ± 1.4	0.84 ± 0.16	44.9 ± 6.4
18RH	2.0 ± 0.3	0.28 ± 0.1	45.7 ± 28.4
60RH	0.7 ± 0.3	0.10 ± 0.04	22.1 ± 15.9

Table 2: Intracellular percentage of glycogen, trehalose and PHB per cellular dry weight (CDW) in the strain *Sphingobium* sp. 22B in control, 18RH (18 % of relativity humidity) and 60RH (60 % of relativity humidity) conditions. Values are means ± SEM.

Product name	Gene	EC Number	Reactions	Scenario reactions	GO	N° Copy
Osmotic stress						
aquaporin family protein	*aqpZ*	-	---	---	GO:0005215 GO:0006810 GO:0016020	1
Trehalose biosynthesis and utilization						
alpha,alpha-trehalose-phosphate synthase	*otsA*	2.4.1.15	R00836 R06043	R02737	GO:0003825	1
trehalose-phosphatase	*otsB*	3.1.3.12	R02778 R06228	R02778	-	1
malto-oligosyltrehalose synthase	*treY*	5.4.99.15	R06243 R01824	-	GO:0047470	1
malto-oligosyltrehalose trehalohydrolase	*treZ*	3.2.1.141	-	-	-	1
glycoside hydrolase family 15 protein (glucoamylase)	*ga*	3.2.1.3	-	-	-	1
trehalase	*treA*	3.2.1.28	R00010 R06103	R00010	GO:0004555	1
glycoside hydrolase family 65 protein (trehalose phosphorylase)	*tp*	2.4.1.64	R02727 R06053	R02727	GO:0047656	1
Glycogen biosynthesis and utilization						
glycogen synthase	*glgA*	2.4.1.21	R02421	R02421	GO:0009011	1
glycogen branching enzyme	*glgB*	2.4.1.18	R02110	R02110	-	1
glucose-1-phosphate adenyltransferase	*glgC*	2.7.7.27	R00948	R00948	GO:0008878	1
glycogen/starch/alpha-glucan phosphorylase	*glgP*	2.4.1.1	R01821	R02111	GO:0004645	1
glycogen debranching enzyme	*glgX*	3.2.1.-	-	-	GO:0003824 GO:0005975 GO:0043169	2
PHB synthesis						
acetyl-CoA acetyltransferase	*phbA*	2.3.1.9	-	R04254	GO:0003985	5
beta-ketoacyl-ACP reductase	*phbB*	1.1.1.36	R01779	R01977	GO:0018454	1
poly-beta-hydroxybutyrate polymerase	*phbC*	-	-	-	-	2
class I poly(R)-hydroxyalkanoic acid synthase	*phaC*	-	+	-	-	1
Exopolysaccharide biosynthesis						
exopolysaccharide biosynthesis glycosyltransferase EpsF	*epsF*	2.4.1.-	-	-	GO:0009058	1
glycosyl transferase, family 4 protein	*lgt1*	-	-	-	-	2
glycosyl transferase, family 2 protein	*lgt2*	-	-	-	-	1
undecaprenyl-phosphate galactosephosphotransferase	*rfbP*	2.7.8.6	-	-	GO:0047360	1

Table 3: Putative genes hypothetically linked to survival under desiccation in *Sphingobium* sp. 22B draft-genome (http://www.ncbi.nlm.nih.gov/genome). Genes showed are related with osmotic stress, trehalose biosynthesis and utilization; glycogen biosynthesis and utilization; PHB and exopolysaccharide biosynthesis in *Sphingobium* sp. 22B genome.

Gene symbol	Encoding gene	EC number	*Sphingobium* sp. C1	*Sphingobium* sp. RAC03	*Sphingobium* sp. EP60837	*Sphingobium* sp. YBL2	*Sphingobium* sp.SYK-6	*Sphingobium* sp. TKS	*Sphingobium japonicum* UT26S	*Sphingobium chlorophenolicum* L-1	*Sphingobium* sp.MI1205	*Novosphingobium* sp. PP1Y	*Novosphingobium aromaticivorans* DSM 12444	*Sphingobium* sp. 22B
Osmotic stress														
aqpZ	aquaporin family protein	-	NF	NF	+	NF	NF	+	+	+	NF	NF	NF	+
Trehalose biosynthesis and utilization														
otsA	alpha,alpha-trehalose-phosphate synthase	2.4.1.15	+	+	+	+	+	+	+	+	+	+	+	+
otsB	trehalose-phosphatase	3.1.3.12	+	+	+	+	+	+	+	+	+	+	+	+
treY	malto-oligosyltrehalosesynthase	5.4.99.15	NF	NF	NF	+	+	NF	NF	NF	NF	NF	NF	+
treZ	malto-oligosyltrehalose trehalohydrolase	3.2.1.141	NF	NF	NF	+	+	NF	NF	NF	NF	NF	NF	+
ga	glycosyldehydrolase family 15 protein (glucoamylase)	3.2.1.3	+	+	+	+	+	+	+	+	+	+	+	+
tre	trehalase	3.2.1.28	NF	NF	NF	+	NF	NF	NF	NF	NF	+	NF	+
tp	glycoside hydrolase family 65 protein (trehalose phosphorylase)	2.4.1.64	NF	NF	+	+	NF	NF	NF	+	+	NF	NF	+
Glycogen biosynthesis and utilization														
glgA	glycogen synthase	2.4.1.21	NF	+	+	+	+	+	+	+	+	+	+	+
glgB	glycogen branching enzyme	2.4.1.18	NF	+	+	+	+	+	+	+	+	+	+	+
glgC	glucose-1-phosphate adenyltransferase	2.7.7.27	+	+	+	+	+	+	+	+	+	+	+	+
glgP	glycogen/starch/alpha-glucan phosphorylase	2.4.1.1	+	+	+	+	+	+	+	+	+	+	+	+
glgX	glycogen debranching enzyme	3.2.1.-	+	+	+	+	+	+	+	+	+	+	+	+
PHB biosynthesis														
phbA	acetyl-CoA acetyltransferase	2.3.1.9	+	+	+	+	+	+	+	+	+	+	+	+
phbB	beta-ketoacyl-ACP reductase	1.1.1.36	+	+	+	+	+	+	+	+	+	+	+	+
phbC	poly-beta-hydroxybutyrate polymerase	-	NF	NF	+	+	+	+	+	NF	+	NF	NF	+
phaC	class I poly(R)-hydroxyalkanoic acid synthase	-	+	+	+	+	+	NF	+	+	+	+	+	+
Exopolysaccharide biosynthesis														
epsF	exopolysaccharide biosynthesis glycosyltransferase EpsF	2.4.1.-	NF	+	NF	+	NF	+	+	NF	NF	NF	NF	+
lgt1	glycosyltransferase, family 4 protein	-	+	+	+	+	+	+	+	+	+	+	+	+
lgt2	glycosyltransferase, family 2 protein	-	+	+	+	+	+	+	+	+	+	+	+	+
rfbP	undecaprenyl-phosphategalactose phosphotransferase	2.7.8.6	NF	NF	NF	NF	+	+	+	NF	NF	NF	NF	+

Table 4: Comparative genome analysis between *Sphingobium* sp. 22B draft-genome and *Sphingomonas ¨sensu latu¨* complete genome projects showing the presence of at least one (+) of putative genes linked with osmotic stress; trehalose and glycogen biosynthesis and utilization; PHB and exopolysaccharide biosynthesis. NF: Putative genes not found.