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Freeze survival of the cyanobacteria *Microcoleus chthonoplastes* without cryoprotector

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Abstract

A *Microcoleus chthonoplastes* strain SC7B9002-1 isolated from microbial mats in tidal channels from San Carlos, Baja California Sur, Mexico was subjected to short- (15 days) and long-term (2 years) conservation assays in liquid nitrogen (–196ºC) using cryoprotective agents, such as 5% DMSO, 20% PVP-40, and 20% glycerol. Survival rate, chlorophyll a, protein, and nucleic acids content were observed in each case. Interesting growth and a significant increase in protein content was observed when no cryoprotectant was used during liquid nitrogen immersion. In the absence of a cryoprotectant, *M. chthonoplastes* lost their typical shape resembled spheroplasts, and recovery cultivation times after freezing were 5 and 25 days (short and long-term, respectively). Recovery from long-term preservation with 5% DMSO took 15 days. PVP and glycerol did not allow recovery of viable cells. The survival of *M. chthonoplastes* to freezing without cryoprotectant and the adaptive mechanisms that allow surviving under freezing conditions are discussed.

Key words

Freezing-survival, Cryopreservation, Cyanobacteria, *Microcoleus chthonoplastes*

Introduction

M. chthonoplastes appears to play an important role in coastal estuaries and salt marsh evaporite interface stratified microbial communities as a primary colonizer (Underwood, 1997; Stolz, 1985; Zvyagintseva *et al*., 1995; Nubel *et al*., 2000). Studies on survival of *Microcoleus*-dominated communities after drying have indicated that rewetting induces complex microbial population to emerge (Brown *et al*., 1985; Abed and García-Pichel, 2001). Also, broad species variability in *M. chthonoplastes* has been recognized, although some molecular studies suggest that all species belong to a common, well-defined taxon (García-Pichel *et al*., 1996). The biochemical (Karsten and García-Pichel, 1996), and physiological responses of the different isolates point to the existence of a wide array of ecotypes (Karsten, 1996).

Because of the potential applications for *M. chthonoplastes*, like mineralizing phosphate (Garasimenko *et al*., 1999), increasing the level of dissolved oxygen in shrimp ponds (López-Cortés, 1999; López-Cortés *et al*., 2001), stabilizing sediment (De Winder *et al*., 1999), transferring nitrogen to enhance mangrove growth (Bashan *et al*., 1998), consuming and oxidizing n-alkanes in contaminated areas (Al-Hasan *et al*., 1998) and producing carotene, superoxide dismutase, ascorbate peroxidase and specific polysaccharide (López-Cortes and Tovar-Ramírez, 1992; Cohen, 1989; Tovar-Ramírez *et al*., 1999), handling pure cultures of *M. chthonoplastes* strains that can preserve their original properties is an important challenge. Efficient biomass production and exploitation of *M. chthonoplastes* (Dubinin *et al*., 1992 a,b) includes finding conditions that promote preservation of microorganisms with specific properties. In this context, long-term cell survival and viability are of major importance (Frosch *et al*., 1987; Vaulot *et al*., 1989; Day and Brand, 2005) to preserve microorganisms based on different cryoprotective agents have been described (Mazur, 1970; Ben Amotz and Gilboa, 1980; James, 1991; Taylor and Fletcher, 1999; Brand and Diller,

2004; Day and Brand, 2005). The use of low molecular weight compounds, capable of permeating the cell membrane and known as permeable cryoprotectants, improves the preservation of cell suspensions by minimizing cell volume changes during freezing (Cañavate and Lubian, 1995; Brand and Diller, 2004; Day and Brand, 2005), or damage from intracellular ice formation. Biological sample preservation by liquid nitrogen immersion is one of the most used techniques in the biological sciences (Ben Amotz and Gilboa, 1980; Brand and Diller, 2004; Day and Brand, 2005), but different survival rates have been reported (Holm-Hansen, 1963; Box, 1988; Romo and Becares, 1992; Bodas *et al*., 1995; Nuñez-Vazquez *et al*, 1996; Day, 1998; Mori *et al*., 2002; Day *et al*., 2005, 2006; Kiyoshi *et al*., 2005; Park, 2006; Takashima *et al*., 2007; Wood *et al*., 2008). The aim of this work was to find suitable conditions for preservation of the cyanobacterium *Microcoleus chthonoplastes* strain SC7B9002-1 using various cryoprotective agents.

Materials and Methods

Microorganisms and growth conditions: *Microcoleus chthonoplastes* strain SC7B9002-1 was isolated from microbial mats in tidal channels from San Carlos, Baja California Sur, México (López-Cortés and Tovar-Ramírez, 1992). The strain was cultured in ASN-III liquid medium illuminated with daylight-cool lamps at 10-12 µE m^{-2} s^{-1} at a constant agitation of 100 rpm in an orbital shaker at 25 ± 2°C, until the middle of exponential phase was reached, as measured by chlorophyll *a*, total protein, and nucleic acids production during growth. Filament number from ten homogenized culture replicates of five 5-µl samples was determined by microscope (Nikon Optiphot-2, Japan). We also made a standard curve with dilution series from 5 to 25 fold to correlate the number of filaments to the absorbance of each diluted sample at 665 nm. Density (cell ml^{-1}) at 665 nm was measured in a Beckman DU-640 spectrophotometer.

Cryopreservation: Short-term cryopreservation was performed with 1 ml of *M. chthonoplastes* culture, or 46,000 ± 5,950 filaments, as determined by microscopy and densitometry. Filaments were poured into cryo-vials, 10 replicates per treatment. After removal of ASN-III liquid medium by centrifugation, the cell pellet of each vial was divided in four, and each quarter resuspended in one of four different 1-ml solution series: vials in series I had 5% Dimethyl sulfoxide (DMSO) grade 1, vials in series II had 20% Poly (vinyl pyrrolidone) (PVP-40), vials in series III had 20% glycerol, and vials in series IV had ASN-III liquid medium (control). Vials were placed in Nalgene freezing containers at –80°C before immersion in liquid nitrogen (–196°C) for 15 days.

For long-term preservation, 7 replicates of *M. chthonoplastes*, each of 172,300 ± 29,400 filaments were introduced into 5% DMSO without cryoprotectant. Vials were shaken in a vortex and gradually frozen starting at 4°C for 20 min, and then at -80°C for 30 min in an ethanol bath. Finally the vials were maintained in liquid nitrogen (-196°C) for 2 years.

Physiological parameters:

Protein, nucleic acid, and chlorophyll a quantification: Protein, nucleic acid, and chlorophyll *a* cell content were measured before and after cryopreservation. Cell samples were centrifuged at 10,000 x *g* for 5 min., and the pellet was separated and washed with 1 ml saline phosphate buffer. Protein was extracted in an ice bath by cell sonication (Cole Parmer 4710 ultrasonicator), with three periodic 20-W pulses. Protein content was calculated according to Bradford (1976) using bovine serum albumin as standard. Nucleic acids were calculated, as described by Warburg and Christian (1941) after extraction, as described by Sanbrook *et al*. (1989) as parameters of cell viability and growth (Tovar *et al*., 1999). Chlorophyll *a* was extracted by cell disruption in cold 90% methanol, and quantified according to Tandeau de Marsac and Houmard (1988).

Survival criteria: After short and long-term cryopreservation, samples were thawed and transferred into a water bath at 38°C, and centrifuged at 10,000 x *g* for 5 min to eliminate the cell suspension medium. The number of filaments were calculated by microscopy as was described above in *Microorganisms and growth conditions section*.

After freezing experiments, cells were recultivated to evaluate their viability under the growth conditions described above (taking in account the number of days).

Statistical analysis: Differences in four samples from each treatment were evaluated by one-way ANOVA and Student *t*-test. The results were expressed as mean ± SD. The level of significant difference was set at 5% ($p<0.05$).

Results and Discussion

The numbers of filaments of *M. chthonoplastes* measured as optical density, and protein, chlorophyll a, and nucleic acid content before and after freezing are summarized in Table 1. The number of filaments obtained by direct count and those from optical density correlate in $r^2 = 0.977$. Nucleic acid, chlorophyll *a,* cell number, and optical density decreased after 15 days of storage under frozen conditions, while protein increased in all the cryoprotectants tested including the control. Protein increase suggest the occurrence of a "cold shock response" which is present in prokaryotes and has been extensively studied in *Escherichia coli* (Phadtare *et al*., 1999) were CspA, the major cold shock protein of *E. coli*, accounts for more than 10% of total protein synthesis during the cold acclimation phase (Goldstain *et al*., 1990). There was an elevated amount of nucleic acid in glycerol and PVP, which could result from interference in the measurements. However, after long-term cryopreservation, cell component content decreased in DMSO and controls.

M. chthonoplastes was observed under the microscope before and after freezing, and some qualitative differences in cell morphology were noticed: with all cryoprotectant treatments after 15 days of freezing *M. chthonoplastes* filaments were shorter than

Table - 1: The effect of short- (15 days, a) and long-term (2 years, b) conservation assays in liquid nitrogen (–196°C) using cryoprotective agents, such as 5% DMSO, 20% PVP-40, and 20% glycerol in filament number, optical density, protein, chlorophyll *a*, and nucleic acid content of *M. chthonoplastes*.

1a- Cryoprotector	Nucleic acids ($\mu g\ ml^{-1}$)	Protein ($\mu g\ ml^{-1}$)	Chlorophyll *a* ($\mu g\ ml^{-1}$)	Cell number (5 µl)	Optical density at 665 nm
Before freezing	15.00 (0.02)^{a}	31.28 (2.03)^{c}	1.80 (0.00)^{a}	230 (4.00)^{a}	0.48 (0.005)^{a}
After freezing					
Control	1.97 (0.20)^{d}	65.05 (8.05)^{b}	1.76 (0.09)^{a}	Nd	Nd
DMSO	2.78 (0.23)^{c}	70.58 (7.46)^{b}	1.7 (0.27)^{a}	110 (3.25)^{b}	0.18 (0.005)^{b}
PVP-40	5.60 (1.13)^{b}	92.37 (2.78)^{a}	1.72 (0.11)^{a}	Nd	Nd
Glycerol	5.15 (0.74)^{b}	90.54 (4.50)^{ab}	1.80 (0.10)^{a}	Nd	Nd
1b- Cryoprotector	**Nucleic acids ($\mu g\ ml^{-1}$)**	**Protein ($\mu g\ ml^{-1}$)**	**Chlorophyll *a* ($\mu g\ ml^{-1}$)**	**Cell number (5 µl)**	**Optical density at 665 nm**
Before freezing	17.52 (2.44)^{a}	74.13 (19.45)^{a}	5.67 (0.14)^{a}	861.60(14.70)	0.84 (0.009)
After freezing					
Control	3.07 (0.12)^{c}	45.81 (9.20)^{b}	4.04 (0.48)^{b}	Nd	Nd
DMSO	6.28 (0.19)^{b}	57.31 (1.40)^{a}	3.04 (0.57)^{c}	Nd	Nd

Nd: Not determined, Numbers in parenthesis are standard deviation of three replicates, and superscripts indicate differences among treatments ($p<0.05$)

Fig. 1: *M. chthonoplastes* cultures after long-term cryopreservation. Only cells cultured in 5% DMSO and in controls grew at different rates

before freezing. In the control assay, the filaments seemed broken and smaller. In contrast, *M. chthonoplastes* filaments in 5% DMSO were larger and thinner than at the start. Long-term cryopreservation of *M. chthonoplastes* without cryoprotectant resulted in cells resembling spheroplasts without well-defined morphology. A cell count was not possible due to the lack of correlation between broken, shorter filaments and their optical density absorbance. No growth was observed after 15 days of storage under frozen conditions in the PVP or glycerol treatments.

After short- and long-term cryopreservation, only cells cultured in 5% DMSO and controls were recovered in ASN-III liquid medium (Fig. 1). Recultivation recovery times after freezing short and long-term without cryoprotectant were 5 and 25 days, respectively taking into account the appearance of large filaments in the liquid medium. However, it took 15 days for recovery from long-term preservation in 5% DMSO.

Our results suggest that the fastest recovery cryoprotectant for short- and long-term cryopreservation of *M. chthonoplastes* strain SC7B9002-1 is 5% DMSO. In previous work by other authors, DMSO was successfully used as a cryoprotectant in liquid nitrogen to preserve *Microcystis aeruginosa* (Box, 1988), and *Pseudanabaena galeata* and *Geitlerinema amphibium* at –30 and –80°C respectively (Romo and Becares, 1992). Survival of freezing by *M. chthonoplastes* has been also previously reported by other authors (Nuñez-Vazquez *et al*., 1996; Urmeneta *et al*., 2003; Hoover *et al*, 2004; Hoover, 2006).

DMSO is well known for preserving cyanobacteria (Bodas *et al*., 1995; Nuñez-Vazquez *et al*, 1996; Day, 1998; Mori *et al*., 2002; Day *et al*., 2005, 2006; Kiyoshi *et al*., 2005; Park, 2006; Takashima *et al*., 2007; Wood *et al*., 2008) and marine algae (Ben-Amotz and Gilboa, 1980; Day *et al*., 1997, Day, 1998; Taylor and Fletcher, 1999; Day *et al*., 2005). Our results, with respect to the use of 5% DMSO as a cryoprotective agent, are consistent with those of Rippka *et al*. (1981), but we used additional gradual freezing steps. DMSO has been used as a cryoprotective agent because it reduces intracellular crystal formation during freezing and avoids cell breakage (Davis *et al*., 1978; Madigan *et al*., 2003).

An important observation in our case was the survival and recovery of *M. chthonoplastes* strain SC7B9002-1 after freezing with no cryoprotectant, which was accompanied by a two fold increase in protein content (Table 1) under all conditions tested, suggesting that the role of proteins during freezing stress is important. It has been demonstrated that microorganisms exposed to

environmental stress synthesize enzymes involved in a particular metabolic pathway as regulatory proteins, or as proteins with specific protective properties (Holmberg and Bulow, 1998; Tovar *et al.,* 1999; Fleck *et al*., 2003). The use of antifreeze proteins have been proposed to protect food (Feeney and Yeh, 1998). Spheroplast-like cells were obtained after storage for two years, as observed by light microscopy. We assume that spheroplasts result from cell wall breakage by water crystallization and that, fortunately, cell wall synthesis occurs again during cell culture, yielding the typical *M. chthonoplastes* morphology. Davey (1989) observed survival of other cyanobacteria after freezing under normal winter conditions in polar regions, and demonstrated that the cyanobacterium *Phormidium* spp. recovers readily from freezing and that photosynthetic respiratory activity resumes immediately after thawing. Survival of this cyanobacterium through the extended winter provides viable inoculum for the next growing season. This capacity reflects the resistance of cyanobacteria to mortality under harsh conditions like desiccation (Potts, 1994); hot springs (Castenholz, 1977); desert (Wyn-Williams, 2000); hypersaline brines (D´Antoni D´Amelio *et al*., 1989; Kumar, 2001); Antarctic ice (Evonne *et al*., 1997) and anoxia (Stal, 1991). Cyanobacteria tolerance to high and low extreme temperatures (Radway *et al*., 1992; Wynn-Williams, 2000), freezing, and desiccation stress has previously observed (Nadeau and Castenhotz, 2000; Lin *et al*., 2004; Sabacka and Elster, 2006)**.** Cyanobacterial life in deserts is poised at the limit of survival by the scarcity of water and its enzymes are stressed in their structural and physiological limits (Wynn-Williams, 2000).

Filamentous *M. chthonoplastes*, *Phormidium ambiguum*, *Oscillatoria neglecta, O. limnetica* and *O. salina* have also been described from the green second layer of mats in hypersaline environments (López-Cortés, 1990; Prufert-Bebout and Gacía-Pichel, 1994; García-Pichel *et al*., 1996; López-Cortés *et al*., 2001 Satyanarayana *et al*., 2005;), hot deserts (Wynn-Williams 2000), the Artic (Callagan *et al*., 2004), and fumaroles on the Antarctic volcano, Mt Erebus (Hoover *et al*., 2004). Thus, this specie is considered as cosmopolitan cyanobacterium (Garcia-Pichel *et al*., 1996) and retains a remarkable ability to adapt to and survive within extreme conditions (Zakhia *et al*., 2008).

Further research, using biochemical or biomolecular approaches to analyze the factors and strategies involved in freeze protection (natural cryoprotector) of cyanobacteria in both field samples and assays *in vitro* is needed to elucidate the adaptive mechanisms that allow the cyanobacteria survive freeze-up.

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